

**UNIVERSITY of  
STIRLING**



## **Profiling (adipo)cytokines in cancer cachexia**

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A thesis submitted to the University of Stirling in partial fulfilment for the degree of  
Doctor of Philosophy

Physiology, Exercise and Nutrition Research Group

Faculty of Health Sciences and Sport

University of Stirling

*February 2023*





## Declaration

I declare that this thesis was composed by myself and that all the data were collected and analysed by myself, under the supervision of Dr Iain Gallagher, Dr Thomas Di Virgilio and Dr Richard Skipworth, with the following exceptions:

- i. The abstract screening, full-text screening and quality assessment from Chapter 2 were completed alongside Rebekah Patton and James McDonald.
- ii. The weight measurements and the blood samples from Chapter 3 were collected by Rebekah Patton, Judith Sayers and Dr Barry Laird.

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## **Acknowledgments**

Completing a doctoral degree was a dream of mine and none of this would have been possible without Dr Iain Gallagher. Iain, thank you for this opportunity! Learning from you and working alongside you has been a privilege. Your guidance and your personality made me enjoy every second of my PhD journey and inspired me to be the best version of myself. You had an unimaginable impact on my development as a researcher and as a person.

I would like to thank Dr Thomas Di Virgilio and Dr Richard Skipworth for their support. It was reassuring to know that I can always reach out to you for advice and assistance. I would also like to thank the members of the Physiology, Exercise and Nutrition Research Group for their friendliness, approachability and willingness to collaborate.

To my colleagues from 3B139 and 3B142, it was a pleasure coming to the office knowing you were there. Ragul and Liivia, I have thoroughly enjoyed our philosophical debates and the knowledge exchange sessions. Milena, Kirstin and Niels – coming to your office for coffee breaks and conversations always made my day better. James, your mentorship was crucial in the early phases of my studies.

Special thanks to a special person, Cristina. Your constant encouragement, patience and positivity enabled me to keep my spirits and motivation high during this process. I'm also extremely grateful to my close friends, Alex E, Ana and Alex S, who were a permanent source of support and entertainment. A heartfelt thank you goes out to Adi, Alex T and Răzvan - your humour and wit brought joy and light-heartedness to every interaction.

Lastly, and most importantly, I would like to thank my parents. Vă mulțumesc pentru sprijinul și dragostea oferite de-a lungul anilor. Mulțumesc, mama, pentru că nu m-ai lăsat să renunț când am vrut să fac asta. Mulțumesc, tata, pentru încrederea permanentă. Nu aş fi reuşit să ajung aici fără susținerea voastră.



## Abstract

Cancer cachexia is an unmet clinical need that affects more than half of patients with cancer. Pro-inflammatory cytokines and adipokines could be involved in the pathogenesis and development of cachexia, but this relationship is not completely understood. This thesis aimed to examine and characterise the role of (adipo)cytokines in cancer cachexia.

A systematic review was conducted to evaluate the relationship between cytokines and cachexia in people with incurable cancer (Chapter 2). Interleukin-6 (IL-6) and tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ) were considerably elevated in cachectic individuals, and a high degree of methodological heterogeneity was observed. Furthermore, data from the REVOLUTION trial were analysed to determine if adiponectin, leptin, intelectin-1 and resistin can predict the modified Glasgow Prognostic Score (mGPS) and cachexia status (Chapter 3). Although adipokines could not predict any of the outcome variables in this study, leptin was negatively associated with mGPS and cachexia, while a positive relationship was identified between resistin and mGPS.

Previous research suggested that intelectin-1 might be involved in cancer cachexia. Given the limited availability of literature, a Bayesian meta-analysis was conducted to evaluate the role of intelectin-1 in cancer and its physiological concentration (Chapter 4). Intelectin-1 levels were substantially higher in patients with gastrointestinal cancer compared to controls. The meta-analysis also estimated that the physiological concentration of intelectin-1 ranges from 193ng/ml to 275ng/ml. Lastly, a cell culture model was designed to evaluate the effect of intelectin-1 on human myotubes (Chapter 5). Increased levels of intelectin-1 diminish insulin-mediated glucose uptake and downregulate the expression of genes involved in myogenesis.

To conclude, IL-6 and TNF- $\alpha$  were highly expressed in cancer cachexia. Leptin and resistin could also contribute to the development of this wasting syndrome, but to a lesser extent. Besides these (adipo)cytokines, intelectin-1 is another potential biomarker of cachexia and future research should focus on elucidating its mechanisms of action.



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## List of publications

Included in the thesis:

- **Paval, D.R.**, Di Virgilio, T.G., Skipworth, R.J.E., Gallagher, I.J., 2022. The Emerging Role of Intelectin-1 in Cancer. *Frontiers in Oncology* 12. DOI:10.3389/fonc.2022.767859.
- **Paval, D.R.**, Patton, R., McDonald, J., Skipworth, R.J.E., Gallagher, I.J., Laird, B.J., 2022. A systematic review examining the relationship between cytokines and cachexia in incurable cancer. *Journal of Cachexia, Sarcopenia and Muscle* DOI: 13, 824–838. DOI:10.1002/jcsm.12912.

Other relevant studies:

- Patton, R., **Paval, D.R.**, McDonald, J.J., Brown, D., Gallagher, I.J., Skipworth, R.J.E., McMillan, D.C., Dolan, R.D., Fallon, M., Laird, B.J.A., 2021. Relationship between cytokines and symptoms in people with incurable cancer: A systematic review. *Critical Reviews in Oncology*, 159, 103222. DOI: 10.1016/j.critrevonc.2021.103222
- Laird, B.J., McMillan, D., Skipworth, R.J.E., Fallon, M.T., **Paval, D.R.**, McNeish, I., Gallagher, I.J., 2021. The Emerging Role of Interleukin 1 $\beta$  (IL-1 $\beta$ ) in Cancer Cachexia. *Inflammation* 44, 1223–1228. DOI: 10.1007/s10753-021-01429-8
- Refalo, M.C., Hamilton, D.L., **Paval, D.R.**, Gallagher, I.J., Feros, S.A., Fyfe, J.J., 2021. Influence of resistance training load on measures of skeletal muscle hypertrophy and improvements in maximal strength and neuromuscular task performance: A systematic review and meta-analysis. *Journal of Sports Sciences* 39, 1723–1745. DOI: 10.1080/02640414.2021.1898094.

In preparation:

- **Paval, D.R.**, Di Virgilio, T.G., Skipworth, R.J.E., Gallagher, I.J., 2023. A Bayesian meta-analysis determining intelectin-1 differences between healthy individuals and patients with cancer. *Frontiers in Oncology*.
- Robertson, T.R., Benzeval, M., Betzer, M., Booker, C., Castagné, R., Delpierre, C., Gallagher, I.J., Kelly-Irving, M., McCrory, C., Morris, T.T., Narimatsu, E., **Paval, D.R.**, 2023. A glossary for social-to-biological research. *Longitudinal and Life Course Studies*

Conference presentations:

- **Paval, D.R.**, Patton, R., McDonald, J., Skipworth, R.J.E., Gallagher, I.J., Laird, B.J., 2021. A systematic review examining the relationship between cytokines and cachexia in incurable cancer. International Seminar of the European Palliative Care Research Centre (11<sup>th</sup> edition): Next generation palliative care research – Oral presentation.
- **Paval, D.R.**, Patton, R., Sayers, J., Fallon, M., Skipworth, R.J.E, Laird, B.J., Gallagher, I.J. 2022. The relationship between cachexia and inflammatory biomarkers in patients with cancer; initial findings from the REVOLUTION cachexia characterisation study. The 15<sup>th</sup> international Conference of the Society on Cachexia, Sarcopenia & Muscle Wasting – Abstract and poster presentation.





# 1. General introduction

## 1.1. Cancer and cancer-associated cachexia

Cancer can be broadly defined as a disease that develops following the uncontrolled growth and spread of cells in the body [1]. More specifically, carcinogenesis involves the mutation of genes responsible for maintaining an equilibrium between cell proliferation and apoptosis, and for the regulation of various molecular pathways that influence cellular structure and function [2]. These genetic alterations could promote unrestricted cellular growth, tissue breakdown, the spread of cancer cells into nearby tissues and metastasis [2,3]. The abnormal behaviour of cells leads to the formation of masses of tissues, known as tumours, that can be benign or malignant. Benign tumours do not invade adjacent tissues and typically do not reoccur after surgery. Conversely, malignant tumours spread into neighbouring tissues and to distant sites in the body through the lymphatic system or the bloodstream, a process that is known as metastasis [4]. There are numerous types of cancer (e.g., sarcoma, lymphoma, melanoma) that can occur in various organs and tissues, of which carcinoma (i.e., arising in the epithelial cells that cover the inner or outer surfaces of the body) is the most common. Additionally, it is believed that a combination of environmental (e.g., smoking, exposure to chemicals, ultraviolet rays, inadequate diet, obesity, radiation) and genetic (e.g., inherited disorders, exposure to certain viruses) factors can increase the risk of developing cancer [1,4]. As indicated by the World Health Organisation [5], cancer is a major cause of death, accounting for approximately 10 million deaths worldwide per year. In the United Kingdom, nearly 375000 individuals are diagnosed with cancer every year, with only 208000 surviving the disease [6]. Similarly, the American Cancer Society [1], reported an estimated 1900000 cancer cases and 610000 cancer-related deaths in 2022. Currently, early detection and treatment are the main methods of diminishing elevated mortality rates. Even though cancer survival improved during the last decade, novel treatment strategies and innovative ways of managing this disease are still required.

One complication that occurs in approximately 80% of patients with advanced types of cancer is the cancer anorexia-cachexia syndrome [7]. Cancer cachexia is a metabolic disorder characterised by the progressive loss of skeletal muscle mass – with or without

the loss of adipose tissue – that cannot be completely treated using standard nutritional interventions. An individual with cancer would be considered cachectic if they unintentionally lost more than 5% body weight in the previous 6 months; or if they had a body mass index (BMI) lower than 20 kg/m<sup>2</sup> and lost more than 2% body weight; or if they have an appendicular skeletal muscle index consistent with sarcopenia (i.e., <5.45 kg/m<sup>2</sup> for women and <7.26 kg/m<sup>2</sup> for men) and lost more than 2% body weight. This consensus definition suggested by Fearon and colleagues [7] has been widely accepted and used in the past ten years, even though other definitions and diagnostic criteria that focus on disease-related malnutrition and inflammation were also proposed [8,9,10]. Table 1.1 highlights all cachexia definitions that were proposed to date.

**Table 1.1.** Cancer cachexia – definitions and diagnostic criteria

Authors	Definition	Diagnostic criteria
Fearon et al. [7]	A metabolic disorder characterised by the progressive loss of skeletal muscle mass – with or without the loss of adipose tissue – that cannot be completely treated using standard nutritional interventions.	<ul style="list-style-type: none"> <li>• more than 5% weight loss in the previous 6 months <b>OR</b></li> <li>• BMI lower than 20 kg/m<sup>2</sup> and more than 2% weight loss <b>OR</b></li> <li>• appendicular skeletal muscle index consistent with sarcopenia (i.e., &lt;5.45 kg/m<sup>2</sup> for women and &lt;7.26 kg/m<sup>2</sup> for men) and more than 2% weight loss</li> </ul>
Evans et al. [8]	A metabolic disorder characterised by loss of muscle mass often associated with inflammation, anorexia and insulin resistance	<ul style="list-style-type: none"> <li>• more than 5% weight loss in the previous 12 months <b>OR</b> BMI lower than 20 kg/m<sup>2</sup> <b>AND</b></li> <li>• met at least three of the following criteria: fatigue, anorexia, abnormal biochemistry (serum albumin &lt;3.2 g/dl, serum CRP &gt;5 mg/l or haemoglobin &lt;12 g/dl), lean tissue depletion or decreased muscle strength.</li> </ul>

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McMillan [9]	A syndrome associated with the systemic pro-inflammatory response.	<p>Individuals assigned to one of the following groups based on CRP and albumin levels (i.e., GPS):</p> <ul style="list-style-type: none"> <li>• no cachexia (CRP <math>\leq</math>10 mg/l and albumin <math>\geq</math> 35 g/l)</li> <li>• malnourished (CRP <math>\leq</math>10 mg/l and albumin <math>&lt;</math>35 g/l)</li> <li>• pre-cachexia (CRP <math>&gt;</math>10 mg/l and albumin <math>\geq</math> 35g/l)</li> <li>• refractory cachexia (CRP <math>&gt;</math>10 mg/l and albumin <math>&lt;</math>35 g/l)</li> </ul>
Cederholm et al. [10]	Disease-related chronic malnutrition with inflammation.	<ul style="list-style-type: none"> <li>• one phenotypic criterion: low muscle mass, low BMI or involuntary weight loss <b>AND</b></li> <li>• one aetiologic criterion: inflammation, reduced food assimilation/intake or disease burden.</li> </ul>

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BMI – body mass index; GPS – Glasgow Prognostic Score; CRP – C-reactive protein.

Despite having several alternatives to the consensus definition elaborated by Fearon and colleagues [7], there are still debates [11] in the field of cancer cachexia regarding the optimal method of classifying cachectic patients.

The pathophysiology of cancer cachexia is multifactorial with several mechanisms thought to contribute to the development of this syndrome [12,13]. Previous research [14,15] suggested that weight loss and muscle wasting are a cause of increased systemic inflammation and negative energy balance. The tumour, the host tissue and the crosstalk between these contribute to increased inflammation [12,15]. Tumour-secreted molecules such as cytokines, eicosanoids and other molecular agents (e.g., activin A, myostatin, lipocalin-2) directly increase catabolism in target tissues. Moreover, they stimulate signalling in the central nervous system and peripheral tissues, leading to elevated catabolism (i.e., increased proteolysis and lipolysis in skeletal muscle, adipose tissue and targeted organs) decreased anabolism and various metabolic alterations [14,15]. Reduced energy intake and elevated energy expenditure also contribute to cancer cachexia. As suggested by Vaughan et al. [16], resting energy expenditure is increased in cancer

cachexia mainly due to ineffective metabolic cycling. This finding is particularly relevant since most cachectic individuals become sedentary and resting energy expenditure (REE) represents a great proportion of the total energy expenditure in this population. Law [15] and Mattox [13] indicated that the reasons contributing to elevated REE levels observed in cachectic patients are complex and include inflammation, tumour metabolism, increased whole-body protein turnover (as opposed to weight-stable cancer patients and healthy individuals) and elevated energy demand by metabolically active organs. Furthermore, the energy intake of numerous cachectic patients is significantly reduced due to a variety of reasons. For example, decreased appetite can occur due to changes in smell and taste following chemotherapy and radiation or as a result of fatigue, pain and inflammation. Other treatment-associated symptoms that can affect individuals' appetite are nausea, diarrhoea, food aversions and vomiting [15]. Changes observed in hunger and satiety signalling in the hypothalamus as well as tumour metabolism are other mechanisms that could contribute to the reduction in energy intake [14]. It is crucial to note that the majority of research examining the pathophysiology of cancer cachexia was conducted on animal models, and longitudinal human studies that monitor the evolution of this syndrome are lacking.

Presently, there is no standard of care for cancer cachexia and nutrition interventions alone are not enough to reverse muscle wasting. A multi-modal approach that includes physical therapy, nutrition and treating the causes behind the reduced food intake (e.g., nausea, pain) is required for the effective management of cachexia [13,14,15]. Recently, a ghrelin receptor agonist, known as anamorelin, was approved for use in Japan as a potential treatment for cancer cachexia [17], but this syndrome remains an unmet medical need for the rest of the world. Most importantly, both the previously discussed multi-disciplinary approach and anamorelin can slow the progression of the disease and ameliorate its severity, but fail to reverse or cure cancer cachexia. This syndrome continues to be a burden for cancer patients and negatively affects their quality of life, prognosis and treatment efficacy.

## **1.2. Muscle biology and sarcopenia**

All types of muscle tissue (i.e., skeletal, cardiac and smooth muscle) are essential for the normal functioning of the human body. Of these, skeletal muscle is particularly relevant for cancer cachexia as it makes up nearly 40% of an individual's body weight, contains approximately two-thirds of the proteins in the body and is responsible for around 40% of the whole-body protein turnover [18]. The roles of skeletal muscle are multifaceted and include producing movement, sustaining posture and maintaining body temperature [19]. Besides these functions, from a mechanical perspective, skeletal muscle generates power and force following the conversion of chemical energy into mechanical energy [19, 20]. From a metabolic standpoint, skeletal muscle is a crucial component of basal energy metabolism, acts as storage for key substrates (e.g., amino acids, carbohydrates) and serves as a major regulator of glucose homeostasis [19,21]. The muscle mass is regulated by the balance between protein degradation and protein synthesis. In turn, this equilibrium depends on various mediators such as hormonal balance, physical activity, nutritional status or the presence of disease. Previous research [18,22] indicated that a decline in muscle mass decreases the body's capacity to maintain health and respond to various illnesses.

The progressive loss of muscle mass combined with reduced muscle strength and altered muscle composition are symptoms of sarcopenia. Sarcopenia is a progressive disorder that is typically determined by ageing but can also occur in association with inflammatory diseases such as cancer [23,24]. This condition that affects the function of skeletal muscle is a public health issue since it leads to adverse outcomes such as frailty, fractures, disability, reduced quality of life, elevated risk of falls and increased mortality [25]. Age-related sarcopenia is a process that often begins after the age of 40, with the most severe symptoms occurring after the age of 65 [26]. The mechanisms that contribute to age-associated sarcopenia are multifactorial and include hormonal changes, anabolic resistance, reduced physical activity levels, imbalanced protein turnover and the transition of muscle fibre types (i.e., decrease in the number and size of type II fibres) [26,27]. At the cellular level, age-related sarcopenia can impair and delay the differentiation of myoblasts into myotubes via altered signalling pathways, reduced availability of satellite cells and the presence of chronic inflammation [28]. Remarkably, more than half of the individuals diagnosed with cancer suffer from cancer-related sarcopenia. Since cancer predominantly

develops in the elderly, older individuals with cancer face a dual risk as they are vulnerable to the adverse outcomes associated with both conditions (i.e., cancer and sarcopenia) [25]. In addition to the age-dependent changes, the factors that contribute to the development of cancer-related sarcopenia are cancer-induced inflammation, the side effects of cancer treatments, malnutrition and increased sedentarism (e.g., attributable to prolonged periods of bed rest) [7]. Due to the additive effects of ageing and cancer, cancer-associated sarcopenia is more severe and develops quicker than age-related sarcopenia. Moreover, it can lead to treatment intolerance, a higher risk of complications during therapy, reduced immune function and increased mortality rates [14,29]. From a molecular perspective, the metabolic alterations caused by the tumour and the presence of additional inflammatory markers (e.g., pro-inflammatory cytokines) could affect myoblasts' differentiation and thus, muscle physiology. It can be argued that cancer-associated muscle loss (as opposed to age-related sarcopenia) has a further negative impact on skeletal muscle cell differentiation. Yet, the available literature is limited and further research should elucidate the cellular differences between age- and cancer-specific muscle loss. To conclude, both types of sarcopenia (i.e., age- and cancer-associated) involve the loss of skeletal muscle mass, strength and function. Nevertheless, the progression rate and the clinical manifestations of sarcopenia are exacerbated by the presence of a tumour.

### 1.3. (Adipo)cytokines

As previously highlighted, several studies [14,30,31] indicated that cytokines might be involved in the formation and development of cancer cachexia. Cytokines are small soluble proteins that can be produced by a variety of cells (e.g., T lymphocytes, macrophages, mast cells, endothelial cells) and are involved in several physiological processes such as immune function, inflammation, and metabolism. They regulate the equilibrium between cell-based and humoral immune responses and contribute to cell growth, maturation and responsiveness [32,33]. The same cytokine can be produced by distinct types of cells and the effect of a given cytokine may be different depending on the targeted cell [32,33,34]. Furthermore, cytokines form complex networks and can affect the same biological process synergistically, antagonistically or additively. They are versatile molecular agents, with both pro-inflammatory and anti-inflammatory functions, that can have paracrine, endocrine and autocrine action [32,34]. Briefly, pro-inflammatory cytokines such as interleukin-1 $\beta$  (IL-1 $\beta$ ), IL-6, tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ) or interferon- $\gamma$  (IFN-  $\gamma$ ) stimulate inflammatory reactions, while anti-inflammatory cytokines (e.g., IL-4, IL-10, IL-1ra) have a role in immunoregulation since they modulate pro-inflammatory responses [21]. Notably, certain cytokines (e.g., IL-6, TGF- $\beta$ , IFN-  $\gamma$ ) can be categorised as both pro- and anti-inflammatory depending on the biological context. The abnormal production of cytokines and any imbalance between pro- and anti-inflammatory cytokines can have negative effects on an individual's health status [34] and consequently, these proteins can be key indicators of various clinical disorders (e.g., cancer, sepsis, acquired immune deficiency syndrome).

Adipocytokines (also known as adipokines) are cytokines produced in the adipose tissue primarily by adipocytes and preadipocytes, but also by immune cells that infiltrated the adipose tissue (e.g., macrophages) or endothelial cells [35,36]. Adipokines such as adiponectin, leptin, resistin or visfatin can influence appetite, inflammation, metabolism, body weight, blood pressure or glucose homeostasis in an autocrine, paracrine or endocrine manner [37]. More recent research evaluated other adipokines such as intelectin-1 (ITLN1), lipocalin-2, vaspin or chemerin and suggested that they can contribute to the interactions between the neuroendocrine and the immune systems as signal molecules, regulate insulin resistance or act as immunomodulatory agents [36]. Additionally, adipocytes and immune cells from the stromal vascular fraction can release

pro- and anti-inflammatory cytokines such as IL-6 and TNF- $\alpha$  [35,36]. As summarised by Mancuso [35], adipokines facilitate the crosstalk between various organ systems and adipose tissue. During caloric restriction, there is a reduction in the level of pro-inflammatory adipokines, while the concentration of anti-inflammatory adipokines increases; this contributes to a decline in immune function and elevated insulin sensitivity. Conversely, in the context of energy excess, anti-inflammatory adipokines levels decrease and the presence of pro-inflammatory adipokines is elevated; this affects glucose homeostasis and leads to inflammation, both locally and systemically. Similar to the 'classical' cytokines that were previously discussed, the balance between pro-inflammatory (e.g., leptin, resistin) and anti-inflammatory (e.g., adiponectin) adipokines can influence inflammation and the development of certain inflammatory conditions such as cancer [37].

The role of multiple (adipo)cytokines in carcinogenesis as well as the relationship between these and cancer development was established by previous literature. IL-6 was identified as an inflammatory cytokine with anti-apoptotic and growth-promoting effects in cancer [38]. A literature review [39] suggested that IL-1, IL-6, TNF and IFN- $\gamma$  induce cell proliferation and induce tumour development through NF- $\kappa$ B signalling. Moreover, TGF- $\beta$ , vascular endothelial growth factor (VEGF) and IL-8 secreted by cancer cells as well as fibroblasts, endothelial cells and tumour-associated macrophages in the tumour microenvironment promote angiogenesis [39]. Other research [40] evaluated the role of TNF- $\alpha$  and highlighted the cytokine's ability to enhance cell proliferation and promote angiogenesis. As opposed to the previously mentioned cytokines that were thoroughly examined by past research, ITLN1 is an adipokine that was not extensively analysed and might have a role in cancer and cancer-associated cachexia. Indeed, previous studies [41,42,43] suggested that ITLN1 concentrations are different between cancer patients and healthy individuals. Moreover, recent work conducted by our research group [44] indicated that ITLN1 was elevated in the visceral adipose tissue (VAT) of cachectic patients compared to healthy individuals. Therefore, the subsequent section will analyse the available literature, discuss the potential roles of ITLN1 in cancer, examine its mechanisms of action and propose pathways for further research.



## 1.4. The emerging role of intelectin-1 in cancer

This section was published in the journal *Frontiers in Oncology* as:

*Paval, D.R., Di Virgilio, T.G., Skipworth, R.J.E., Gallagher, I.J., 2022. The Emerging Role of Intelectin-1 in Cancer. Frontiers in Oncology 12. DOI:10.3389/fonc.2022.767859.*

### 1.4.0. Abstract

**Background:** Intelectin (ITLN) is an adipokine with two homologs – ITLN1 and ITLN2 – that has various physiological functions. Studies analysing the relationship between ITLN and cancer are focused on ITLN1; the available literature on ITLN2 and cancer is limited. This review aims to evaluate the role of ITLN1 in cancer without imposing any inclusion criteria, to examine pro- and anti-cancer roles for ITLN1 and to discuss whether the relationship between ITLN and cancer is mediated by obesity.

**Findings:** Overall, ITLN1 level was highly variable in cancer patients but different from healthy individuals. Compared to control groups, patients with gastrointestinal and prostate cancer showed increased concentrations of circulating ITLN1, while patients with gynaecological, breast, bladder and renal cancer had lower ITLN1 levels. Several studies also evaluated tissue and tumour expression of ITLN1. In gastrointestinal cancer, ITLN1 was increased in tumour tissue compared to adjacent healthy tissue and elevated in the visceral adipose tissue of patients compared to controls. Consequently, the high levels of circulating ITLN1 might be determined by the tumour and the cancer-associated weight loss in gastrointestinal cancer. ITLN1 can activate the phosphoinositide-3-kinase-protein kinase B/Akt (PI3K/Akt) pathway. The improper regulation of this pathway may contribute to a series of cellular events that favour tumour development and progression. Obesity has been linked with an increased risk of developing some cancers. Indeed, low circulating ITLN1 levels may be a marker of the metabolic effects of obesity, rather than obesity per se, and might contribute to a deregulation of the PI3K/Akt pathway.

**Conclusions:** ITLN1 could be associated with cancer formation and progression. Since circulating ITLN1 levels are highly variable and differ between cancer types, the local tumour production of ITLN1 could be more relevant in determining malignant behaviour.

Future research should aim to identify the source of ITLN1 variability, to understand the differences in ITLN1 between distinct tumour types and to further explore the signalling pathways through which this adipokine influences cancer biology.

### 1.4.1. Introduction

Intelectin (ITLN), also known as omentin, is a 34KDa lectin that contains a fibrinogen-like domain and a unique intelectin-specific region that makes it distinct from other immune lectins [45,46]. There are two homologs with 83% amino acid identity termed intelectin-1 (omentin-1) and intelectin-2 (omentin-2). ITLN is mainly produced by stromal vascular fraction cells, but not by adipocytes, in VAT, while its levels are very low in subcutaneous adipose tissue (SAT) [47]. Moreover, ITLN1 expression has also been found in epicardial fat, the small intestine, colon, ovary, lungs and renal collecting tubes, whereas ITLN2 is expressed in intestinal Paneth cells [46,48,49]. Both ITLN homologs can bind microbial glycan chains but not human glycans and thus, the adipokine may have a role in anti-microbial defence [46,50]. Previous work has also identified possible roles for ITLN1 in polyspermy prevention and iron metabolism via interaction with lactoferrin [46].

Interest in the role of ITLN in cancer has been driven by the observation that ITLN1 levels differ between healthy individuals and patients with various types of cancer [41,42,43,51]. We recently identified ITLN1 and ITLN2 mRNA as increased in the VAT of gastrointestinal cancer patients and found that local but not circulating ITLN1 protein demonstrated a relationship with cancer cachexia [44]. It has also been suggested that the relationship between ITLN1 and cancer might be influenced by overweight and obesity [52]. Collectively, these findings indicate that ITLN might have a role in cancer biology and that it could be used as a biomarker for cancer itself or cancer progression. Yet, the exact mechanisms through which this adipokine induces physiological changes are not completely understood and the variable levels of ITLN described in the literature have not been fully explained by previous research. Therefore, we will review the available literature on ITLN in cancer without imposing any inclusion criteria, discuss proposed pro- and anti-cancer roles for ITLN and finally analyse whether the relationship between ITLN and cancer is mediated by BMI. Furthermore, this review will identify current knowledge gaps and propose pathways for future work. Most of the studies analysing the relationship between ITLN and cancer focus on ITLN1. Although ITLN2 is likely to influence various physiological processes, there are little published data on this topic and therefore, the present review will focus on ITLN1.

## 1.4.2. Discussion

### *ITLN1 & Gastrointestinal Cancers*

A recent systematic review examining circulating ITLN1 levels in cancer found that ITLN1 was often increased in individuals with colorectal cancer compared to healthy controls [52]. Another study evaluating a cohort of Chinese patients identified increased ITLN1 levels as a potential risk factor for colorectal cancer [53]. Higher levels of circulating ITLN1 were also found to increase the probability of recurrence or death in colorectal cancer after surgery; yet, the difference in circulating ITLN1 between the groups was very small [54]. A study by Uyeturk and colleagues [55] examined circulating ITLN1 levels after surgery and chemotherapy in colorectal cancer and found that the levels remained elevated compared to a healthy control group who did not have surgery or chemotherapy. However, the reported range of results in this study was very low (pg/ml). Circulating ITLN1 levels also seem to be increased in pancreatic cancer patients compared to healthy controls [42,56]. In comparison, two studies have found that higher tumour expression (rather than circulating levels) of ITLN1 was associated with a good prognosis in colorectal cancer [57,58]. Unfortunately, circulating ITLN1 was not assessed in these studies. Zheng and colleagues [59] emphasised that ITLN1 levels were greater in gastric cancer tissue compared to normal gastric mucosa. We previously identified higher ITLN1 expression in the VAT of patients with upper gastrointestinal cancer compared to healthy controls, whilst expression of ITLN1 mRNA in SAT and circulating ITLN1 levels did not differ between groups [44].

Although the evidence is not strong enough to generate a definitive conclusion, it can be argued that ITLN1 expression is increased locally and perhaps systemically in gastrointestinal cancers. The elevated circulating levels of this adipokine might be partly determined by the tumour [60] and the degree of cancer-associated weight loss [44,61]. Whilst the function of ITLN1 in these cancers is unknown, some of the evidence presented above would suggest that higher local tumour levels may be a good prognostic indicator. However, the reported circulating levels of ITLN1 are extremely variable. Thus, whilst ITLN1 may have potential as a biomarker for the diagnosis or progression of gastrointestinal cancers, circulating levels may be too variable to be useful. This question should be investigated with appropriately powered studies.

### ***ITLN1 & Urological Cancers***

Levels of ITLN1 are also dysregulated in prostate cancer. Arjmand and colleagues [52] suggested that prostate cancer patients had significantly higher circulating ITLN1 levels compared to control groups in all the studies included in their review. Although the stated aim of their review was to compare cancer patients with healthy controls, not all of the included studies respected this criterion. In the prostate cancer subgroup analysis, two studies compared cancer patients against individuals with benign prostatic hyperplasia (BPH). In both studies, circulating ITLN1 was higher in prostate cancer than in BPH [62,63]. Similarly, a more recent study observed that prostate cancer patients had greater concentrations of circulating ITLN1 compared to individuals with BPH [64]. Zhang et al. [65] examined circulating ITLN1 levels in bladder cancer and found reduced levels in patients compared to healthy controls. However, the reported ITLN1 levels were outside the assay range indicated in the study's methodology. Circulating ITLN1 was also lower in patients with renal cell carcinoma than in matched controls [66]. In summary, the available literature suggests that circulating ITLN1 is elevated in prostate cancer and reduced in bladder and renal cancer. Further evidence is required to strengthen these interim conclusions.

### ***ITLN1, Breast & Gynaecological Cancers***

Three reports examined circulating levels of ITLN1 in breast cancer compared to healthy controls. Patients with breast cancer had lower circulating ITLN1 levels in all three studies [43,67,68]. Tahmasebpour and colleagues [43] also observed that ITLN1 gene expression was significantly downregulated in breast cancer tissue compared to adjacent normal tissue. Several studies assessed the relationship between ITLN1 and gynaecological cancers. In a subgroup meta-analysis by Arjmand et al. [52], circulating ITLN1 did not significantly differ between women with ovarian cancer and healthy controls. Another study evaluating the same topic [69] indicated that circulating ITLN1 was lower in patients with high-grade ovarian cancer as opposed to healthy women and women with benign gynaecological disease. Moreover, these authors observed that ITLN1 mRNA was expressed at a lower level in the omental adipose tissue of patients with high-grade ovarian cancer compared to women with benign disease [69]. Interestingly, this finding contradicts our study in upper gastrointestinal cancer [44] where disease severity increased omental

ITLN1 expression. Holman et al. [70] and Cymbaluk-Ploska et al. [71] also observed lower circulating ITLN1 in women with endometrial cancer. Although both studies were analysed in the systematic review by Arjmand and colleagues [52], only Holman et al. [70] compared cancer patients with healthy controls. The comparison group from the other study [71] included women with endometrial polyps (benign endometrial changes). In summary, despite the fact that only a limited amount of data were available, a tendency for lower circulating ITLN1 levels in breast and gynaecological cancers was observed. Future studies should aim to explore this relationship and develop a better understanding of the role of ITLN1 in these types of cancer.

### ***Other Cancers***

In an examination of 42 neuroblastoma tumours, Li et al. [72] found that ITLN1 protein was expressed in 33% of the tumour specimens with variable degrees of staining, from weak to intense. Higher levels of ITLN1 staining tended to occur in tumours with more favourable features [72]. Furthermore, data mining of the R2 microarray genomics and visualisation resource suggested that higher ITLN1 mRNA expression was associated with a greater probability of survival [72]. Another study noted a lower level of circulating ITLN1 in Iranian male smokers with lung cancer compared to apparently healthy smokers. Notably, circulating ITLN1 in smokers with lung cancer was not different from that of healthy non-smokers [73]. An in-silico meta-analysis of RNA-Seq data noted that ITLN1 mRNA expression was consistently lower in lung tumours compared to healthy lung tissue across 7 data sets and that higher ITLN1 mRNA levels in tumours predicted better survival [74]. In malignant pleural mesothelioma (MPM), early work by Wali et al. [75] found that ITLN1 mRNA and protein were highly expressed in tumours as opposed to normal tissue. Subsequently, increased expression of ITLN1 was observed in mesothelioma cell lines and epithelioid mesotheliomas [76,77,78]. ITLN1 levels were elevated in the pleural fluid of MPM patients compared to the levels seen in lung cancer, tuberculosis or pneumonia [76]. The mean differences were driven by a few individuals with very high levels of pleural ITLN1 (>3000ng/ml), while plasma ITLN1 concentrations did not differ between MPM patients and healthy controls [76]. Although immunoreactivity for ITLN1 in epithelioid mesothelioma tumours was very strong, ITLN1 seemed to be absent from other tumours (both mesothelioma and non-mesothelioma) unless they were mucus-producing

[76,77,78]. Overall, the available data on the previously discussed types of cancer are limited but generate pathways for further research. The direction of the difference in circulating ITLN1 between cancer patients and control groups depends on cancer type. Similarly, different tumours were found to express distinct concentrations of ITLN1. Table 1.2 includes a summary of the studies from this review that measured ITLN1 in human participants diagnosed with cancer. The source of ITLN1 variability between different types of cancer should be one of the main areas explored by future studies.

**Table 1.2.** Summary table - characteristics of studies that examined human participants with different types of cancer

Study (year)	Cancer type	Participants	Findings	ITLN1 source	ITLN1 level in cancer
<b>Gastrointestinal cancers</b>					
Zhao et al. (2019) [53]	Colorectal	358 patients 286 controls	Increased ITLN1 associated with a higher risk of developing colorectal cancer.	Circulating	↑
Feng et al. (2020) [54]	Colorectal	319 patients 300 controls	Higher levels of ITLN1 increase the probability of recurrence or death after surgery. The difference between patients and controls was small.	Circulating	↑
Um. Uyeturk et al. (2014) [55]	Colorectal	45 patients 35 controls	Increased ITLN1 levels after surgery and chemotherapy compared to a healthy control group.	Circulating	↑
Karabulut et al. (2016) [42]	Pancreatic	33 patients 30 controls	ITLN1 levels increased in patients compared to healthy controls.	Circulating	↑
Kiczmer et al. (2018) [56]	Pancreatic	20 patients 18 controls	ITLN1 elevated in patients compared to healthy controls.	Circulating	↑
Miller et al. (2020) [44]	Upper gastrointestinal	16 patients 8 controls	ITLN1 levels did not differ between groups.	Circulating	↔
			Higher ITLN1 expression in the visceral adipose tissue of patients compared to healthy controls. No difference in subcutaneous adipose tissue expression.	Tissue	↑
Kim et al. (2012) [57]	Colorectal	6 patients	Higher tumour expression of ITLN1 was associated with good prognosis.	Tissue	↓
Katsuya et al. (2020) [58]	Colorectal	148 patients	Increased tumour expression of ITLN1 was associated with good prognosis.	Tissue	↓
Zheng et al. (2012) [59]	Gastric	196 patients	ITLN1 level was greater in gastric cancer tissue compared to normal gastric mucosa.	Tissue	↑



### Urological cancers

Ug. Uyeturk et al. (2014) [62]	Prostate	50 patients 30 controls	ITLN1 was higher in patients than in controls suffering from benign prostate hyperplasia.	Circulating	↑
Fryczkowski et al. (2018) [63]	Prostate	40 patients 40 controls	ITLN1 was higher in patients compared to controls with benign prostate hyperplasia.	Circulating	↑
Borowski and Sieminska (2020) [64]	Prostate	72 patients 65 controls	Patients had greater ITLN1 concentrations compared to individuals with benign prostate hyperplasia.	Circulating	↑
Zhang et al. (2016) [65]	Bladder	42 patients 42 controls	ITLN1 levels were decreased in patients compared to healthy controls.	Circulating	↓
Shen et al. (2016) [66]	Renal cell carcinoma	41 patients 42 controls	ITLN1 levels were lower in patients as opposed to healthy controls.	Circulating	↓

### Breast and gynaecological cancers

Alaee et al. (2016) [67]	Breast	30 patients 30 controls	Patients with breast cancer had lower ITLN1 levels compared to healthy controls.	Circulating	↓
Nourbakhsh et al. (2018) [68]	Breast	45 patients 45 controls	Patients with breast cancer had lower ITLN1 levels compared to healthy controls.	Circulating	↓
Holman et al. (2014) [70]	Endometrial	74 patients 74 controls	Lower ITLN1 levels in patients compared to healthy controls.	Circulating	↓
Cymbaluk-Ploska et al. (2018) [71]	Endometrial	92 patients 76 controls	Decreased ITLN1 in patients compared to controls. The control group included 32 women with endometrial polyps and 44 with normal endometrium.	Circulating	↓
Tahmasebpour et al. (2002) [43]	Breast	88 patients 86 controls	Patients had lower ITLN1 levels compared to healthy controls.	Circulating	↓
			ITLN1 gene expression was significantly downregulated in breast cancer tissue compared to adjacent normal tissue	Tissue	↓
Au-Yeung et al. (2020) [69]	Ovarian	147 patients 147 controls	ITLN1 was lower in patients as opposed to healthy women (n=99) and women with benign gynaecological disease (n=48).	Circulating	↓
			ITLN1 mRNA was expressed at a lower level in the omental adipose tissue of patients compared to women with benign disease.	Tissue	↓

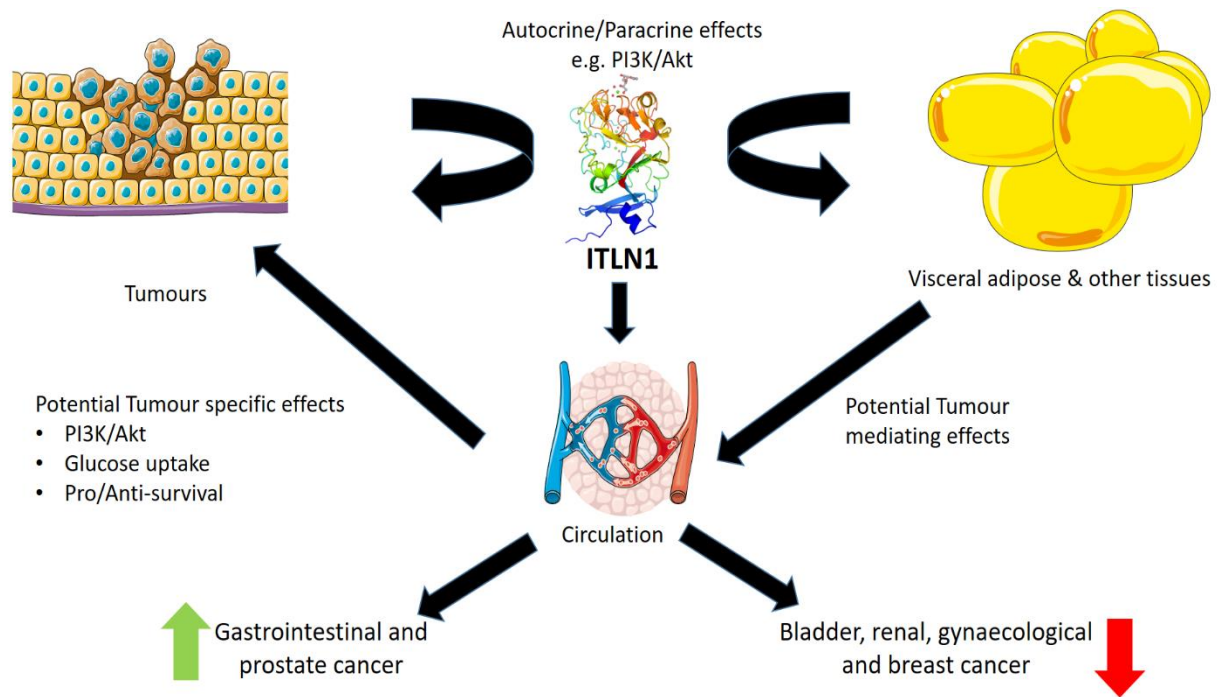
**Other cancers**

Ansari et al. (2018) [73]	Lung	45 patients 61 controls	ITLN1 levels did not differ between patients and healthy non-smokers.	Circulating	↔
Tsuji et al. (2010) [76]	Malignant pleural mesothelioma	8 patients	ITLN1 concentrations did not differ between patients and healthy controls.	Circulating	↔
Li et al. (2015) [72]	Neuroblastoma	42 patients	Higher levels of ITLN1 observed in tumours with more favourable characteristics. Higher ITLN1 mRNA expression associated with a greater probability of survival.	Tissue	↓

The arrows are used to indicate the level of ITLN1 in patients (or cases with a worse prognostic/ higher risk of cancer) compared to controls (or cases with improved prognostic/ lower risk of cancer).

### ***Mechanisms for Intelectin Effects in Cancer***

Figure 1.1 highlights the potential roles of ITLN1 in cancer and its probable mechanisms of action. Previous work has indicated that ITLN1 can influence Akt-mediated growth pathways. The phosphoinositide-3-kinase-protein kinase B/Akt (PI3K/Akt) signal transduction pathway has a crucial role in various cellular functions that contribute to cancer, including metabolism, growth, motility, proliferation, and angiogenesis [51]. ITLN1 increases Akt phosphorylation in adipocytes, osteoblasts and mesenchymal cells [47,79,80,81]. Wu and colleagues [80] also indicated that the inhibition of Akt prevents the proliferative effect of ITLN1 in osteoblasts, suggesting that ITLN1 signals through the Akt pathway in these cells. In mesenchymal stem cells, ITLN1 also leads to Akt-mediated proliferation, resistance to oxidative stress and secretion of pro-angiogenic factors [81]. These actions may be particularly important in tumours as mesenchymal stem cells are part of the tumour microenvironment and can favour tumour growth [82]. Activation of mesenchymal gene programmes is also a characteristic of advanced tumours [83]. Overall, these findings suggest that ITLN1 could locally potentiate PI3K/Akt signalling and glucose uptake in cancer cells to promote survival. The ITLN1/TMEM207 axis is another pathway that may influence carcinogenesis [84]. TMEM207 is a transmembrane protein that has a role in ITLN1 processing. It was suggested that low levels of TMEM207 contribute to a decrease in ITLN1 concentrations and consequently, promote colorectal carcinogenesis [84,85]. The available research on this pathway is still limited and future studies are needed to confirm the relationship between TMEM207 and ITLN1. Given that circulating ITLN1 varies between different types of cancer, the local level or the tumour production of ITLN1 may be more important than the circulating level in contributing to malignant behaviour.



**Figure 1.1.** Potential roles for intelectin-1 (ITLN1) in cancer. ITLN1 produced by tumours may have pro- or anti-survival autocrine and/or paracrine effects on these tumours. Simultaneously, ITLN1 produced by normal tissues such as the visceral adipose tissue or the pre-malignant/malignant tissue may enter the circulation and affect tumour biology via PI3/Akt or other pathways. Circulating ITLN1 is reduced with increased adiposity and therefore, adiposity may act as a mediator of ITLN1's effects on tumours or tumour formation. Results in the literature suggest that, as a biomarker, circulating ITLN1 is increased in gastrointestinal and prostate cancer and decreased in bladder, renal, gynaecological and breast cancer. The clinical relevance of these findings requires further investigation. Image credits: ITLN1 structure – Kwangkanont on Wikimedia, CC-BY-SA; other images provided by Servier Medical Art under CC.BY 3.0. <https://smart.servier.com>

Conversely, other groups have found that ITLN1 may play an anti-tumour role. In colorectal cancer cell lines, the reduction of ITLN1 expression by short-hairpin RNA increased cell growth and proliferation as well as Akt, extra signal-regulated kinase (ERK) and epidermal growth factor receptor (EGFR) phosphorylation [58]. ITLN1 treatment reduced the proliferation and the migratory ability of gastric cancer cells by upregulating hepatocyte nuclear factor 4 $\alpha$  (HNF4a) via increased inhibition of nuclear factor kappa B (NF $\kappa$ B) [86]. Furthermore, a supraphysiological dose of ITLN1 (1-2 $\mu$ g/ml) led to the arrest of cell cycling and subsequent apoptosis in hepatocellular carcinoma cell lines [87]. Supraphysiological

ITLN1 also inhibited proliferation and promoted apoptosis of colon cancer stem cells in a time-dependent manner [88]. In neuroblastoma cell lines and human tumours, increased ITLN1 seemed to have a PI3K/Akt-mediated protective effect by increasing the expression of N-myc downstream-regulated gene 2 (NDRG2) and by reducing tumour volume and metastatic potential [72]. Notably, higher ITLN1 expression in human neuroblastoma tumours improved the probability of survival [72]. Similarly, higher tumour expression of ITLN1 was associated with improved prognosis in patients with colorectal cancer [57,58]. These results suggest that ITLN1 may have potential as an anti-cancer agent. However, the levels of ITLN1 used in many of these studies were in the ug/ml range whereas the normal physiological concentration of ITLN1, even in cancer or cell culture supernatant, is in the ng/ml range. Therefore, there may be unanticipated physiological effects if elevated levels of ITLN1 are used as an anti-cancer agent.

### ***Intelectin, Obesity and Cancer Risk***

VAT is the primary source of ITLN suggesting that the relationship between this adipokine and various cancer mechanisms could be mediated by BMI or adiposity. The presence of excess adipose tissue alters adipokine production which may contribute to the development of cancer, indicating a potential role of ITLN1 [84,89]. Also, it has been previously demonstrated that obese individuals present a higher risk of developing cancer compared to people with normal weight [90]. Obesity has been linked to lower levels of circulating ITLN1 in several studies [49,91]. However, low circulating ITLN1 levels in people with impaired glucose regulation and untreated type 2 diabetes [92], women with metabolic syndrome secondary to polycystic ovary syndrome [93], Japanese men with a higher number of metabolic risk factors [94] as well as in women with gestational diabetes and BMI < 30 [92] suggests that low ITLN1 may be a marker for the metabolic effects of obesity rather than obesity itself. Although a recent meta-analysis of studies examining the relationship between body weight and circulating ITLN1 levels found an overall effect, there was very high study heterogeneity (93%) and evidence of publication bias [95]. Furthermore, subgroup analysis revealed that higher-quality studies suggested no relationship and that serum ITLN1 level was significantly lower in overweight but not obese individuals [95]. In summary, the available evidence suggests that circulating ITLN1 may be a marker of metabolic dysregulation rather than overweight/obesity per se.

In their review, Arjmand and colleagues [52] found no overall relationship between circulating ITLN1 and cancer. As we note above evidence suggests that the direction of change for circulating ITLN1 may be different in the presence of different tumours. Notably, levels of circulating ITLN1 were consistently higher than control groups in gastrointestinal and prostate cancer and lower than control groups in renal, lung, bladder, endometrial and breast cancer [52]. Arjmand et al. [52] also suggested that ITLN1 levels were higher in studies examining cancer patients with a mean BMI>25 as opposed to studies that included individuals with an average BMI<25. This could indicate an interaction between cancer presence, adiposity and ITLN. However, as noted above, the evidence base for an association between circulating ITLN1 and overweight/obesity is weak [95]. Arjmand et al. [52] also grouped studies based on average BMI values but did not consider the variation around the mean for several studies [41,51,62,66,73,96]. Not all of the patients included in these studies had a BMI>25 according to their published variance statistics. Including these studies in the 'BMI>25' subgroup therefore does not represent a valid statistical approach. Thus, tumour-specific directional changes in circulating ITLN1 levels, local versus global changes in ITLN1 as well as aggregate views of overweight/obesity or metabolic status in patient groups could lead to misinterpretations of the relationship between circulating ITLN1 and cancer risk or status.

### ***Future Directions***

Changes in circulating ITLN1 levels have been associated with several cancers. Tumour site and/or type seems to be important in the direction of the change. Further work is required to identify whether ITLN1 has utility as a biomarker for cancer occurrence or re-occurrence. This work would have to further confirm and take account of the possible tumour-specific direction of change in ITLN1. As we noted in our work on adipose tissue in upper gastrointestinal cancer, increased production of VAT ITLN1 is a feature of cancer-induced weight loss [44]. Future studies with larger cohort sizes should also investigate whether cancer-induced weight loss leads to increased circulating ITLN1. The mechanisms by which ITLN1 may lead to either positive or negative changes in cancer also deserve research attention. Further work on the signalling pathways and the roles of ITLN1 in both normal and dysregulated metabolism should help identify the mechanisms through which this adipokine influences tumour biology and the metabolic consequences of cancer.

### **1.4.3. Conclusion**

The available literature indicates that ITLN1 might have a role in cancer formation and development since ITLN1 level was highly variable but different from healthy controls when patients with various cancer types were examined. High concentrations of ITLN1 were found in patients with gastrointestinal [42,53] and prostate cancer [63] compared to control groups. Conversely, women with breast [67] and gynaecological cancer [69] expressed less ITLN1 as opposed to healthy individuals. Although these are relevant observations, a meta-analysis with a reliable methodological approach is required to quantify ITLN1 levels in cancer patients and healthy participants. Most available studies, including a recent systematic review [52], only measured differences between groups. To date, no study discussed the variability in ITLN1 levels observed in both healthy and diseased individuals. For instance, in the review by Arjmand et al. [52] mean ITLN1 varied from 1.8 to 618.0 ng/ml in the cancer groups, while the range in the control groups was 1.6 to 756.4 ng/ml. Therefore, the source of ITLN1 variability and its physiological concentration should be determined by future research.

Several studies [80,81] assessed ITLN1's mode of action and observed that it activates the PI3k/Akt pathway. The improper regulation of this pathway could lead to the proliferation of mesenchymal cells that favour the progression and development of cancer. Low ITLN1 levels are a marker of the metabolic effects of obesity and might contribute to a deregulation of the PI3k/Akt pathway. Otherwise, local changes in ITLN1 could favour a pro-tumour environment. Further research is required to clarify the role of ITLN1 in carcinogenesis and to describe the pathways through which this adipokine interacts with other cells. Moreover, future studies should aim to assess ITLN1 expression in cancer patients by measuring its concentration in blood, cancer tissue as well as SAT and VAT. Animal models and in vitro investigations might focus on determining the direction of the causal relationship between ITLN1 and cancer. This area of research has been emerging in recent years and numerous promising pathways can be explored.

## 1.5. Aims and objectives

Besides their role in cancer, cytokines and adipokines could be involved in the pathophysiology of cancer cachexia. The relationship between (adipo)cytokines and the cachectic phenotype was discussed by previous literature [18,31,97,98]. Numerous studies, including cell and animal models, examined this relationship but no definitive conclusions have been agreed upon. The (adipo)cytokine network is complex and more research is required to better map its influence on cancer cachexia. To date, no systematic review analysed the relationship between cytokines and cancer cachexia and this is a fundamental step towards progress in this field. Furthermore, several studies [11,53,64] proposed ITLN1 as an adipokine with a potential role in cancer and cancer cachexia. However, there is still scope for additional research as ITLN1's behaviour in different types of cancer as well as its mechanisms of action remain unclear. Therefore, the current thesis aims to:

- evaluate the relationship between (adipo)cytokines and cancer cachexia in people with incurable cancer by conducting a systematic review of the literature (Chapter 2);
- further explore the relationship between adipokines (i.e., adiponectin, leptin, resistin, ITLN1) and the cachectic phenotype by analysing data collected from a cohort of patients with incurable cancer (Chapter 3);
- examine the role of ITLN1 in cancer and its physiological levels by conducting a systematic review and a Bayesian meta-analysis of all available data (Chapter 4);
- investigate the effect of different ITLN1 doses on human skeletal muscle myotubes by assessing the global transcriptome, gene expression, protein synthesis and glucose uptake (Chapter 5).



## 2. A systematic review examining the relationship between cytokines and cachexia in incurable cancer

This chapter was published in the *Journal of Cachexia, Sarcopenia and Muscle* as:

Paval, D.R., Patton, R., McDonald, J., Skipworth, R.J.E., Gallagher, I.J., Laird, B.J., Collaborative, the C.C., 2022. A systematic review examining the relationship between cytokines and cachexia in incurable cancer. *Journal of Cachexia, Sarcopenia and Muscle* DOI: 13, 824–838. DOI: 10.1002/jcsm.12912.

### 2.0. Abstract

**Background:** Cancer cachexia is an unmet clinical need that affects more than 50% of patients with cancer. The systemic inflammatory response, which is mediated by a network of cytokines, has an established role in the genesis and maintenance of cancer as well as in cachexia; yet, the specific role of the cytokine milieu in cachexia requires elucidation. This systematic review aims to examine the relationship between cytokines and the cachexia syndrome in patients with incurable cancer.

**Methods:** The databases MEDLINE, EMBASE, CINAHL, CENTRAL, PsycINFO and Web of Science were searched for studies published between 01/01/2004 and 06/01/2020. Included studies measured cytokines and their relationship with cachexia and related symptoms/signs in adults with incurable cancer. After title screening (n=5202), the abstracts (n=1264) and the full-text studies (n=322) were reviewed independently by two authors. The quality assessment of the selected papers was conducted using the modified Downs and Black checklist.

**Results:** Overall, 1277 patients with incurable cancer and 155 healthy controls were analysed in the 17 eligible studies. The mean age of the patients was 64±15 (mean ± standard deviation). Only 34% of included participants were female. The included studies were assessed as moderate-to-high quality evidence (mean quality score: 7.8; range: 5-10). A total of 31 cytokines were examined in this review, of which IL-6 (14 studies) and TNF-α (12 studies) were the most common. The definitions of cachexia and the weight-loss thresholds were highly variable across studies. Although the data could not be meta-

analysed due to the high degree of methodological heterogeneity, the findings were discussed in a systematic manner. IL-6, TNF- $\alpha$  and IL-8 were greater in cachectic patients compared to healthy individuals. Also, IL-6 levels were higher in cachectic participants as opposed to non-cachectic patients. Leptin, interferon- $\gamma$  (IFN- $\gamma$ ), IL-1 $\beta$ , IL-10, adiponectin and ghrelin did not demonstrate any significant difference between groups when individuals with cancer cachexia were compared against non-cachectic patients or healthy participants.

**Conclusions:** These findings suggest that a network of cytokines, commonly IL-6, TNF- $\alpha$  and IL-8 are associated with the development of cachexia. Yet, this relationship is not proven to be causative and future studies should opt for longitudinal designs with consistent methodological approaches, as well as adequate techniques for analysing and reporting the results.

## 2.1. Introduction

Cancer cachexia is a complex syndrome characterised by the loss of skeletal muscle mass – with or without loss of fat mass – which cannot be fully reversed using standard nutritional care [7]. This multifactorial syndrome that leads to progressive functional impairment occurs at different rates depending on the type of cancer, affects more than 50% of the patients and accounts for 20% of cancer-related deaths [99]. Furthermore, it has been established that cachexia diminishes the effectiveness of anti-cancer treatments [100] and negatively affects patients' quality of life [101]. To date, there is no licensed treatment and no standard of care [12].

Cancer cachexia results from a combination of reduced energy intake, excess energy expenditure, elevated catabolism and increased systemic inflammation [14]. Previous research suggested that the systemic inflammatory response has a role in the progression of both cancer [102] and cancer-related cachexia [14,103].

Inflammation is mediated by a network of pro- and anti-inflammatory cytokines that are normally in equilibrium. In the cancer state, the equilibrium is disrupted, resulting in a dysfunctional state of simultaneous immune stimulation and suppression [104]. Cytokines operate both within the tumour microenvironment and by interacting with other tissues in the body to generate a systemic response [105]. Indeed, a considerable amount of evidence indicates the contribution of cytokines in cellular events that determine the initiation, promotion, invasion and metastasis of cancer [106]. Similarly, Fearon and colleagues [107] highlighted that the production rate of several cytokines is associated with the prevalence of cachexia in multiple types of cancer. Even though cytokine levels were correlated with cancer and cachexia in numerous studies, the mechanisms through which these substances act upon the tumour and other body systems are not completely understood.

Multiple systematic reviews [108,109] have evaluated the relationship between cytokines and cancer. Likewise, the role of cytokines in cachexia was previously examined [67,107,110], but none of the investigations used a systematic approach to appraise the available evidence. Moreover, very few studies [111] assessed the relationship between cytokines and cachexia in individuals suffering from incurable cancer. If the relationship between cytokines and the development of cancer cachexia was elucidated, this may identify key therapeutic targets that could be translated into clinical therapies. To date, no systematic review evaluated the relationship between cytokines and cachexia in patients

with cancer. Therefore, this systematic review aimed to explore the relationship between cytokines and the cachexia syndrome (including related symptoms such as weight loss, anorexia and reduced physical function) in people with incurable cancer.

## **2.2. Methods**

### **2.2.1. Search strategy**

The following databases were searched for studies published in English between 01/01/2004 and 06/01/2020: MEDLINE, EMBASE, CINAHL, CENTRAL, PsycINFO and Web of Science. The search strategy was verified by a subject librarian and included (but was not limited to) the following terms: cytokine, interleukin, interferon AND cancer, metastasis, neoplasm AND cachexia, weight loss, anorexia (Appendix A).

### **2.2.2. Inclusion and exclusion criteria**

Eligible studies met the following criteria: adults (>18 years old); diagnosed with incurable cancer, defined as metastatic cancer or locally advanced cancer treated with palliative intent; measured the level of one or more cytokines; assessed at least one symptom and/or sign associated with cachexia. Studies examining all primary cancer types were included to ensure that as much information as possible regarding cytokines and cachexia was retrieved. Diagnosis of cachexia was based on the criteria reported by primary authors rather than any specific definition, allowing the inclusion of studies conducted before 2011, the year when the Fearon definition was published [7]. This ensures that as many studies as possible were included, regardless of the definitions or the weight loss thresholds used to diagnose cachexia.

Studies were excluded if the participants were cancer survivors or being treated with curable intent. Additionally, the studies were not considered for inclusion if patients' symptoms were attributed directly to a form of therapy or medication. Although no criterion regarding the study design was imposed, the current review did not consider case studies, animal models, protocols or conference abstracts.

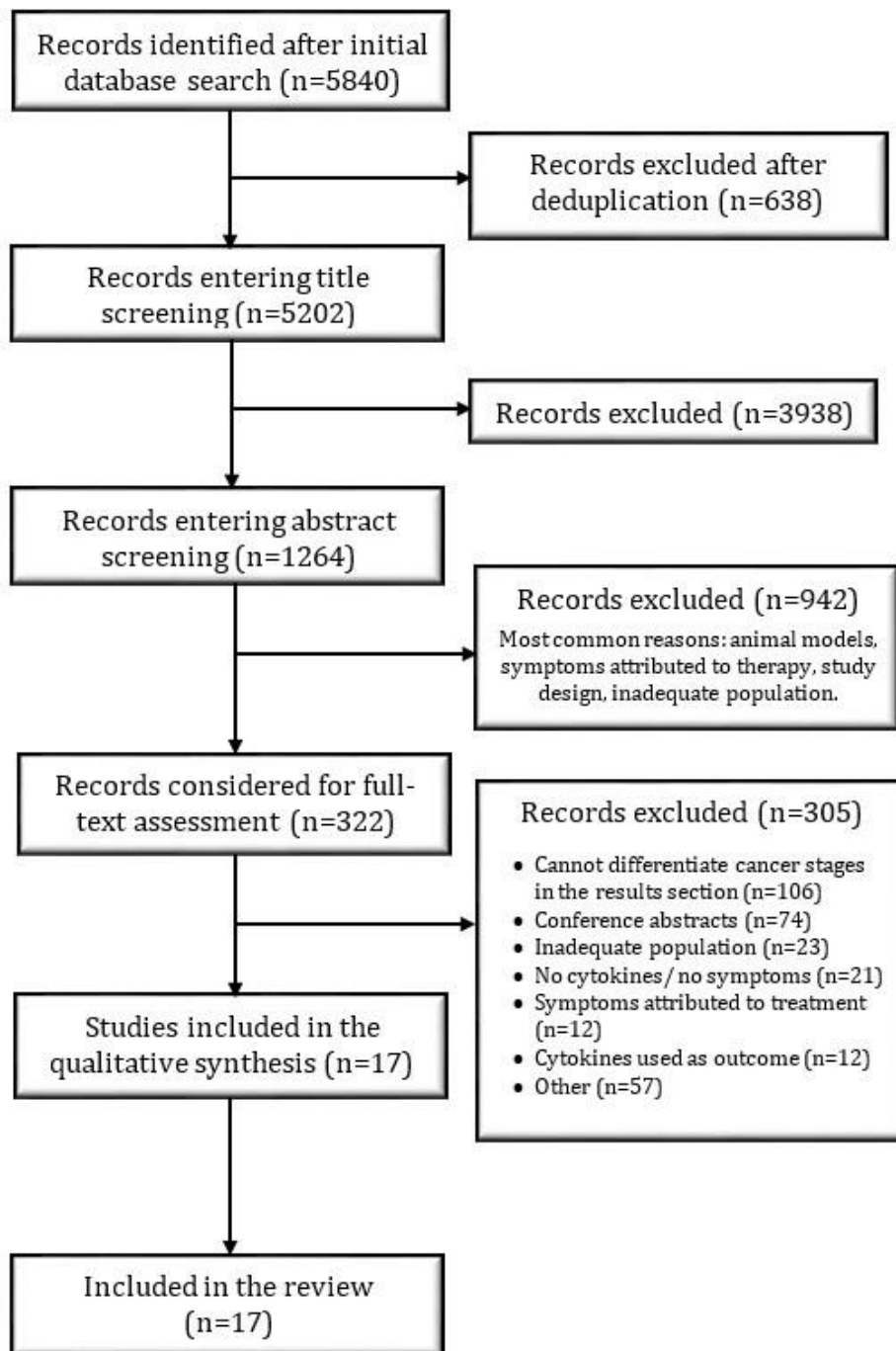
### **2.2.3. Study selection and quality assessment**

Figure 2.1 highlights the PRISMA flow diagram of study selection. The titles of the studies were screened independently by RP using a conservative approach – whenever the title did not provide enough information, the study was included in the next selection phase. Abstract screening was conducted by DRP and RP in a similar manner and the studies

identified as relevant were accepted for full-text assessment. Following full-text assessment (DRP and RP), the quality of the included studies was appraised by DRP, RP and JM using ten relevant questions (Appendix B) selected from the modified Downs and Black (MDB) checklist [112]. Studies were rated on a scale from 0 to 10 (i.e., one point per question) using standardised criteria that focused on data reporting, internal validity and external validity. The quality of the evidence was classified as follows: 0-4 low quality, 5-7 moderate quality and 8-10 high quality.

#### **2.2.4. Data extraction, management and analysis**

A specifically designed collection form was used to systematically capture all the relevant information from the eligible studies. Where studies measured cytokines at multiple time points (2/17 studies) only baseline data were included. No statistical analyses were conducted due to the great level of heterogeneity in study design and data reporting identified between the included studies. Thus, the findings are presented in a descriptive manner, highlighting similarities and discrepancies as well as strengths against weaknesses from the available literature. Lastly, no ethical approval was required for this systematic review.



**Figure 2.1.** Flow diagram of the study selection process

## 2.3. Results

### 2.3.1. Study characteristics

A total of 5202 studies were identified after removing the duplicates from the database search (Figure 2.1). After evaluating the titles, 1264 studies were included in the abstract screening phase, of which 322 were selected for full-text screening. At the end of the study selection process, 17 studies met the inclusion criteria of this systematic review. Table 2.1 summarises the main characteristics of the eligible studies. Overall, 1277 patients with incurable cancer and 155 healthy controls from 13 different middle-income and high-income countries [113] were recruited from both inpatient and outpatient settings. The mean age of the patients was  $64 \pm 15$  (mean  $\pm$  standard deviation), with female participants making up only a third (34%) of the sample. The most common types of cancers were lung and pancreatic cancer, although various other types such as colorectal, breast, gastric or oesophageal cancer were evaluated.

The majority of the studies (15/17) measured and reported cytokine levels at a single time-point. One study [114] measured intra-day cytokine variation. In this instance, the morning measurements were used in this systematic review as they were taken after an overnight fast. One study [115] measured patients' cytokine levels at enrolment and every four weeks until death. This study reported baseline and end-point data. The baseline measurements were extracted and used in the present review as the end-point data were not reported separately for cachectic and non-cachectic patients. The mean quality score of the papers from the current review was 7.8 (range 5 to 10), indicating that the studies incorporated evidence of moderate-to-high quality (Table 2.1). Although six studies were of moderate quality and eleven were of high quality, several methodological weaknesses were persistent across study reports. The majority of the included studies were marked down as they failed to meet various methodological norms that had an impact on both internal and external validity. Most commonly, the data were not fully reported for all the measured cytokines – some studies specified central tendency values and measures of dispersion only for statistically significant relationships, while other papers only reported p values (Table 2.2). Additionally, several studies did not accurately describe participants' selection criteria and/or the sample collection methodology.

A methodological characteristic that played a pivotal role in the included studies was the timing of blood sampling as previous research [116,117] suggested that cytokine levels



show intra-day variation. Only eight studies indicated that blood was collected in the morning after an overnight fast, while the others provided relatively vague information about this matter (i.e. before chemotherapy, using standard methods) or failed to specify the period of the day when blood sampling was performed (Table 2.1). Furthermore, almost all studies (16/17) reported the assay used to quantify cytokine levels, but only five reported the sensitivity of the assay. The enzyme-linked immunosorbent assay (ELISA) was the most-used quantification method, whereas other validated methods such as the electrochemiluminescence immunoassay (ECLIA) and the radioimmunoassay (RIA) were used in some studies.

**Table 2.1.** Characteristics of the included studies

Author (year)	Country	Participants	Cancer type	Cytokines	Collection method	Assay method	Sensitivity reported	MDB score
Fortunati et al. (2007) [118]	Italy	33 patients 23 controls	Lung cancer (non-small cell, small cell and adenocarcinoma)	TNF- $\alpha$ , IL-6	Morning, overnight fast	ELISA	Yes	6
Grim-Stieger et al. (2008) [119]	Austria	61 patients	Breast, colorectal, lung, pancreatic, gastric and renal cancer	TNF- $\alpha$ , IL-6	Morning, overnight fast	ELISA	No	8
Takahashi et al. (2009) [120]	Japan	16 patients 10 controls	Oesophageal, gastric, colorectal cancer	TNF- $\alpha$ , IL-6, IFN- $\gamma$ , IL-1Ra, Leptin, Ghrelin	Morning, overnight fast	ELISA	No	5
Gioulbasanis et al. (2011) [121]	Greece	115 patients	Lung cancer (non-small cell and small cell)	Leptin, Adiponectin, Ghrelin	N/R	RIA	Yes	8
Scheede-Berghdal et al (2012) [122]	Canada	83 patients	Gastrointestinal and non-small cell lung cancer	IL-1 $\beta$ , IL-6, IL-8, TNF- $\alpha$	Morning, overnight fast	Bio-Plex Cytokine Assay	No	7
Den Kamp et al. (2013) [123]	Netherlands	26 patients 22 controls	Non-small cell lung cancer	IL-6, IL-8, IL-10, TNF- $\alpha$ , IFN- $\gamma$	N/R	Multiplex Antibody Assay	Yes	7
Fujiwara et al. (2014) [114]	Japan	21 patients	Pancreatic cancer	IL-6, TNF- $\alpha$ , Leptin	Morning, overnight fast	RIA and ELISA	Yes	10
Lu et al. (2014) [124]	China	110 patients	Oesophageal squamous cell carcinoma	MIC-1	N/R	ELISA	No	7
Bilir et al. (2015) [125]	Turkey	46 patients 34 controls	Gastroesophageal, pancreatic, colorectal, ovarian, breast and laryngeal cancer	IL-1 $\alpha$ , IL-1 $\beta$ , IL-6, TNF- $\alpha$	Morning, overnight fast	ELISA	No	8
Srdic et al. (2016) [126]	Croatia	100 patients	Advanced non-small cell lung cancer	IL-6	N/R	ECLIA	No	8
Penafuerte et al. (2016) [127]	Canada	122 patients	Head, neck, breast, upper gastrointestinal, lung, hepatobiliary, prostate and colorectal cancer	IL-1 $\alpha$ , IL-1 $\beta$ , IL-3, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, IL-15, IL-18, IFN- $\gamma$ , MCP-1, TNF- $\alpha$ , Leptin, Ghrelin, Adiponectin, TRAIL, TGF- $\beta$ 1	N/R	Bio-Plex Cytokine Assay	No	9
Lerner et al. (2016) [128]	USA	218 patients	Lung and pancreatic cancer	IL-1, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12, IL-13, IL-17, IFN- $\gamma$ , GDF-15, MCP-1, IP-10	N/R	Bio-Plex Cytokine Assay	No	9
Bye et al. (2016) [115]	Norway	20 patients 40 controls	Pancreatic cancer	IL-6, IL-10, TNF- $\alpha$ , Adiponectin, Leptin, IFN- $\gamma$	Non-fasting	ELISA	Yes	8
Fogelman et al. (2017) [129]	USA	89 patients 6 controls	Pancreatic cancer	IL-1 $\beta$ , IL-6, IL-8, TNF- $\alpha$ , Leptin, Adiponectin, Ghrelin	N/R	N/R	N/R	7
Demiray et al. (2017) [130]	Turkey	67 patients 20 controls	Non-small-cell lung cancer	Leptin, Resistin	Morning, overnight fast	ELISA	No	8
Murton et al. (2017) [131]	United Kingdom	4 patients 4 controls	Advanced non-small-cell lung cancer	IL-6, TNF- $\alpha$	Morning, overnight fast	ELISA	No	6
Hou et al. (2018) [132]	Taiwan	146 patients	Pancreatic cancer	IL-1 $\beta$ , IL-6, IL-8, TNF- $\alpha$	N/R	ELISA	No	10

ELISA - enzyme-linked immunosorbent assay; ECLIA - electrochemiluminescence immunoassay; RIA - radioimmunoassay; MDB - modified Downs and Black; N/R - not reported.

### **2.3.2. Main findings**

Table 2.2 highlights the main findings of the included studies as well as relevant data and grouping criteria. The studies included in the current review analysed the relationship between cytokine levels and cachexia or the degree of weight loss experienced by cancer patients. A great level of variation was observed between the definitions of cachexia and the weight loss thresholds used across the studies to classify and group participants. A third of the included studies defined cachexia as suggested by Fearon and colleagues [7], while several studies referred to cachexia as a syndrome that implies losing more than 5% [118] or 10% [114] body weight. Furthermore, some authors did not use the term 'cachexia' but instead classified the participants according to the amount of weight lost during a period of three to six months before the study. The criteria according to which participants were grouped are not homogenous across studies. Although the data could not be subject to a meta-analysis due to the methodological differences, a systematic summary of the findings is subsequently described and discussed.

**Table 2.2** Main findings of the included studies

Authors (year)	Variable measured	Grouping criteria	Findings	Data			
					CC	NC	Control
Fortunati et al. (2007) [118]	Cachexia	Cachexia defined as more than 5% weight loss in the previous 6 months.	TNF- $\alpha$ levels were greater in CC patients compared to NC individuals ( $p<0.05$ ) and controls ( $p<0.01$ ). IL-6 was greater in CC compared to controls ( $p<0.01$ ) but did not significantly differ from NC patients ( $p>0.05$ ).		4.2 $\pm$ 0.6	2.7 $\pm$ 0.5	1.9 $\pm$ 0.3
				IL-6	11.6 $\pm$ 1.6	8.2 $\pm$ 1.8	0.4 $\pm$ 0.1
				Values are shown as mean $\pm$ SD.			
Grim-Stieger et al. (2008) [119]	Weight loss	Evaluated whether participants suffered any weight loss since diagnosis or in the past 3 months.	No significant correlations found between TNF- $\alpha$ and WL since diagnosis ( $p=0.19$ ) or during the last 3 months ( $p=0.11$ ). No significant correlation found between IL-6 and WL since diagnosis ( $p=0.13$ ) or during the last 3 months ( $p=0.12$ ).	Only p values.			
Takahashi et al. (2009) [120]	Cachexia	No definition.	CC patients expressed greater levels of TNF- $\alpha$ , IL-6, IL-1Ra ( $p<0.01$ ) and ghrelin ( $p=0.04$ ) compared to healthy participants. No difference in IFN- $\gamma$ was observed between groups ( $p=0.27$ ), while leptin was significantly higher in healthy controls ( $p=0.02$ ).	Only p values.			
Gioulbasanis et al. (2011) [121]	Weight loss and nutritional sufficiency	Patients assigned to group: A - nutritional sufficiency (15% lost more than 5% body weight), B - risk of malnutrition (63% lost weight) or C - malnourished (83% lost weight).	The mean levels of leptin were significantly higher ( $p<0.01$ ) in group A compared to group B and group C. Less adiponectin ( $p=0.06$ ) was detected in group A compared to group B and group C. Ghrelin levels did not significantly differ ( $p>0.05$ ) between group A, B and C.		A	B	C
				Leptin	5.3 $\pm$ 4.2	3.6 $\pm$ 3.2	1.5 $\pm$ 1.7
				Adiponectin	10.0 $\pm$ 4.2	12.9 $\pm$ 6.9	13.4 $\pm$ 5.6
				Ghrelin	761 $\pm$ 809	818 $\pm$ 1081	817 $\pm$ 514
				Values are shown as mean $\pm$ SD.			
Scheede-Berghdal et al. (2012) [122]	Weight loss and sarcopenia	Participants grouped based on the degree of weight loss 6 months prior to enrolment - more or less than 5% weight loss. The presence of sarcopenia assessed by calculating the appendicular lean mass index (Baumgartner, 2000).	The study compared high versus low levels of cytokines. Higher levels of IL-1 $\beta$ and TNF- $\alpha$ were significantly ( $p<0.01$ ) associated with the presence of more than 5% weight loss. The levels of IL-6 and IL-8 could not significantly predict ( $p>0.05$ ) weight loss.  Similarly, IL-1 $\beta$ and TNF- $\alpha$ were positively associated ( $p<0.05$ ) with the presence of sarcopenia, while a trend existed for both IL-6 ( $p=0.06$ ) and IL-8 ( $p=0.09$ ).		OR	95%CI	
				IL-1 $\beta$	7.14	1.87 to 27.26	
				TNF- $\alpha$	7.15	1.87 to 27.32	
				IL-6	2.13	0.65 to 6.95	
				IL-8	2.24	0.68 to 7.31	
					OR	95%CI	
				IL-1 $\beta$	5.35	1.37 to 20.97	
				TNF- $\alpha$	5.56	1.47 to 20.97	
				IL-6	3.52	0.65 to 6.95	
				IL-8	3.03	0.85 to 10.76	
Den Kamp et al. (2013) [123]	Cachexia	Participants grouped based on the 2011 consensus definition of cancer cachexia.	Significantly ( $p<0.05$ ) higher levels of IL-6 and IL-8 were observed in the plasma of CC patients compared to individuals with PC and controls. IFN- $\gamma$ was significantly higher ( $p<0.05$ ) in controls compared to PC patients. The levels of TNF- $\alpha$ and IL-10 did not differ between groups.	Only p values.			

Fujiwara et al. (2014) [114]	Cachexia	Cachexia defined as ECOG PS 1 to 4, grade 1 to 4 anorexia and more than 10% weight loss over the past 6 months.	IL-6 (p=0.35), TNF- $\alpha$ (p=0.27) and leptin (p=0.27) levels did not differ between CC and NC patients.		CC	NC
				IL-6	13.8 (2.6-23.4)	6.8 (1.4-28.7)
				TNF- $\alpha$	7.1 (1.2-30.2)	3.3 (1.2-30.0)
				Leptin	2.4 (1.0-9.8)	4.0 (2.0-9.9)
Values are shown as median (range).						
Lu et al. (2014) [124]	Weight loss	Participants divided based on the degree of weight loss before chemotherapy – more or less than 5%.	MIC-1 levels were significantly higher (p=0.01) in patients with >5% weight loss compared with those with $\leq$ 5% weight loss.		>5% WL	$\leq$ 5% WL
				MIC-1	1560 (1090-2141)	1124 (776-1560)
				Values are shown as median (interquartile range)		
Bilir et al. (2015) [125]	Cachexia	Cachexia – BMI<20, weight loss during treatment or weight loss of more than 5% prior to illness in the past 6 months and continuing in the last few months. Refractory cachexia – patients unresponsive to treatment with a life expectancy lower than 3 months and reduced performance status.	IL-1 $\alpha$ (p=0.03), IL-6 (p<0.01) and TNF- $\alpha$ (p<0.01) were higher in people suffering from CC compared to controls. IL-1 $\beta$ (p=0.6) did not differ between groups.  IL-1 $\alpha$ was higher in individuals with CC compared to patients with RC (p=0.02). IL-1 $\beta$ was greater in patients with RC compared to individuals with CC (p=0.01). IL-6 (p=0.70) and TNF- $\alpha$ (p=0.12) did not differ between groups.		CC	Control
				IL-1 $\alpha$	14.0 $\pm$ 9.9	9.9 $\pm$ 7.5
				IL-1 $\beta$	3.7 $\pm$ 0.8	3.6 $\pm$ 0.7
				IL-6	16.5 $\pm$ 4.0	5.7 $\pm$ 3.6
				TNF- $\alpha$	15.9 $\pm$ 7.1	12.0 $\pm$ 5.2
					CC	RC
				IL-1 $\alpha$	17.0 $\pm$ 11.0	10.0 $\pm$ 5.0
				IL-1 $\beta$	3.4 $\pm$ 0.4	3.9 $\pm$ 0.9
				IL-6	17.4 $\pm$ 8.0	15.4 $\pm$ 5.4
				TNF- $\alpha$	17.0 $\pm$ 7.0	14.0 $\pm$ 7.0
Values are shown as mean $\pm$ SD.						
Srdic et al. (2016) [126]	Cachexia	Participants grouped based on the 2011 consensus definition of cancer cachexia.	Patients with CC had significantly higher levels of IL-6 compared to patients with NC (p=0.04).		CC	NC
				IL-6	15.2	7.5
Means with no measure of dispersion.						
Penafuerte et al. (2016) [127]	Cachexia	Participants grouped based on the 2011 consensus definition of cancer cachexia.	TGF- $\beta$ 1: patients with CC (p<0.01) and PC (p=0.04) expressed higher levels compared to NC patients; no difference between PC and CC (p>0.05). IL-8: Patients with CC showed greater levels than individuals with PC (p<0.01) and NC (p<0.01); no difference between PC and NC (p>0.05). IL-6: greater in patients with CC compared to NC (p<0.01); no difference between PC and CC and between PC and NC (p>0.05). TRAIL: levels higher in patients with CC compared to NC individuals; no difference between PC and CC and between PC and NC (p>0.05).	Only p values. Data were not reported for all cytokines.		

Lerner et al. (2016) [128]	Weight loss	Participants divided based on the degree of weight loss – more than 5%, between 0-5% and no weight loss.	<p>GDF15 was greater in patients with &gt;5%WL (p&lt;0.01) and with ≤5% WL (p&lt;0.01) compared to individuals with no WL.</p> <p>IL-12 levels were greater in individuals with &gt;5% WL compared to both ≤5% WL (p=0.03) and no WL (p&lt;0.01).</p> <p>IL-10 levels were higher in patients with &gt;5% WL compared to both ≤5% WL (p=0.05) and no WL (p=0.05).</p> <p>IL-7 was greater in participants with &gt;5% WL compared to both ≤5% WL (p=0.08) and no WL (p=0.06).</p> <p>Participants with &gt;5% WL showed greater IL-6 (p=0.06) and IL-2 (p=0.07) levels as opposed to patients with no WL.</p> <p>The levels of IL-13 (p=0.04), IL-8 (p=0.06) and IL-9 (p=0.08) were higher in participants with &gt;5% WL compared to individuals with ≤5% WL.</p> <p>All other relationships showed greater, non-significant p values.</p>		No WL	≤5% WL	>5% WL			
				GDF15	1000	2000	2000			
				IL-12	43	47	84			
				IL-10	29	29	71			
				IL-7	19	19	29			
				IL-6	23	26	39			
				IL-2	36	39	64			
				TNF-α	99	99	154			
				IL-17	143	139	215			
				IL-13	28	25	39			
				IL-8	79	69	124			
				IFN-γ	199	177	282			
				IL-4	8	7	10			
				IL-5	24	25	33			
								Median values with no measure of dispersion.		
Bye et al. (2016) [115]	Cachexia	Participants grouped based on the 2011 consensus definition of cancer cachexia and on the modified Glasgow Prognostic Score (mGPS)	<p>CC and NC was determined according to the 2011 consensus and no difference in cytokine levels was observed between the groups (p&gt;0.05).</p> <p>IL-6 was greater in CC patients compared to NC individuals, when the disease was assessed according to mGPS (p&lt;0.01). The other cytokines did not differ between groups (p&gt;0.05)</p>		CC	NC				
				IL-6	4.4 (2.2-34.6)	2.2 (0.5-5.3)				
				IL-10	0.7 (0-3.4)	0.9 (0.2-7.7)				
				TNF-α	7.5 (4.1-22.7)	8.4 (3.3-11.9)				
				IFN-γ	0.13 (0-13.6)	0.12 (0.1-0.5)				
				Adiponectin	7.2 (5.9-15.6)	10.1 (4.6-26.0)				
				Leptin	1.0 (0.3-23.9)	2.69 (0.3-9.8)				
								CC	NC	
				IL-6	2.2 (0.5-5.3)	12.4(3.2-34.6)				
				IL-10	0.8(0-7.7)	0.7(0-3.4)				
				TNF-α	9.2(3.3-22.7)	6.3(4.1-17.9)				
				IFN-γ	0.1(0.4-0.8)	0.(0.1-0.5)				
				Adiponectin	8.2(3.9-26.0)	8.4(0.3-9.8)				
				Leptin	2.3(0.3-23.9)	1.0(0.3-9.8)				
								Values are shown as median (range)		

Fogelman et al. (2017) [129]	Weight loss	The participants in the weight loss group had either 10% weight loss or died at 60 days after baseline. The non-weight loss group failed to meet any of the aforementioned criteria.	<p>IL-1<math>\beta</math>: levels were greater in the control group compared to both no WL and WL (p=0.07); the levels were higher in the no WL group compared to WL (p=0.03).</p> <p>IL-6: levels were smaller in the control group compared to both WL (p&lt;0.01) and no WL (p&lt;0.01); the levels were higher in the WL group compared to no WL (p=0.03).</p> <p>TNF-<math>\alpha</math>: levels were smaller in the control group compared to both WL (p&lt;0.01) and no WL (p&lt;0.01); levels were higher in the WL group compared to no WL (p=0.03).</p> <p>IL-8: levels were smaller in the control group compared to both WL (p&lt;0.01) and no WL (p&lt;0.01); no significant differences observed between the WL and no WL groups (p&gt;0.05).</p> <p>All other relationships showed greater, non-significant p values.</p>		Control	No WL	WL
				IL-1 $\beta$	34.3 (0-79.4)	4.4 (0-633.3)	0.1 (0-299.7)
				IL-6	5.8 (2.9-6.3)	14.5 (4.7-1593)	37 (5.6-524.3)
				TNF- $\alpha$	2.1 (1.5-3.3)	3.3 (1.5-16.2)	4.1 (2.2-18.9)
				IL-8	10.8 (4.6-14.4)	37.1 (5.2-3185)	56.8 (6.7-2204.8)
				Adiponectin	15.3 (8.7 - 27)	18.4 (7.5-59.4)	19.5 (8.9-599.8)
				Leptin	8.9 (2.4-91.1)	7.9 (0.2-134.3)	8.8 (0.7-91.1)
				Ghrelin	0 (0-4.6)	0 (0-3351.9)	2.7 (0-3351.9)
Demiray et al. (2017) [130]	Weight loss	Weight loss at the time of diagnosis defined as more than 10% weight loss within the past 6 months.	The levels of leptin (p=0.44) and resistin (p=0.54) did not differ between patients with and without WL.		No WL	WL	
				Leptin	10.7 $\pm$ 2.5	7.2 $\pm$ 2.1	
				Resistin	5.6 $\pm$ 0.2	6.7 $\pm$ 0.3	
				Values are shown mean $\pm$ SD.			
Murton et al. (2017) [131]	Cachexia	Participants grouped based on the 2011 consensus definition of cancer cachexia.	Cachectic individuals showed higher levels of IL-6 (p<0.05) and TNF- $\alpha$ (p=0.06) compared to healthy controls.	Only p values.			
Hou et al. (2018) [132]	Cachexia	Participants grouped based on the 2011 consensus definition of cancer cachexia.	<p>There was a positive (p=0.03) relationship between IL-8 and WL. Also, a positive relationship (p=0.07) was observed between IL-6 and WL. The other correlations were less strong and showed greater p values.</p> <p>IL-8 levels were greater in CC compared to NC (p=0.01). IL-1<math>\beta</math> (p=0.95), IL-6 (p=0.16) and TNF-<math>\alpha</math> (p=0.84) levels did not differ between CC and NC patients.</p>		Pearson's coefficient (r)		
				IL-1 $\beta$	0.10		
				IL-6	0.24		
				IL-8	0.28		
				TNF- $\alpha$	0.08		

CC – cancer cachexia; NC – cancer non-cachexia; PC – pre-cachexia; RC – refractory cachexia; CTR – control; WL – weight loss; SD – standard deviation; ECOG PS – Eastern Cooperative Oncology Group Performance Status.

A total of 31 different (adipo)cytokines were measured across the 17 studies included in the present review (Table 2.1). The most frequently analysed cytokines were IL-6 (14), TNF- $\alpha$  (12), leptin (7), IL-8 (6), IL-1 $\beta$  and IFN- $\gamma$  (5), IL-10, ghrelin and adiponectin (4).

The majority (11/14) of the studies analysing IL-6 indicated the presence of a relationship between high levels of IL-6 and cachexia or weight loss. Cachectic (weight-losing) patients showed significantly more IL-6 compared to healthy control groups in six out of six studies. When cachectic (weight-losing) individuals were compared with non-cachectic (weight-stable) cancer patients, five out of eight studies indicated that the levels of IL-6 were significantly higher in cachectic participants. A study that compared pre-cachectic patients against those with cancer cachexia observed greater levels of IL-6 in the latter group [123], while two other studies did not find any differences between pre-cachexia and cachexia [124,127]. Furthermore, two studies [119,122] did not find any statistically significant relationship between IL-6 and weight loss, while Hou et al. [132] indicated the presence of a medium association ( $r=0.24$ ,  $p=0.07$ ). Interestingly, Scheede-Berghdal and colleagues [123] indicated that higher IL-6 levels were positively associated with the presence of sarcopenia. Thus, the evidence suggests higher IL-6 expression in cachectic patients compared to non-cachectic counterparts and healthy individuals.

Another cytokine showing a relationship with the presence of cancer cachexia and weight loss was TNF- $\alpha$ . The levels of TNF- $\alpha$  were significantly higher in cachectic (weight-losing) patients compared to healthy controls in five out of six studies. The sixth study [128] also found a greater concentration of TNF- $\alpha$  in the cachectic group, but the difference was not statistically significant. Only two out of six papers indicated that cachectic (weight-losing) patients expressed more TNF- $\alpha$  than non-cachectic (weight-stable) counterparts, while the other studies did not find any statistically significant differences between groups. Likewise, no difference was observed between pre-cachectic and cachectic patients, while two other studies did not find any significant correlation between TNF- $\alpha$  and weight loss. Therefore, TNF- $\alpha$  levels are elevated in cachectic patients compared to healthy controls, while no significant distinction was noticed between weight-stable and weight-losing cancer patients.

Similar to the aforementioned cytokines, but with fewer studies to support the findings, IL-8 levels were overall higher in cachectic (weight-losing) patients. The studies that compared healthy controls against individuals with cancer cachexia ( $n=2$ ) reported that the



levels of IL-8 were significantly higher in the diseased group. Additionally, two out of three studies that examined IL-8 levels in cachectic (weight-losing) and non-cachectic (weight-stable) cancer patients found that IL-8 was increased in cachectic participants. Lastly, individuals with cancer cachexia had more IL-8 compared to pre-cachectic patients in both studies that examined this comparison. Overall, IL-8 showed increased levels in participants with cancer cachexia and weight loss compared to non-cachectic, pre-cachectic and healthy groups, but the strength of these observations is limited given the small number of studies analysing this cytokine.

Leptin, IFN- $\gamma$ , IL-1 $\beta$ , IL-10, adiponectin and ghrelin did not demonstrate any significant difference between groups when cachectic (weight-losing) patients were compared against non-cachectic (weight-stable) counterparts or healthy participants (Table 2.2). However, a study worth mentioning was conducted by Scheede-Berghdal and colleagues [110] who observed that higher levels of IL-1 $\beta$ , as opposed to low IL-1 $\beta$  concentrations, were significantly associated with the presence of more than 5% weight loss (OR=7.14,  $p < 0.01$ ) and sarcopenia (OR=5.35,  $p < 0.05$ ). The other cytokines listed in Table 2.1 are not discussed since they were analysed by two or fewer studies and not enough information was available.

## **2.4. Discussion**

### **2.4.1. Main findings**

The aim of the current review was to examine the relationship between cytokines and the cachexia syndrome (including related symptoms such as weight loss, anorexia and reduced physical function) in people with incurable cancer irrespective of tumour type. Overall, IL-6, TNF- $\alpha$  and IL-8 were present in greater concentrations in patients losing weight as opposed to healthy individuals. Leptin, IFN- $\gamma$ , IL-1 $\beta$ , IL-10, adiponectin and ghrelin were also evaluated, but no relationship was observed between the cytokines' circulating levels and the degree of weight loss. Moreover, the definitions of cachexia and the weight loss thresholds used across the studies to categorise participants were heterogeneous and a more consistent approach should be adopted for future studies.

The levels of circulating IL-6 were elevated in weight-losing and cachectic patients compared to healthy controls in all studies that analysed this cytokine. Furthermore, more than half of the studies that compared cachectic and weight-losing patients with non-cachectic or weight-stable counterparts indicated the presence of higher IL-6 concentrations in cachectic individuals. The direction of these relationships was also observed by other research and it has been previously suggested that IL-6 is a central regulator of the progression of cancer and cancer-associated cachexia [133,134,135]. Several studies examined the effect of IL-6 inhibitors on cachexia. Clazakizumab, an anti-IL-6 antibody, was tested in patients with non-small cell lung cancer and improved cachexia and anaemia in phase I and II trials [136]. Despite the fact that the drug seemed well tolerated, there is no phase III trial ongoing. Furthermore, various case reports [137,138] and animal models [139] indicated that tocilizumab might ameliorate cancer-associated cachexia. Often used in patients with rheumatoid arthritis, tocilizumab was associated with increased weight and BMI in a recent systematic review [140]. Although the previously mentioned reports suggest a potential positive effect of tocilizumab, no clinical trials are currently examining its effect on cancer cachexia. To conclude, assessing the circulating levels of IL-6 could be a useful method of monitoring the development of cancer cachexia and future trials should aim to integrate the cytokine in the multi-factorial management of this disorder.

Circulating TNF- $\alpha$  was expressed in higher concentrations in cachectic and weight-losing patients as opposed to healthy individuals. There was no difference in TNF- $\alpha$  when

cachectic and weight-losing patients were compared to non-cachectic and weight-stable patients. The available literature highlights the role of TNF- $\alpha$  as a key mediator of cachexia given the cytokine's ability to activate NF $\kappa$ B, one of the main pathways that determine skeletal muscle atrophy [141,142]. Various studies focused on analysing the effectiveness of TNF- $\alpha$  inhibitors such as etanercept and infliximab [143,144,145]. In a cohort of patients with incurable cancer, etanercept only produced a small level of weight gain and failed to treat cachexia [143]. Similarly, pancreatic cancer patients receiving infliximab gained an insignificant amount of weight compared to counterparts receiving a placebo. Another trial analysing the effectiveness of OHR/AVR118, an agent targeting both IL-6 and TNF- $\alpha$ , indicated that cancer patients with cachexia improved anorexia, strength and dyspepsia [146]. This finding reinforces the idea that not one, but multiple cytokines could be responsible for the onset and progression of cancer cachexia and a multimodal approach is required in the management of this disorder.

The majority of the studies analysing IL-8 indicated that the cytokine's expression was greater in patients with cancer cachexia and weight loss compared to non-cachectic, weight-losing, pre-cachectic and healthy individuals. Although the strength of this observation is limited given the small number of papers examining this cytokine, future research might evaluate the direction of the relationship between IL-8 and cachexia since this matter was not thoroughly explained by the available literature. Furthermore, none of the other cytokines analysed in the current review showed any relationship with the amount of weight lost by patients. However, previous research linked cytokines such as IL-1 $\alpha$  [147], IL-1 $\beta$  [148] and IFN- $\gamma$  [149] with the occurrence and development of weight loss. Overall, there is not enough evidence available regarding the previously mentioned cytokines to reach a definitive conclusion and future studies should aim to explore this knowledge gap.

#### **2.4.2. Inconsistencies in grouping criteria**

The studies included in the current review used distinct methods of defining cachexia and various weight loss thresholds to group participants (Table 2.2). Some studies used the consensus definition from 2011 [7] or the modified GPS (mGPS) [150] to assess and diagnose cachexia. Multiple studies [124,128] used a 5% weight loss limit as the main grouping criterion and only discussed patients' weight without referring to cachexia as a

disorder. Interestingly, various papers classified participants using weight-loss thresholds that appeared to be chosen arbitrarily (i.e. 10%), while others used cachexia definitions that were not validated by previous literature (Table 2.2). Thus, the results could not be meta-analysed due to the lack of a consistent method of grouping participants. The current review presented findings in a descriptive manner, giving a useful indication of the trajectory of the available evidence. However, conducting a meta-analysis would provide a more precise and reliable summary of the included studies and should allow an effective comparison between them [151]. Consequently, practitioners could make well-informed decisions based on high-quality evidence with a lower risk of bias [152] and this would have a positive impact on patients' treatment and quality of life. Future studies should adhere to definitions and thresholds that are already established by the literature in order to promote uniformity and consensus in the field of cancer cachexia. Otherwise, any novel method for defining and assessing cachexia should be accompanied by a thorough rationale.

#### **2.4.3. Limitations and directions for future research**

Most of the studies analysed in this review had a cross-sectional design and do not allow the inference of a causal relationship between cytokines and cachexia. A limitation of the present findings is that only two studies reported multiple cytokine measurements and only the baseline data were used in the current review. Future work in this area should assess cytokine levels longitudinally to fully elucidate their effect on the cachexia phenotype. Moreover, the relationship between cytokines and cachexia was examined in all primary tumour types. Although this may be considered a limitation since cachexia is less common in some cancers, failing to include all primary tumour types means that minimal data would be available and important studies might be omitted.

Numerous papers were excluded from the present review as the data of patients with early and advanced forms of cancer were combined in the analysis. Although relevant evidence might have been left out of this study, the information about individuals with incurable malignancies could not be differentiated from the data of patients with operable forms of cancer. Additionally, the assay used to measure cytokine levels is an important methodological factor and it was reported in all but one investigation. However, less than a third of the studies indicated the sensitivity of the assay and thus, the validity of the results that failed to consider this parameter was low.

Several other errors were observed in the statistical analysis of the results and in the methods used to report findings. In the present systematic review, the available body of literature could not be meta-analysed due to the high degree of methodological heterogeneity as well as the lack of transparency and failure to meet basic standards of data reporting. Specifically, five studies [119,120,123,127,131] only reported p-values, while two studies [126,128] did not report any measure of dispersion (i.e. standard deviation, interquartile range). Several studies examined multiple cytokines and only displayed data for statistically significant relationships. The use of these practices in the literature leads to biased reporting and inflation of type I errors in systematic reviews. One study [132] examined the correlation coefficient between cytokines and weight loss, while the other nine studies used different methods of reporting data (i.e., measure of central tendency, dispersion or effect sizes). The remaining studies have major inconsistencies in grouping criteria. Only one study [115] used the Fearon definition [7], while another classified participants based on nutritional sufficiency [124]. Two studies [114,130] grouped participants based on a 10% weight-loss threshold in the last 6 months, while another study [129] used the same threshold but measured at 60 days prior to enrolment. The last four studies [115,118,122,124] grouped patients based on a 5% weight loss limit. Yet, not even these studies could be meta-analysed since they measured different cytokines and use dissimilar methods of reporting data (mean and standard deviation, odds ratio and confidence interval as well as median and interquartile range). To enable meta-analyses in the future, consensus on cachexia definition, detailed reporting as well as the standardisation of cytokines measured and assays used would be optimal.

Although this review provided useful information, it also highlighted areas where research could be optimised. Future studies should be longitudinal, with an extensive characterisation of the cachexia phenotype (including loss of lean mass/weight, patient-reported outcomes of anorexia, fatigue and quality of life, physical activity and other measures of function), allowing a better understanding of the relationship between cytokines and the phenotype. Additionally, future studies should incorporate surrogate markers of the inflammatory response such as acute-phase proteins (i.e. CRP, serum amyloid A) and also cytokine receptors (i.e. sIL-6R, sIL-2R, IL1-R1, IL1-R2, TNF-R1 and TNF-R2). Adding these markers as a complementary measurement would generate a more accurate overview of the inflammatory state and the cascade of immune events underlying cancer cachexia. As previously mentioned, increasing homogeneity in study design should

be a priority for future research. This can be achieved by grouping participants according to established criteria such as the Fearon definition [7] or the mGPS [150]. Most importantly, regardless of the study design chosen by researchers, it is crucial to describe the methodology and the results in a transparent manner. Specifically, all measured variables should be reported and not only the significant results (complete datasets can be added as supplementary material to increase a manuscript's reliability); authors should go beyond p-values and must report data using central tendency values or effect sizes alongside measures of dispersion; the blood collection methods, the type of assay used to measure biomarkers and the sensitivity of the assay should be described in the methods section.

#### **2.4.4. Conclusions**

A relationship between cytokines, cachexia and weight loss was observed in the current review. The levels of IL-6 and TNF- $\alpha$  were greater in cachectic patients compared to healthy individuals. A similar result was obtained for IL-8, but fewer studies supported the finding. IL-6 was the only cytokine expressed in higher concentrations in cachectic participants compared to non-cachectic cancer patients. The other cytokines analysed did not show any notable relationship with cachexia or the amount of weight lost by cancer patients. These findings indicate that a network of cytokines including IL-6, TNF- $\alpha$  and IL-8 are associated with the development of cancer cachexia. An index created from multiple cytokines might serve as a 'biomarker' that could be used to analyse the onset and progression of cancer cachexia. However, this relationship is not causal and future work should opt for longitudinal designs with consistent methodological approaches, as well as adequate mechanisms of analysing and reporting results

### **3. The relationship between adipokines and the cachectic phenotype in people with incurable cancer (REVOLUTION study)**

#### **3.0. Abstract**

**Background:** Cancer cachexia is an unmet clinical need that affects more than 50% of patients with cancer. The production of various (adipo)cytokines might influence the pathophysiology of cancer cachexia due to their involvement in the systemic inflammatory response as well as their ability to influence insulin sensitivity, appetite, and muscle mass. REVOLUTION is a trial that assesses various biological components of cancer cachexia. This study aims to examine whether the adipokines leptin, intelectin-1, adiponectin, resistin and visfatin can predict the modified Glasgow Prognostic Score (mGPS) and cachexia in patients with incurable cancer.

**Methods:** 23 females and 15 males with various types of incurable cancer were assessed at admission in a palliative care centre and every 6 weeks until death. Blood samples were collected at every timepoint to determine the mGPS and quantify adipokine levels. Additionally, weight was measured to calculate individuals' body mass index (BMI) and assess cachexia. Bayesian ordinal regression was used to analyse the predictive value of adipokines.

**Results:** Overall, 10 individuals survived until the first timepoint and only 3 were still alive 12 weeks after enrolment. At baseline, 52% of participants were cachectic, and 71% were highly inflamed according to the mGPS. Visfatin was below the level of detection in all samples. Statistical modelling suggested that the adipokines could not be used as predictors of mGPS and cachexia in this cohort. Leptin showed a small negative relationship with mGPS and cachexia, while resistin was positively associated with mGPS. The level of none of the adipokines was different between timepoints.

**Conclusion:** This study provides further insights into the role of adipokines in cancer cachexia. Leptin and resistin should be integrated alongside other inflammatory markers in future models that aim to predict cachexia and mGPS. Further research should increase reliability and methodological quality by including larger sample sizes and more frequent measurements.

### 3.1. Introduction

Cancer cachexia is a multifactorial syndrome characterised by the continuous loss of skeletal muscle mass, with or without adipose tissue wasting [7], that affects more than half of cancer patients [16]. This complex syndrome diminishes the effectiveness of anti-cancer therapies [100], leads to progressive functional impairment [99], has a negative effect on patients' quality of life and diminishes survival rates [101]. Most importantly, cancer cachexia cannot be entirely reversed with conventional nutritional support [7]. This condition remains an unmet clinical need as no licensed treatment and no standard of care are currently available.

Besides weight loss, inflammation is a factor that has a major influence on cancer cachexia pathogenesis [153]. Previous research suggested that the mGPS, a score determined based on albumin and CRP levels, could be used in assessing cachexia and determining survival (i.e., time to death) in several cancer types [150]. Recent work by McGovern et al. [11] also highlighted the role of systemic inflammatory response in the development and progression of cancer cachexia. Typically, a network of cytokines is responsible for mediating the inflammatory response. Yet, in cancer, the equilibrium between pro- and anti-inflammatory cytokines is affected, leading to an imbalance that determines both immune suppression and stimulation [104]. Previous studies indicated that the production of various cytokines could influence the pathophysiology of cancer-associated cachexia [107]. Indeed, IL-6, TNF- $\alpha$  and IL-8 were associated with cachexia and weight loss in a recent systematic review conducted by our research group (Chapter 2). Furthermore, various adipocytokines such as adiponectin, leptin, ITLN1 and resistin were identified as biomarkers with a potential role in cancer cachexia [44,98,154,155] due to their ability to regulate appetite, body weight, protein synthesis and insulin sensitivity. However, given the limited availability of literature and the presence of methodological limitations, the relationship between these biomarkers, the clinical phenotype and the development of cancer cachexia needs further clarification. This can be achieved through longitudinal studies that include a comprehensive description of the cachexia phenotype (i.e., systemic inflammatory response, body composition, symptom phenotype, quality of life and physical function).

One such trial is REVOLUTION – Routine EValuatiOn of people LivIng with caNcer [156] – a prospective study examining clinical parameters, body composition, the biological



components of cancer cachexia and various patient-reported outcomes. The objectives of this trial were to characterise individuals with cancer (cachexia) that were approaching the end of life, to set priorities for future research and to enhance patient care. Recent work by Cederholm and colleagues [10] defined cachexia as chronic diseases-related malnutrition with inflammation and identified both phenotypic (i.e., decreased BMI, decreased muscle mass and involuntary weight loss) and tumour aetiologic criteria (i.e., diminished food intake or assimilation and inflammation or disease burden). The authors also emphasised the importance of simultaneously examining all components of this multifaceted disease [10]. Although the presence and the relevance of the factors that contribute to the cachectic phenotype were acknowledged by most authorities, the relationship between those components requires additional elucidation. Therefore, the current study aims to examine if adiponectin, leptin, ITLN1, resistin and visfatin can predict cachexia and mGPS in a cohort of patients with incurable cancer from the REVOLUTION trial.

## **3.2. Methods**

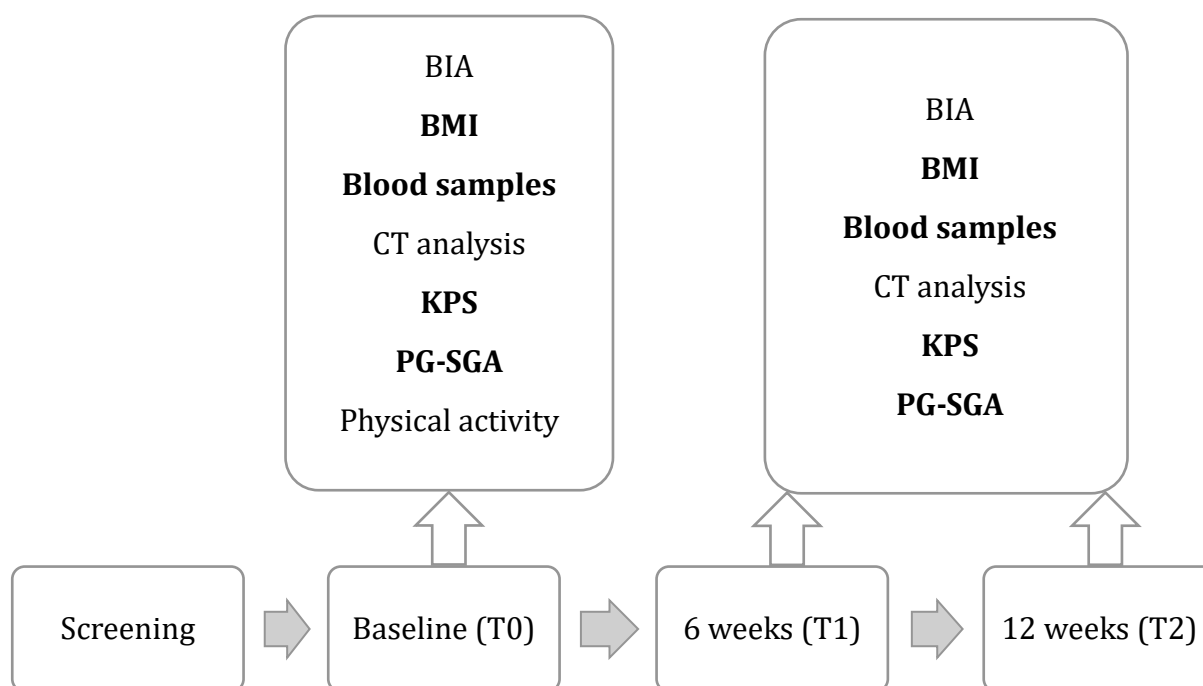
The complete protocol of the trial was described by Patton and colleagues [156]. This section highlights the methods that were relevant for the data examined in the current study. Ethical approval was obtained (20/WS/0043) before the start of this trial, which was conducted in accordance with the Declaration of Helsinki (2013).

### **3.2.1. Patient selection criteria**

REVOLUTION is a prospective observational cohort study. Participants were recruited from a palliative care unit in the United Kingdom. Patients were included in the study if they were adults ( $\geq 18$  years old) diagnosed with incurable cancer (clinical, histological or radiological evidence of metastatic cancer and individuals receiving therapy with palliative intent). Patients were excluded if the cancer was potentially curable or if they had another condition that could increase the risks of complications for the patient or for the investigators.

### **3.2.2. Study design and measurements**

After admission in the palliative care centre, patients were given information about the trial. Those who offered consent to participate in this study were subsequently enrolled. Various measurements and/or samples were taken at admission and every 6 weeks until the end of life (Figure 3.1). The following demographic characteristics were collected at baseline: age, gender, diagnosis, tumour site, medication and therapies received. The international classification of disease for oncology (ICD-O) proposed by the World Health Organisation [157] was used to classify cancer and tumour types. The design of the trial (i.e., enrolling participants when they are admitted to the palliative care clinic and taking serial measurements) facilitates the assessment of the longitudinal changes that occur in patients with incurable cancer as they are approaching the end of life.



**Figure 3.1.** Study flow diagram. The text in bold represents the measurements that were analysed in the current study; the other measurements were conducted to meet additional objectives of the REVOLUTION trial. BIA – bio-impedance analysis; BMI – body mass index; CT – computerised tomography; KPS – Karnofsky Performance Scale; PG-SGA – Patient-Generated Subjective Global Assessment.

At every timepoint, patients’ height and weight were recorded and used to calculate BMI as kg/m<sup>2</sup> [158]. Moreover, the Fearon consensus definition [7] was used to assess whether participants were cachectic. The mGPS was used to determine the level of systemic inflammation based on the concentration of circulating CRP (mg/l) and albumin (g/dl) [150]. Previous research has indicated that the mGPS can be used as a predictor of survival in various types of cancer [159,160]. A score of 0 (CRP ≤ 10mg/l, regardless of albumin levels) indicates good prognosis, a score of 1 (CRP > 10mg/l and albumin ≥

3.5g/dl) indicates intermediate prognosis, while a score of 2 (CRP > 10mg/l and albumin < 3.5g/dl) suggests poor prognosis.

Two questionnaires were used to evaluate patients' ability to perform ordinary tasks and their nutritional sufficiency. Firstly, the Karnofsky Performance Scale (KPS) was used to assess the functional status of patients with incurable cancer. The KPS is a validated instrument [161] that examines patients' performance on a scale ranging from full wellbeing (100%) to death (0%). Following the completion of the questionnaire, participants were assigned to one of the three groups: group A (80%-100%) – can perform daily tasks without requiring any help, group B (50%-70%) – can perform daily tasks with assistance and group C (<40%) – requires ongoing help and approaches death gradually. Additionally, the Patient-Generated Subjective Global Assessment (PG-SGA) was used to assess the nutritional status of participants. This validated instrument is widely used in both clinical practice and academic research [162] to assess nutritional status, as an interventional triage method and to examine the effectiveness of interventions. An additive score was calculated following the completion of the questionnaire and patients were assigned to one of the following groups: no intervention required (0-1), patient and family nutrition education in conjunction with an eventual pharmacological intervention as suggested by the section of the survey that examined symptoms (2-3), requires an intervention delivered by a dietitian alongside symptom management as indicated by box 3 in the survey (4-8) and critical need for improved symptom management and/or nutritional intervention ( $\geq 9$ ).

At each time point, venous blood samples were collected between 09:00 and 16:00 due to availability of patients and priorities of patient care. Consequently, not all patients were fasted at the time of sample collection. For cytokine analysis, blood was collected in tubes containing EDTA (i.e., Sarstedt S-Monovette) and kept on ice until processing (i.e., within 30 minutes of collection). Subsequently, samples were centrifuged at 4000rpm for 10 minutes at room temperature on a Hettich EBA 20 centrifuge (Sigma Aldrich, Z601020, UK) and plasma aliquots were immediately stored at -20°C. Samples were transferred on dry ice to -80°C storage within four weeks of collections. For the analysis of routine cellular markers, blood samples were collected in tubes containing EDTA (for white blood cells, neutrophils and lymphocytes) or serum clotting gel (for albumin, CRP

and other soluble markers). Afterwards, the samples were sent to a partner clinical laboratory for analysis as routine blood samples.

A Human Luminex Discovery Assay (R&D Systems, L140655, UK) was used to measure the plasma levels of the following circulating analytes: adiponectin (sensitivity 148.0 pg/ml, standard curve 823 – 200000 pg/ml), leptin (sensitivity 10.2 pg/ml, standard curve 494 – 120000 pg/ml), visfatin (sensitivity 2243.0 pg/ml, standard curve 12200 – 2975000 pg/ml), resistin (sensitivity 3.0 pg/ml, standard curve 53.5 – 13000 pg/ml). The assay was conducted in accordance with the manufacturer's instructions. Briefly, the standards and the samples were prepared using calibrator diluent RD6-52 and added to a 96-well plate with 50µl of diluted microparticle cocktail. Following incubation on a horizontal orbital microplate shaker (2 hours, 800rpm, room temperature), the plate was washed three times using a handheld magnetic device and wash buffer. A total of 50µl of biotin-antibody cocktail was subsequently added to the plate and incubated for one hour under the same conditions as before (800 rpm, room temperature). The plate was washed as previously and incubated for 30 minutes (800 rpm, room temperature) with 50µl of streptavidin-PE. After another wash step, 100µl of wash buffer was added to each well and the plate was incubated for 2 minutes on the shaker (800 rpm, room temperature) to resuspend the microparticles prior to reading the plate using the Luminex 100/200 system (Luminex xMAP Technologies, UK). All samples and standards were analysed in triplicate. The concentration of circulating ITLN1 was determined with an ELISA kit (Abcam, ab269545, UK, sensitivity 0.21 ng/ml, range 0.3 – 20 ng/ml). The assay was conducted according to the manufacturer's directions. In summary, samples and standards were prepared using sample diluent NS. A total of 50µl of antibody cocktail was added to each well and the plate was incubated for one hour at room temperature on a plate shaker set to 400 rpm. Subsequently, the wells were washed three times with 350µl wash buffer and then incubated for 10 minutes (room temperature, 400rpm) with 100µl of TMB development solution. Following this step, 100µl of stop solution was added to each well and mixed on an orbital shaker for one minute (room temperature, 400rpm). Lastly, the optical density was recorded at 450nm on a Synergy LX multi-mode reader (Biotek, USA). Both standards and samples were analysed in triplicate.

### 3.2.3. Data analysis

Patients' data were collected on case record forms and stored using a secure online database. Each participant was identified with a unique trial number. The statistical analysis was performed using the programming language R (version 4.2.1). The R script used to analyse the data can be found in Appendix C.

A 5-parameter log-logistic curve was fit to the standards for each analyte and used to quantify analyte levels in the samples collected from the Luminex instrument. A linear model was used to normalise the optical density values recorded from the ITLN1 ELISA and to determine sample concentrations. In this study, data were reported as mean  $\pm$  standard deviation unless specified otherwise. Bayesian high density intervals (HDI) were used to describe the posterior distributions generated by the statistical models. For example, a 90% HDI refers to 90% of the values from the posterior distribution of a given variable. A Bayesian ordinal probit regression model was developed using the library *rstanarm* to assess whether the continuous variables adiponectin, resistin, ITLN1 and leptin can predict mGPS and cachexia status. The coefficients and the HDIs generated by the model can be used to analyse the relationship between adipokines and outcome variables, to make predictions and to establish the certainty of the estimations [163]. Specifically, a positive coefficient suggests a positive relationship with the response category (i.e., an increase in the predictor determines a rise in the odds of a higher response variable), while a negative coefficient indicates a negative correlation between the predictors and the ordinal variables. The value of the coefficients and the width of the HDIs are key parameters in assessing the precision of the estimates, with narrower HDIs suggesting stronger relationships. Lastly, *rstanarm* was used to design a Bayesian generalised linear model that evaluated if adipokine levels are different between timepoints.

### 3.3. Results

Overall, 23 females and 15 males with a mean age of  $66 \pm 13$  were recruited in the current study. Participants were diagnosed with the following types of cancer: bladder (n=2), breast (n=7), colorectal (n=7), gallbladder (n=1), neck (n=1), lung (n=6), mesothelioma (n=1), pancreas (n=3), prostate (n=3), renal (n=3), skin (n=2) and upper gastrointestinal (n=2). Of these, 4 individuals had locally advanced cancer, while 34 were diagnosed with metastatic cancer. Participants' characteristics are further described in Table 3.1.

**Table 3.1.** Participants' characteristics. Data were collected at baseline and every 6 weeks until the end of life.

	Timepoint 0 (n=38)		Timepoint 1 (n=10)		Timepoint 2 (n=3)	
Weight (kg)	74.1 $\pm$ 19.8		76.3 $\pm$ 16.9		73.3 $\pm$ 16.5	
BMI (kg/m <sup>2</sup> )	25.7 $\pm$ 6.5		26.8 $\pm$ 6.1		26.3 $\pm$ 4.5	
mGPS (n and %)	0	7 (18%)	0	1 (10%)	0	1 (33%)
	1	4 (12%)	1	2 (20%)	1	0 (0%)
	2	27 (71%)	2	7 (70%)	2	2 (67%)
Cachexia status (n and %)	NC	10 (26%)	NC	3 (30%)	NC	1 (33%)
	CC	20 (52%)	CC	2 (20%)	CC	0 (0%)
	UA	8 (22%)	UA	5 (50%)	UA	2 (67%)
Type of therapy received (n and %)	Immuno- therapy	7 (18%)	Immuno- therapy	2 (20%)	Immuno- therapy	2 (67%)
	Chemo- therapy	19 (50%)	Chemo- therapy	1 (10%)	Chemo- therapy	1 (33%)
	Hormonal	8 (21%)	Hormonal	1 (10%)	Hormonal	1 (33%)
Type of medication received (n and %)	Steroids	16 (42%)	Steroids	2 (20%)	Steroids	1 (33%)
	NSAIDS	4 (11%)	NSAIDS	2 (20%)	NSAIDS	1 (33%)
	Antibiotics	6 (16%)	Antibiotics	1 (10%)	Antibiotics	0 (0%)
CRP (mg/l)	77.9 $\pm$ 78.2		69.5 $\pm$ 76.7		25.7 $\pm$ 27.2	
Albumin (g/l)	27.2 $\pm$ 6.0		29.3 $\pm$ 6.6		33.3 $\pm$ 4.7	

Adiponectin (ng/ml)	323.4 ± 124.1	251.8 ± 123.8	280.4 ± 102.7
Leptin (ng/ml)	22.1 ± 27.0	15.1 ± 5.5	10.4 ± 0.8
Intelectin-1 (ng/ml)	19.5 ± 15.1	11.3 ± 5.6	23.0 ± 17.2
Resistin (ng/ml)	14.7 ± 7.1	11.6 ± 6.6	11.1 ± 4.9

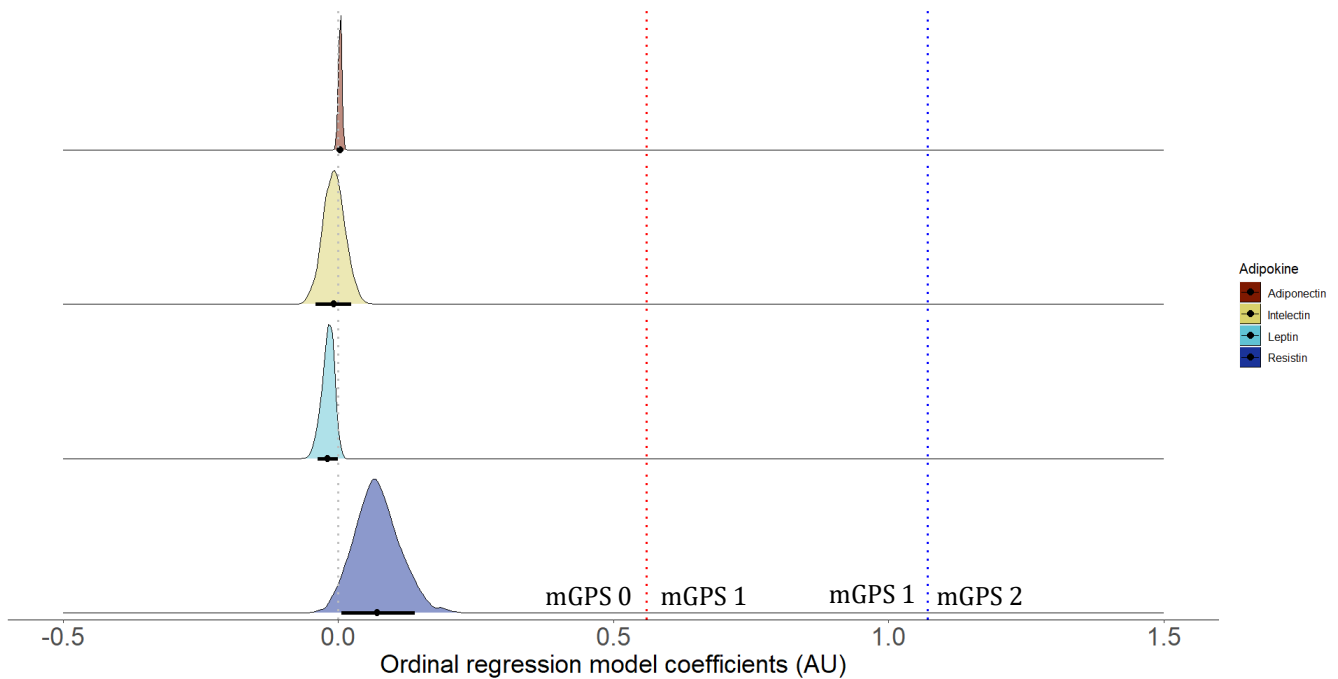
Data are presented as mean± standard deviation. mGPS was assessed using the guidelines proposed by Proctor et al. [138]. Cachexia was determined as per the Fearon et al. [7] consensus: NC = non-cachectic, CC = cancer cachexia, UA = unable to assess. NSAIDS = non-steroidal anti-inflammatory drugs. CRP = C-reactive protein.

As highlighted in Table 3.1, 27% of the individuals that were initially enrolled in the study survived until T1 and only 8% were still alive 12 weeks after the baseline measurements. At each timepoint, approximately 70% of the participants had an mGPS of 2, indicating constant levels of high inflammation and poor prognosis. Furthermore, the baseline measurements suggested that 52% of the individuals were cachectic according to the degree of weight loss. The weight of several participants could not be determined since the palliative care centre had access to a standing scale that could not be used by individuals that were not able to stand up due to their poor condition. Remarkably, cachexia status could not be assessed in half of the patients that were examined at T1. Throughout the study, participants received a variety of therapies (i.e., immunotherapy, chemotherapy, hormonal therapy) and used a wide range of medication (i.e., steroids, antibiotics and non-steroidal anti-inflammatory drugs). Only six of the individuals enrolled in the trial did not receive any form of therapy or medication at baseline. Lastly, multiple adipokines (i.e., adiponectin, leptin, ITLN1, resistin and visfatin) were examined in the current study. Visfatin was excluded from the statistical analysis since all samples were below the lowest level of detection. Moreover, 12 leptin samples were below detection levels (i.e., 9 from T0 and 3 from T1), while two baseline resistin samples exceeded the superior limit of detection.

A Bayesian ordinal regression model was used to determine the relationship between baseline adipokine levels (i.e., adiponectin, leptin, ITLN1 and resistin) and mGPS (Figure 3.2). Overall, adiponectin (0.003±0.002) and resistin (0.071±0.041) were positively associated with mGPS, meaning that a rise in the level of any of these adipokines could

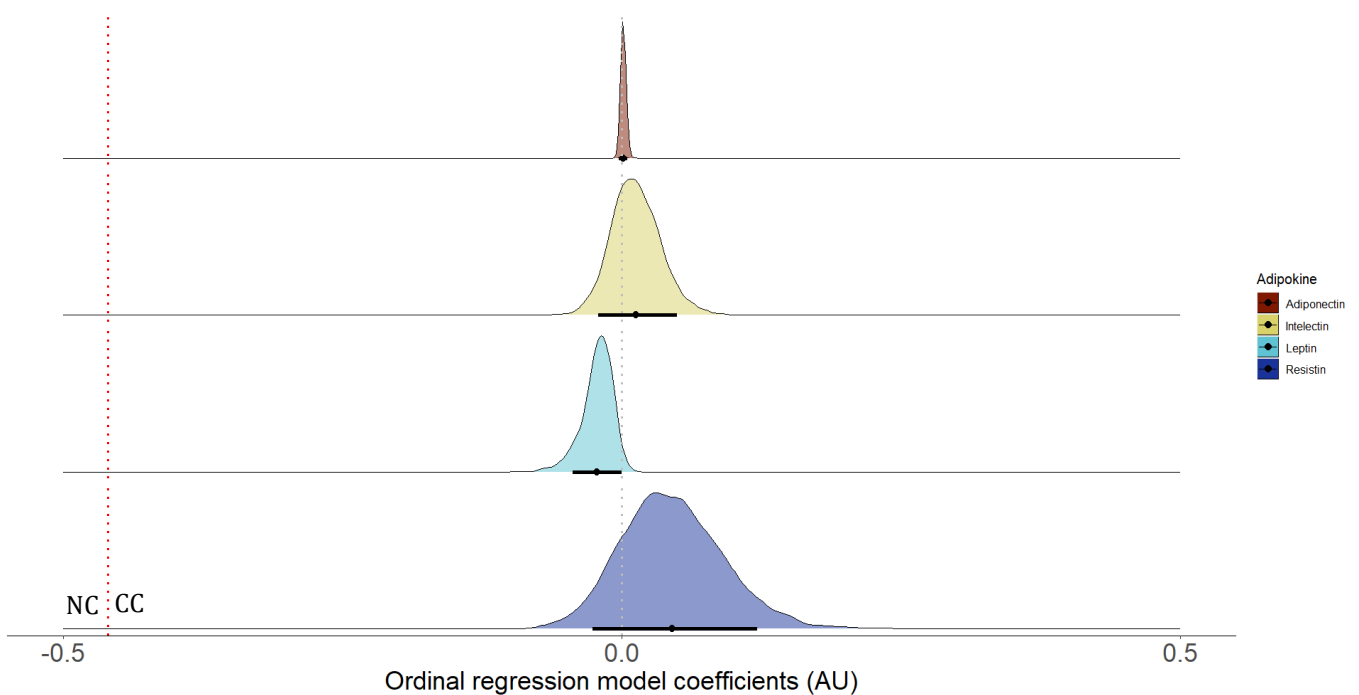


contribute to an increase in mGPS. Conversely, mGPS had a negative relationship with ITLN1 ( $-0.008 \pm 0.019$ ) and Leptin ( $-0.019 \pm 0.011$ ), suggesting that elevated levels of these adipokines could lead to a decline in mGPS. As highlighted in Figure 3.2 and detailed in Appendix D, the 90% HDIs of the posterior distributions of adiponectin and ITLN1 contain 0. This indicates a high degree of uncertainty for the estimates generated by the model. Furthermore, the HDIs of the posterior distribution of resistin and leptin did not include 0, making them the only adipokines that had a considerable effect on mGPS. The mean estimates of the model's intercepts (i.e., 0.56 and 1.07) are represented by the dotted lines in Figure 3.2 (i.e., red and blue). The level of uncertainty around the intercepts was also elevated as indicated by the considerably wide HDIs (Appendix D). Despite the relationships observed between resistin, leptin and the outcome variable, the adipokines examined in the current study cannot be used as predictors of mGPS in this group of patients due to the high level of variability detected for most of the model's parameters.



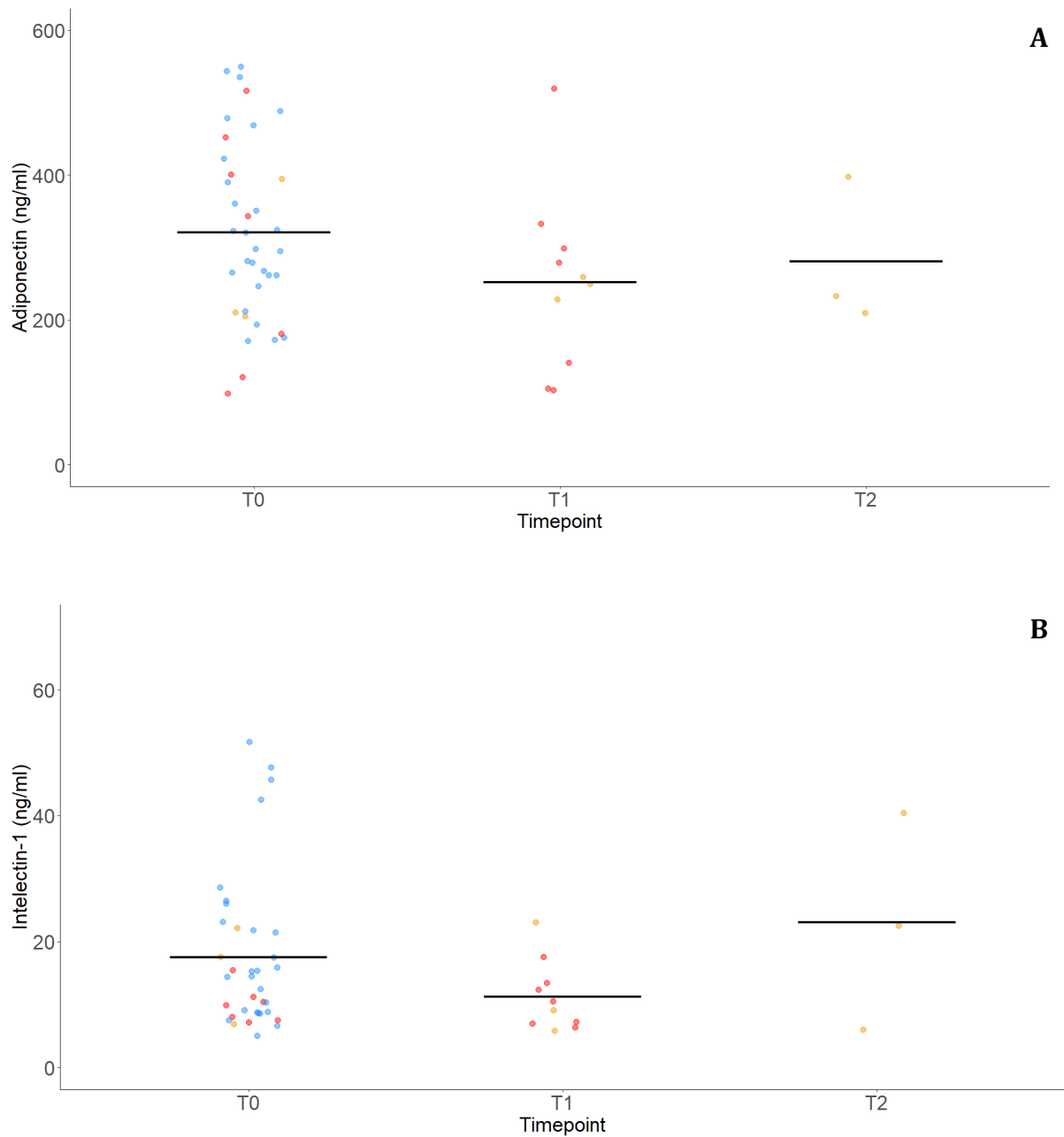
**Figure 3.2.** Posterior distributions of coefficients generated by the model that analysed the relationship between adipokines and mGPS. The red and blue dotted lines represent the boundaries between different mGPS categories (i.e., 0, 1 and 2). The points on the density plots represent the mean of the posterior distribution and the thick lines represent the 90% HDI.

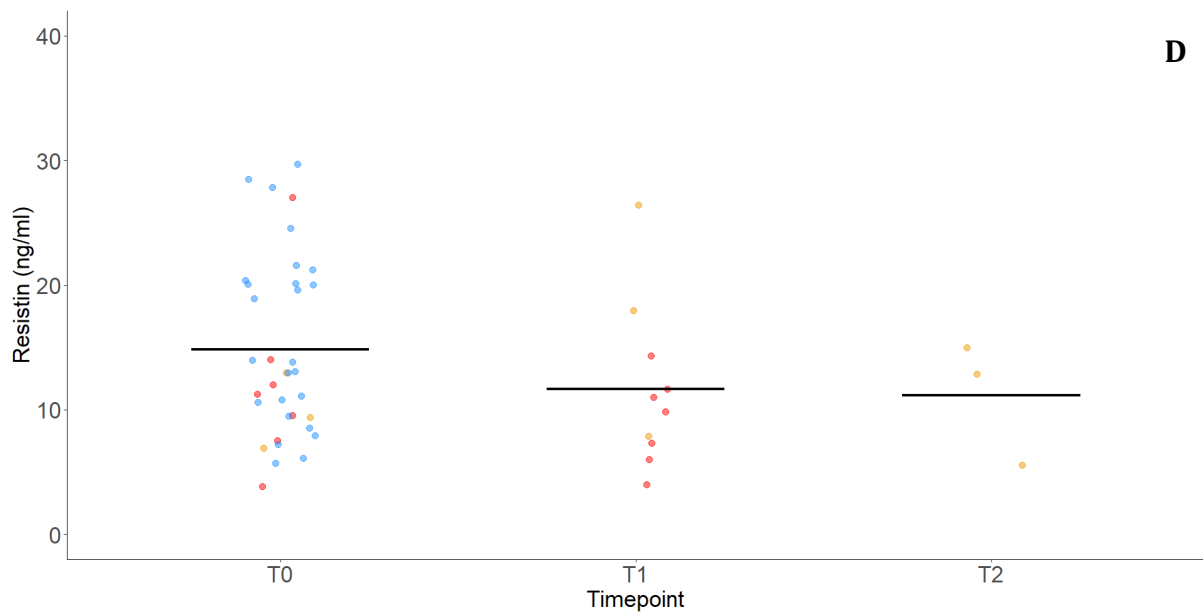
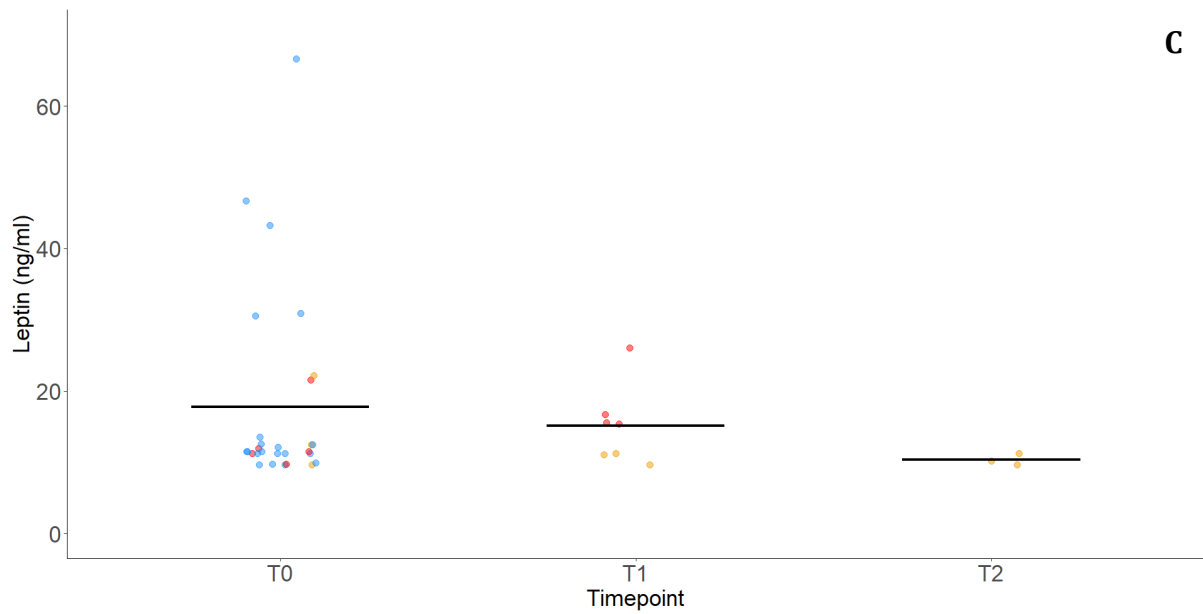
The relationship between cachexia defined according to Fearon et al. [7] and adipokines was also examined using a Bayesian ordinal regression model (Figure 3.3). Adiponectin ( $0.001\pm 0.002$ ), resistin ( $0.045\pm 0.045$ ) and ITLN1 ( $0.013\pm 0.021$ ) showed a positive association with cachexia, suggesting that a rise in the level of these adipokines would contribute to an increase in the probability of being cachectic. The opposite was observed for leptin ( $-0.022\pm 0.014$ ), which showed a negative relationship with cachexia status. The 90% HDIs of the posterior distributions of adiponectin, resistin and ITLN1 contain 0 (Appendix D), indicating that the relationship between these variables and cachexia is weak. However, leptin's posterior distribution does not include 0, making it the only analyte that was considerably associated with cachexia. The high degree of uncertainty around the intercept of this ordinal model (Appendix D) and the elevated variability of most posterior distributions indicate that the examined adipokines could not be used to predict cachexia in this cohort of patients with incurable cancer.



**Figure 3.3.** Posterior distributions of coefficients generated by the model that analysed the relationship between adipokines and cachexia status. The red dotted line represents the boundary between different cachexia categories (i.e., NC = non-cachectic and CC = cancer cachexia). The points on the density plots represent the mean of the posterior distribution and the thick lines represent the 90% HDI.

Figure 3.4 highlights the evolution of adipokines throughout the trial. Although this was beyond the aim of the current study, a Bayesian generalised linear model was used to determine if adipokine levels were different between timepoints. Since the number of patients that survived 12 weeks was low (Table 3.1), T2 was excluded from the analysis. Overall, none of the adipokines was substantially different between T0 and T1 (Figure 3.4). A detailed description of the posterior distributions was reported in Appendix D.





**Figure 3.4.** The levels of adiponectin (A), intelectin-1 (B), leptin (C) and resistin (D) measured at baseline (T0), 6 weeks (T1) and 12 weeks (T2) after enrolment. The blue points represent individuals that were evaluated only at T0, the red dots represent individuals that survived until T1, while the yellow dots represent individuals that survived until T2. The black line indicates the mean level of the analyte at each timepoint.

### 3.4. Discussion

The present study aimed to examine if adipokines could be used as predictors of mGPS and cachexia in patients with incurable cancer. An additional analysis was conducted to determine if the concentration of adiponectin, leptin, ITLN1 and resistin varied between timepoints.

Bayesian ordinal regression indicated that the adipokines analysed in the current study could not predict mGPS in this group of patients due to the high degree of uncertainty observed in most of the model estimates. Interestingly, leptin showed a small negative relationship with mGPS, suggesting that higher inflammation levels are associated with lower leptin concentrations. Leptin is a metabolic hormone with an established role in the regulation of appetite that also contributes to the control of the hypothalamic-pituitary-adrenal (HPA) axis, insulin secretion and energy homeostasis [164]. In addition to these physiological functions, previous research also evaluated the role of leptin in the immune response and its relationship with inflammation. Several rodent models highlighted that a decrease in leptin concentrations led to an impaired immune response [165,166]. In a sample of patients with advanced ovarian cancer, Maccio and colleagues [167] observed a negative correlation between leptin and two markers of inflammation, namely IL-6 and CRP. Another large observational study of patients with various types of cancer [168] observed that mGPS was negatively associated with leptin levels. Although the amount of research that examines the relationship between leptin and inflammation is limited, the direction of the available evidence follows what was reported in the present study. Future work should monitor and report leptin and inflammatory markers in patients with cancer. The role of leptin in the immune response and the causality of the relationship between leptin and inflammation should be further explored by cell culture and animal models. Additionally, statistical modelling suggested the presence of a positive relationship between resistin and mGPS, indicating that increased concentrations of this adipokine can be found in individuals with elevated inflammation levels. The physiological roles of resistin include the regulation of glucose homeostasis, insulin sensitivity and inflammation [169]. Previous in vitro models argued that resistin can act as a pro-inflammatory marker that contributes to an increase in IL-6, IL-1 and TNF- $\alpha$  mRNA expression [170,171]. Additionally, several studies that evaluated individuals with cancer and diabetes [172,173,174] reported a positive correlation

between circulating resistin levels and CRP, TNF- $\alpha$  and IL-6. Although previous literature did not assess the association between resistin and mGPS, the findings of the current study align with reports that suggested the presence of a relationship between resistin and inflammation. To conclude, future predictive models could include leptin and resistin, but should also aim to find other biomarkers that might have a stronger influence on mGPS in patients with cancer.

The current study also examined if adiponectin, leptin, ITLN1 and resistin can be used to predict cachexia status. The uncertainty around the ordinal model coefficients suggested that the adipokines were not accurate predictors of cachexia in this cohort of individuals with incurable cancer (Figure 3.3). The small negative relationship between leptin and cachexia was the only statistically meaningful interaction observed in this analysis. Specifically, the posterior distribution highlighted that lower levels of this adipokine could be found in cachectic patients. In healthy individuals, a decline in leptin concentrations would typically determine an increase in appetite. The current study and past reports [154,175] identified that leptin levels were downregulated in cancer cachexia. Yet, the appetite of cachectic patients is typically low and negative energy balance (i.e., low food intake and high energy expenditure) is amongst the main drivers of this wasting syndrome. Bing and colleagues [176] suggested that the lack of a compensatory improvement in appetite in cancer cachexia (despite low leptin concentrations) could be determined by the elevated levels of hypothalamic leptin receptors that are the result of the action of various tumour products. Although this is a reasonable hypothesis, our systematic review (Chapter 2) indicated that previous studies [115, 121] failed to observe a relationship between leptin and cachexia in patients with incurable cancer. The research on leptin's role in cachexia is still in the early phases. More clinical data are required to better understand the fluctuations of this adipokine and rodent models could be developed to provide further insights into its mechanisms of action.

Various additional observations can be made in relation to the data examined in the current study. The level of adiponectin, leptin, ITLN1 and resistin was compared between timepoints using Bayesian linear modelling. It was observed that none of the analytes was considerably different between T0 and T1. Although data were insufficient to generate a comprehensive overview of the adipokines' trajectory across all timepoints, conducting

prospective studies that include multiple measurements of relevant cachexia biomarkers is an essential step towards a better understanding of this wasting syndrome. Another notable aspect is the fact that the concentration of visfatin was below the level of detection in all samples. It could be argued that a faulty assay led to the abnormally low visfatin levels, but other samples (i.e., leptin and resistin) also expressed values that were not within the detection range. Previous research [177] indicated that is not uncommon for circulating cytokines to be undetectable. Anderson and colleagues [178] reported that IL-1 $\beta$  was not detectable in a subgroup of cancer patients, while Christian et al. [179] suggested that the same cytokine was below the level of detection in 39% of the individuals examined in their study. Another investigation [180] reported that IL-6, IFN- $\gamma$  and IL-10 were not detectable in approximately 13% of the participants. Indeed, it is essential to report and discuss analytes that were outside the detection limits of an assay. However, a great proportion of the available literature fails to report insignificant and undetectable variables (Chapter 2). Changing this modus operandi would enable future research to determine which cytokines can provide reliable data (i.e., by selecting the analytes that show the lowest level of variability) and to identify technologies (i.e., assays and machinery) that can be used for optimal results.

This study also has several limitations that are subsequently discussed. There was a drastic decrease in the number of participants, with only 3 individuals examined 12 weeks after baseline. The limited availability of T2 data led to the exclusion of this timepoint from the analysis and hindered the possibility of statistically interpreting the longitudinal evolution of adipokines. Although it is expected to see a decline in the number of patients given the population analysed in the present study, a better characterisation of the cachectic phenotype could be obtained by including more frequent measurements. Moreover, the blood samples were collected between 09:00 and 16:00 due to the availability of patients and priorities of patient care and thus, a mix of fasted and non-fasted individuals was included in the current study. As emphasised in Chapter 2, (adipo)cytokines can be affected by food intake and show diurnal variation. This had a negative effect on the reliability of the data and future trials should aim to improve the methodological quality by imposing stricter blood collection protocols.

To conclude, adiponectin, leptin, ITLN1 and resistin could not be used as predictors of mGPS and cachexia in this study that examined a heterogeneous cohort of patients with

various types of cancer. Leptin's negative relationship with mGPS and cachexia as well as the positive correlation observed between resistin and mGPS should be explored by additional studies. Although statistically relevant, these relationships are not considerably strong and future analyses should aim to find other biomarkers that can be integrated into a predictive model alongside leptin and resistin. Future research should also aim to include multiple and more frequent measurements, to increase the initial sample size and to improve methodological quality.



## 4. A Bayesian meta-analysis determining intelectin-1 differences between healthy individuals and patients with cancer

Understanding the behaviour of different biomarkers in various types of cancer is essential. The following section does this by meta-analysing the available literature on the adipokine ITLN1. Examining ITLN1 differences between cancer patients and healthy individuals as well as estimating the adipokine's physiological concentration are crucial steps towards mapping its role in cancer. This chapter was submitted to the *Molecular Targets & Therapeutics* section in the journal *Frontiers in Oncology*.

### 4.0. Abstract

**Background:** Intelectin-1 (ITLN1) is an adipokine with multiple physiological functions, including a role in tumour formation and development. Previous research reported variable ITLN1 levels for cancer patients and healthy individuals. This study aimed to compare ITLN1 concentrations between controls and cancer patients and to determine the adipokine's physiological level.

**Methods:** Five databases were searched for studies that measured the level of ITLN1 in adults that were healthy or diagnosed with any type of cancer. After title, abstract and full-text screening, the methodological quality of the studies was assessed. The extracted data were meta-analysed using the R language and Bayesian statistical techniques.

**Results:** Overall, 15 studies compared circulating ITLN1 levels between healthy individuals (n=3424) and cancer patients (n=1538), but no differences were observed between these studies. ITLN1 was not different between groups in an analysis that evaluated high-quality studies only (n=5). The meta-analysis indicated considerably higher ITLN1 levels in gastrointestinal (i.e., colorectal, pancreatic, gastric) cancer compared to controls, while the other cancer types did not demonstrate differences between groups. The mean ITLN1 level of healthy individuals was  $234 \pm 21$  ng/ml (n=136), while the average value in high-quality studies (n=52) was  $257 \pm 31$  ng/ml.

**Conclusion:** Different types of cancer showed different circulating ITLN1 patterns. Circulating ITLN1 concentration was higher in gastrointestinal cancer compared to controls, with strong support from the meta-analytical model. Our analysis also determined the mean ITLN1 level in healthy individuals; this is a crucial starting point for understanding how this cytokine associates with diseases. Two-thirds of the studies were of low methodological quality and thus, future work in this field must focus on improved methods.

## 4.1. Introduction

ITLN is an immune lectin that contains a fibrinogen-like domain and a unique intelectin-specific region [45,46]. ITLN1 and ITLN2 are two homologs that share 83% amino acid identity. Both ITLN1 and ITLN2 can bind to microbial glycan chains but not to human glycans and thus, ITLN may have a role in antimicrobial defence [46,50]. ITLN is largely produced by stromal vascular fraction cells in VAT [181]. Furthermore, low levels of ITLN1 were found in SAT, epicardial fat, lungs, renal collecting tubes, colon and the small intestine, while ITLN2 was primarily expressed in intestinal Paneth cells [46,47,48,49].

Previous research indicated that circulating ITLN1 concentrations differ between cancer patients and healthy individuals [41,42,43,51]. Indeed, in our recent narrative review (Chapter 1.3), we observed a difference in circulating ITLN1 levels when people with cancer were compared to healthy individuals. The difference was influenced by cancer type since patients with gastrointestinal and prostate cancer showed higher concentrations of circulating ITLN1, while individuals with breast, bladder, renal and gynaecological cancer expressed lower circulating ITLN1 levels. We also noted a relationship between cancer cachexia and local but not circulating ITLN1 [44]. The same study indicated that ITLN1 mRNA and protein concentrations were substantially elevated in the VAT of patients with gastrointestinal cancer. Other research that measured gene expression suggested that tumour levels of ITLN1 were significantly different from the concentrations observed in healthy tissue [43,59]. ITLN1 can activate the PI3K/Akt pathway [80,81] and the improper regulation of this pathway can determine a cascade of events that favour cancer development and progression. Also, obesity was associated with an increased risk of tumour formation [90] and proposed as a factor that influences the relationship between ITLN1 and cancer [84,89]. The decrease of ITLN1 levels observed in overweight and obese individuals could be a marker of the metabolic effects of obesity, contributing to a deregulation of the PI3K/Akt pathway [155]. Therefore, ITLN1 could be a potentially important biomarker in cancer biology. Its mechanisms of action as well as concentration differences between cancer patients and healthy individuals should be further explored and explained.

Importantly, the physiological level of ITLN1 remains uncertain as the adipokine's reported concentrations are highly variable in both cancer patients and healthy individuals. Some variability is to be expected when ITLN1 is measured in cancer

patients. Indeed, Arjmand et al. [52] examined the relationship between ITLN1 and cancer in a systematic review and observed that concentrations ranged from 2 to 1100 ng/ml. Whilst comprehensive, their review had some methodological weaknesses (Chapter 1.3). In healthy people, Watanabe et al. [49] reported that circulating ITLN1 levels vary from 5 to 800 ng/ml. This range is close to that reported by Arjmand et al. [52] in people with cancer. There has been no discussion so far about this wide range of ITLN1 values observed across different studies. Determining the source of variability and the physiological concentration of ITLN1 would allow useful comparisons between healthy individuals and patients with different conditions, in which adipokine levels are typically dysregulated. Quantification of a mean physiological concentration would also set the benchmark for future cell culture experiments and animal studies. Therefore, this systematic review aims to compare ITLN1 concentrations between cancer patients and healthy controls and to determine the mean level of ITLN1 in healthy individuals.

## **4.2. Methods**

The protocol of this systematic review is described below and was also registered on PROSPERO on 18/01/2022 (registration number CRD42022303406). Ethical approval was not required for this study.

### **4.2.1. Search strategy**

The following databases were searched on 18/01/2022: MEDLINE, EMBASE, CENTRAL, CINAHL and Web of Science. The search strategy (i.e. intelectin\* OR omentin\*) was designed to capture all studies measuring intelectin or omentin (including related terms), without imposing any additional criteria. Appendix E includes a detailed version of the search strategy and the number of studies extracted from each database.

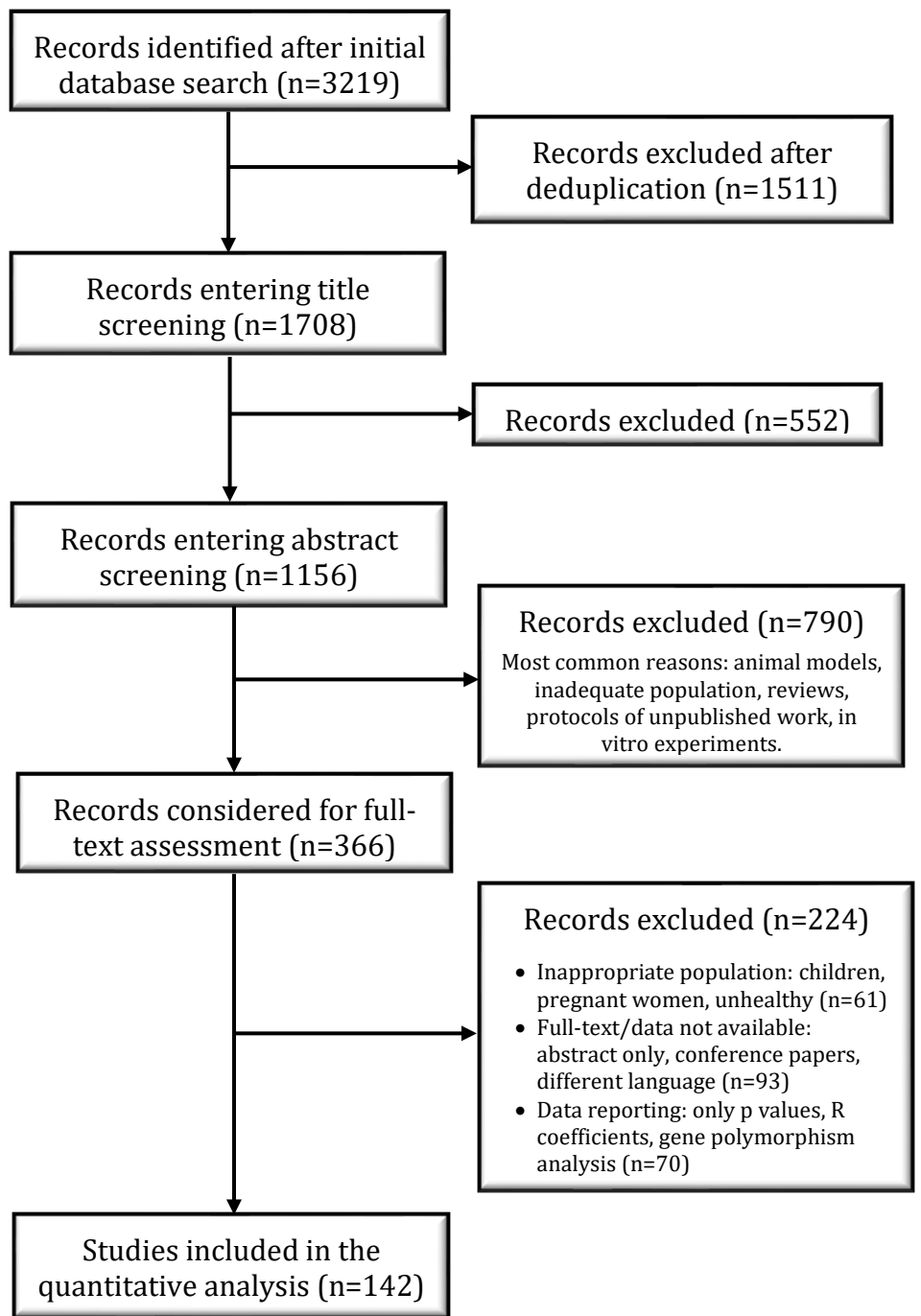
### **4.2.2. Inclusion and exclusion criteria**

Eligible studies measured circulating and tissue ITLN1 protein levels of adults that were either healthy or diagnosed with any type of cancer. All types of cancer were included in the current review. Studies that examined healthy participants were included if enough details were given to make an objective assessment of participants' health status. Healthy participants did not have any medical conditions that could affect ITLN1 levels. Studies were not included in the analysis if participants' health status could not be accurately assessed or if the details given were limited or unclear. No criteria were imposed on the design of the studies as long as a measurement of ITLN1 was included. This review did not include any studies that measured ITLN1 protein in pregnant women or children. Moreover, animal models, protocols or conference abstracts were excluded from the current review.

### **4.2.3. Study selection and data extraction**

The PRISMA flow diagram (Figure 4.1) highlights the study selection process that was independently conducted by RP. The titles were screened in a conservative manner – if the title did not provide enough information, the study was included in the next phase of

selection. A similar approach was used for abstract screening; after this step, all suitable studies were considered for full-text screening. At the end of the full-text screening, relevant data were extracted from the included studies with a specifically designed collection form (Appendix F). The number of participants, the mean age and BMI, the method used to collect ITLN1, the description of the assay used to quantify ITLN1 and recorded ITLN1 levels were extracted from all studies. Additionally, the type of cancer, the stage of the disease as well as the treatment received by participants were extracted from the studies that evaluated people with cancer. The authors of eligible primary studies were contacted whenever additional data were required.



**Figure 4.1.** PRISMA flow diagram of the study selection process

#### 4.2.4. Study quality and data analysis

Only a small number of studies measured tissue concentration of ITLN1 and thus, the current meta-analysis focused on circulating levels. The timing of the blood sample and the characteristics of the assay used to quantify ITLN1 were extracted and used to assess study quality. Firstly, for the purpose of this meta-analysis, an essential criterion was that studies collected blood in the morning after an overnight fast, as previous research [116,117] indicated that (adipo)cytokines are affected by diurnal variation. Secondly, given the variable concentration of ITLN1, it was crucial that studies mentioned the assay used to quantify ITLN1, described its characteristics (e.g., sensitivity, range) and reported data that were in accordance with the assay parameters (e.g., within detection limits). The data extracted from studies that adhered to the previously mentioned methodological standards were considered high-quality data. Conversely, studies that failed to report all or any of the characteristics described earlier were not included in the high-quality studies group.

All data were analysed using the R programming language, version 4.2.1 [182]. Most of the studies reported ITLN1 in the ng/ml range and used the mean and standard deviation (SD) to report the concentrations. When this was not the case, data were converted to mean and SD [183] and to ng/ml. Throughout this study, data are reported as mean  $\pm$  SD unless otherwise specified.

The present study used a Bayesian approach to analyse the available data. The R package RoBMA (Robust Bayesian Meta-Analysis) was used to compare standardised mean differences (SMDs) between cancer patients and healthy individuals [184]. The package accounts for potential publication bias in the statistical model and uses Bayesian model averaging to compare meta-analytic models that assume the presence or the absence of an effect, heterogeneity and publication bias. Bayes' factors (BFs) and model-averaged values determined from posterior model probabilities are used to indicate the magnitude of the relationships [185]. BFs compare two models and express the relative strength of the evidence in favour of one of the two models that are compared (i.e., null model versus alternative model, experimental group versus control group). Kass and Raftery [186] provide a scale for the interpretation of BFs. In this review, the BF indicates the relative evidence that e.g., ITLN1 levels are higher in one group compared to the other. Bayesian model averaging provides a principled method to make probability-weighted averages



from several possible models [187]. In this study, the meta-analytic estimate of ITLN1 level was made by averaging over each of the models considered by RoBMA. Furthermore, the package brms [188] was used to determine mean ITLN1 levels in healthy individuals by fitting a normal-normal hierarchical model to the available data [177]. Bayesian HDIs were also used to describe and summarise the uncertainty associated with the model-estimated parameters. For example, a 95% HDI represents the range of the posterior distribution that contains 95% of the values determined by the meta-analytical model. The dataset and the R code used to examine the data are included in Appendix F.

## 4.3. Results

### 4.3.1. Study characteristics

A total of 1708 studies that measured ITLN1 levels were identified after the removal of duplicates and were included in the title screening phase (Figure 4.1). Subsequently, 1156 abstracts were examined, of which 366 records were considered for the full-text assessment. At the end of the screening phases, data from 142 studies were extracted and statistically examined. Of these, 15 studies were included in a meta-analysis comparing ITLN1 levels between healthy individuals and cancer patients. A further 5 studies measured ITLN1 levels in cancer patients but did not include a healthy control group – these were not examined in the meta-analysis but were used to characterise the role of ITLN1 in different types of cancer. Additionally, data from 138 studies that measured circulating ITLN1 in apparently healthy individuals were used to estimate a Bayesian HDIs for the physiological level of circulating ITLN1 in healthy individuals.

The characteristics of the studies that measured ITLN1 in cancer patients are described in Table 4.1. Overall, 1538 patients and 3424 controls were included in the meta-analysis that compared ITLN1 levels between cancer patients and apparently healthy individuals. A further 342 patients were recruited in the 5 additional studies that measured circulating ITLN1 in cancer patients but did not include a control group. The mean age of individuals with cancer was  $60 \pm 7$  (mean  $\pm$  SD), with female patients representing 50% of the sample. The most common types of cancer were breast, colorectal, prostate and pancreatic cancer, although other types such as lung and ovarian cancer were also assessed. Since some cytokines can show intra-day variation [116,117] and having consistent methodological approaches is essential for meta-analyses, the timing of blood sampling was a factor that played a key role in assessing the quality of the included studies. A total of 10 studies collected blood in the morning after an overnight fast, 3 studies reported that blood was collected in a fasted state (without mentioning the period of the day), 1 study suggested that blood was collected in the morning (but did not indicate whether participants fasted), 3 studies stated non-specific/opportunistic time of blood collection without giving further details (i.e., pre-operative, at hospital admission) and 3 studies did not report collection methods. Furthermore, the majority (19/20) of the studies used an ELISA to quantify the level of circulating ITLN1, with only one study [71] using a multiplex immunoassay (Table 4.1). Overall, only 8 studies adequately

described the characteristics of the assay (Table 4.1), while 5 studies did not report any characteristics (e.g., sensitivity, range) and 7 were inconsistent (e.g., reported data were outside the assay range) or failed to give sufficient details (e.g., the manufacturer's reported intra- and inter-assay coefficient of variation). Interestingly, only 5 studies passed the methodological quality control and provided high-quality data [56,66,73,190,191] as the assay characteristics were reported adequately and the blood was collected in the morning, after an overnight fast.

**Table 4.1.** Characteristics of studies that measured circulating ITLN1 in cancer patients.

Study (year)	Cancer type	Participants (n)	ITLN1 level (ng/ml)	Blood collection method	Assay method	Assay details reported
Alaee et al. (2016) [67]	Breast	Patients (30)	73.1±29.7	N/R	ELISA	Inconsistent
		Controls (30)	108.8±65.4			
		Data shown as mean ± SD				
Aleksandrova et al. (2016) [41]	Colorectal	Patients (251)	459 (379-570)	N/R	ELISA	Yes
		Controls (2295)	396 (328-486)			
		Data shown as median (IQR)				
Karabulut et al. (2016) [42]	Pancreatic	Patients (33)	9.6 (3.6-219.5)	At hospital admission	ELISA	No
		Controls (33)	1.6 (0.8-5.0)			
		Data shown as median (range)				
Shen et al. (2016) [56]	Renal	Patients (41)	3.6±0.8	Fasting	ELISA	Yes
		Controls (42)	9.9±1.4			
		Data shown as mean ± SD				
Zhang et al. (2016) [55]	Bladder	Patients (42)	1.8 (0-9.2)	Overnight fast	ELISA	Inconsistent
		Controls (42)	5.2 (2.3-13.2)			
		Data shown as median (IQR)				
Yildiz et al. (2017) [161]	Ovarian	Patients (41)	43.8 ± 19.1 <sup>^</sup>	N/R	ELISA	Yes
		Controls (41)	37.4±12 <sup>^</sup>			
		Data shown as mean ± SD				
Khademi-Ansari et al. (2018) [73]	Lung	Patients (45)	3.6 ± 0.7*	Fasting	ELISA	Yes
		Controls (31)	3.6±0.6*			
		Data shown as mean ± SD				
Kiczmer et al. (2018) [56]	Pancreatic	Patients (20)	582.5 (422.6-663.7)	Overnight fast	ELISA	Yes
		Controls (18)	461.7 (345.4-494.4)			
		Data shown as median (IQR)				
Nourbakhsh et al. (2018) [68]	Breast	Patients (45)	157±66*	Overnight fast	ELISA	No
		Controls (45)	217±75*			
		Data shown as mean ± SD				
Zhao et al. (2019) [53]	Colorectal	Patients (358)	67.3±32.3	Overnight fast	ELISA	Insufficient
		Controls (286)	33.2±20.0			
		Data shown as mean ± SD				

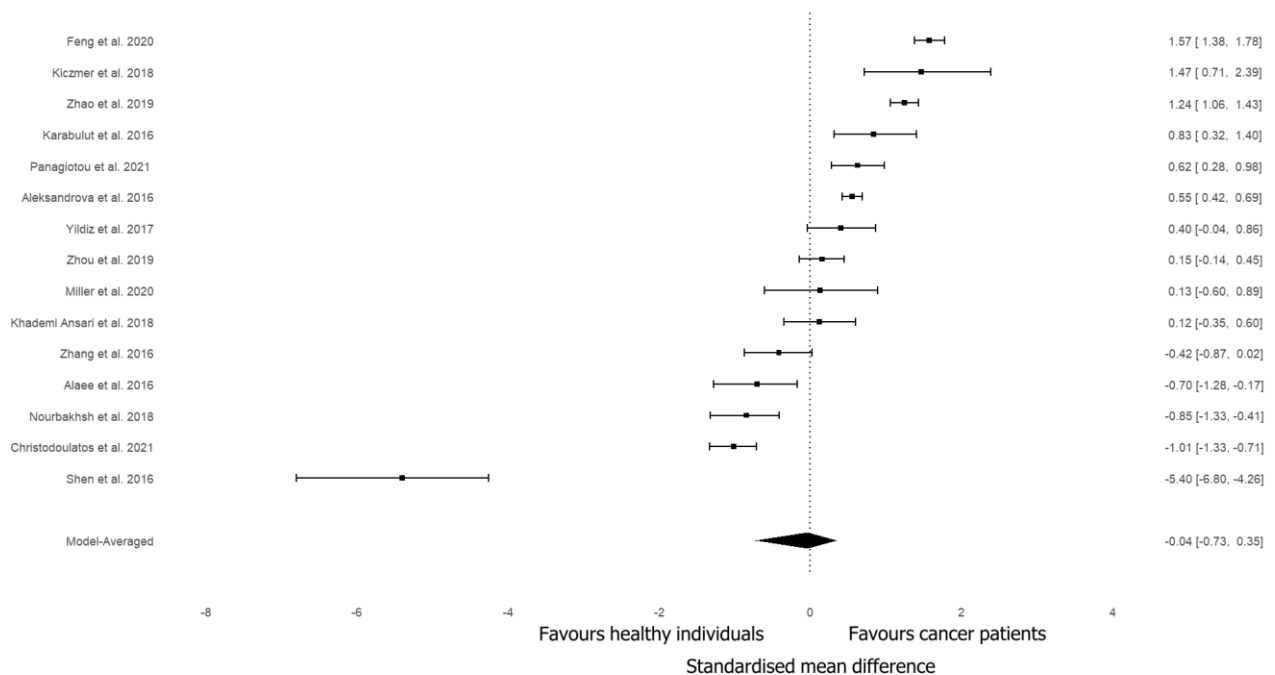
Zhou et al. (2019) [51]	Prostate	Patients (90)	12.9±6.15	Overnight fast	ELISA	Inconsistent
		Controls (90)	5.0±4.7			
		Data shown as mean ± SEM				
Feng et al. (2020) [54]	Colorectal	Patients (319)	69.3±23.5	Overnight fast	ELISA	No
		Controls (300)	37.9±15.4			
		Data shown as mean ± SD				
Miller et al. (2020) [44]	Gastrointestinal	Patients (12)	3.8±6.4	At induction of anaesthesia	ELISA	Yes
		Patients (12)	1.9±0.8			
		Controls (12)	2.3±1			
		Data shown as mean ± SD				
Christodoulatos et al. (2021) [190]	Breast	Patients (103)	340.5±109.3	Overnight fast	ELISA	Yes
		Controls (103)	476.7±156.1			
		Data shown as mean ± SD				
Panagiotou et al. (2021) [191]	Breast	Patients (72)	567.7±236.2	Overnight fast	ELISA	Yes
		Patients (24)	589.0±256.3			
		Controls (56)	436.6±173.7			
		Data shown as mean ± SD				
<b>No control group</b>						
Uyeturk et al. (2014) [62]	Prostate	Patients (50)	547.8 (297.1-945.7)	Overnight fast	ELISA	Inconsistent
		Data shown as median (range)				
Cymbaluk-Ploska et al. (2018) [71]	Endometrial	Patients (92)	610.1 (218.5-13377)	Pre-operative	Multiplex immunoassay	No
		Data shown as mean (range)				
Fryczkowski et al. (2018) [63]	Prostate	Patients (40)	478.8 (398.2-584.7)	Overnight fast	ELISA	Inconsistent
		Data shown as median (IQR)				
Borowski and Sieminska (2020) [64]	Prostate	Patients (72)	594.3±266.9	Morning	ELISA	No
		Data shown as mean ± SD				
Tahmasebpour et al. (2020) [43]	Breast	Patients (88)	132.3±9.1*	Fasting	ELISA	Insufficient
		Data shown as mean ± SD				

SD = standard deviation; IQR = interquartile range; ELISA = enzyme-linked immunosorbent assay

\*Data reported as ng/l; ^Data reported as pg/ml

### 4.3.2. ITLN1 differences between cancer patients and healthy individuals

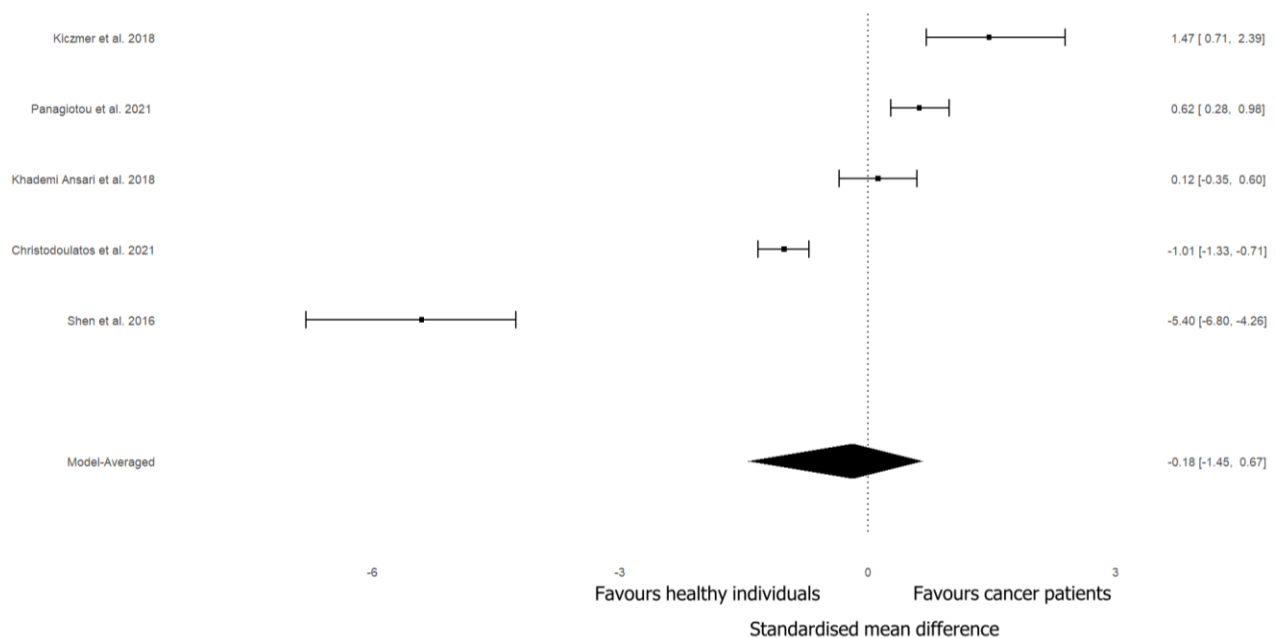
A meta-analysis was conducted to evaluate whether the circulating levels of ITLN1 varied between healthy individuals and cancer patients (Figure 4.2). The analysis found weak evidence [186] in favour of higher ITLN1 values in the control group (BF=0.4) when all types of cancer were examined (SMD=-0.04; 95%HDI: -0.74 to 0.35). Additionally, there was a very high level of evidence in favour of heterogeneity (BF>10000) but low evidence of publication bias (BF=0.5).



**Figure 4.2.** Forest plot showing meta-analytic results for the comparison of ITLN1 levels in cancer patients and healthy individuals. Model-averaged data are presented as SMD and 95% HDI.

A sensitivity analysis was conducted to check if an outlying study [66] had an impact on the overall results (Appendix G, Figure 4.9). Although a minor change was observed in the SMD between groups (SMD=0.06, 95%HDI: -0.15 to 0.52) after removing the study, the evidence in favour of a difference between groups was still weak (BF=0.4). The level of heterogeneity was consistently high (BF>10000), while the evidence of publication bias remained weak

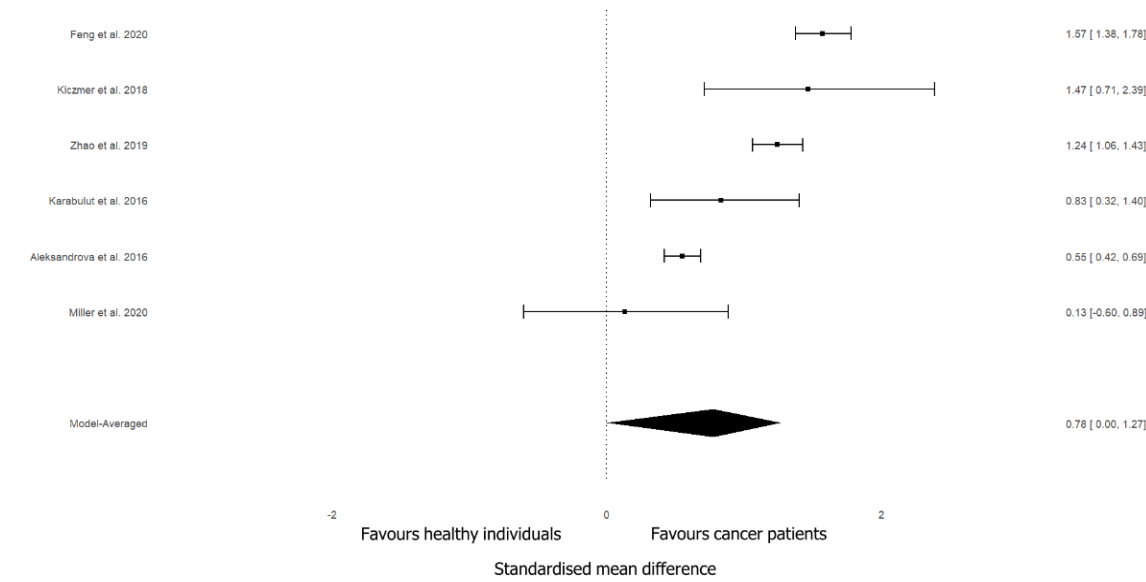
(BF=0.9). Consequently, the study by Shen and colleagues [66] did not have a considerable impact on the overall results of the current meta-analysis. High-quality studies were extracted and analysed separately to examine if study quality had an impact on the meta-analytic results. The mean effect size (SMD=-0.18, 95%HDI: -1.45 to 0.67) and the strength of the available evidence (BF=0.8) was not substantially different from the original model, indicating that ITLN1 levels are not different when all people with cancer are compared to healthy individuals (Figure 4.3). Similar to the previous models, there was strong evidence in favour of a high degree of heterogeneity (BF>10000) and low support for publication bias (BF=0.7).



**Figure 4.3.** Forest plot showing meta-analytic results for high-quality studies that compared ITLN1 levels in people with cancer and healthy individuals. Model-averaged data are presented as SMD and 95% HDI.

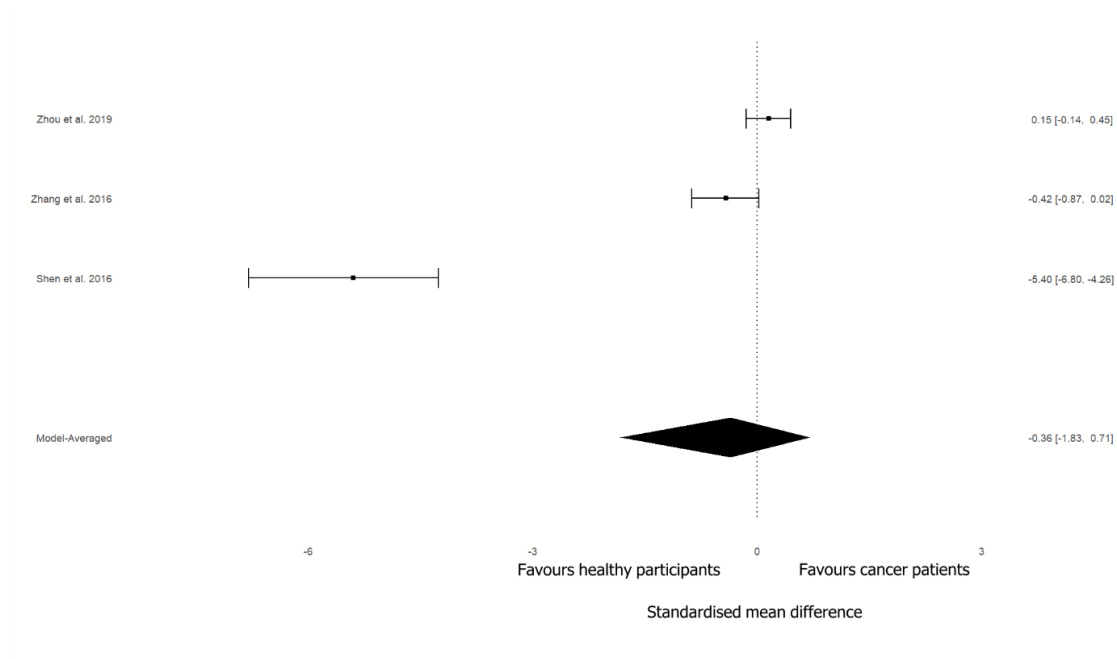
In our previous narrative review [155] we described how the level of ITLN1 varies depending on cancer type. Consequently, a subgroup analysis was conducted to evaluate circulating ITLN1 differences between healthy participants and people with various types of cancer (i.e., gastrointestinal, urological and breast). As observed in Figure 4.4, there was

strong evidence (BF=10.5) in favour of higher levels of ITLN1 in people with gastrointestinal cancer (i.e., colorectal, pancreatic, gastric) compared to healthy controls (SMD=0.77, 95%HDI: 0.00 to 1.27). However, there was only weak evidence that the concentration of ITLN1 was different between groups when individuals with urological cancer (Figure 4.5) were compared to healthy participants (SMD=-0.36, 95%HDI: -1.83 to 0.71, BF=1.2). Furthermore, women with breast cancer (Figure 4.6) had lower ITLN1 levels than healthy individuals (SMD=-0.20, 95%HDI:-1.01 to 0.17), but the available evidence was again (BF=0.9). All subgroup analyses showed very strong evidence in favour of high heterogeneity levels (BF>10000). The evidence for publication bias was weak for patients with gastrointestinal (BF=0.8), urological (BF=0.5) and breast cancer (BF=1.0).

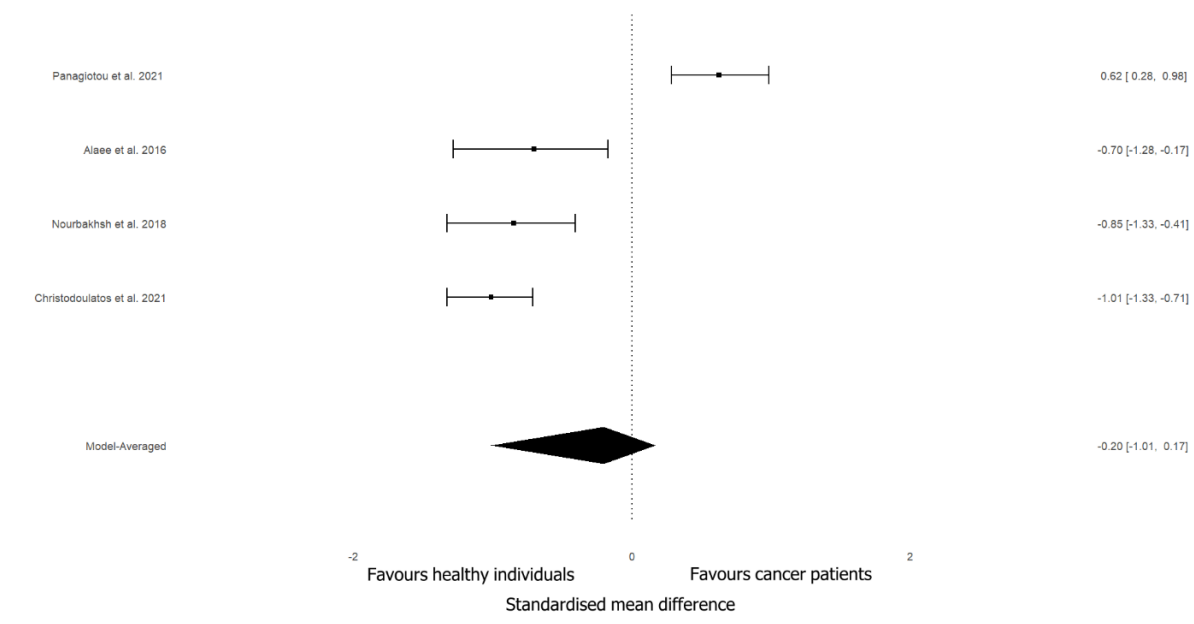


**Figure 4.4.** Forest plot showing meta-analytic results for studies that compared ITLN1 levels in people with gastrointestinal cancer and healthy individuals. Model-averaged data are presented as SMD and 95% HDI.





**Figure 4.5.** Forest plot showing meta-analytic results for studies that compared ITLN1 levels in people with urological cancer and healthy individuals. Model-averaged data are presented as SMD and 95% HDI.



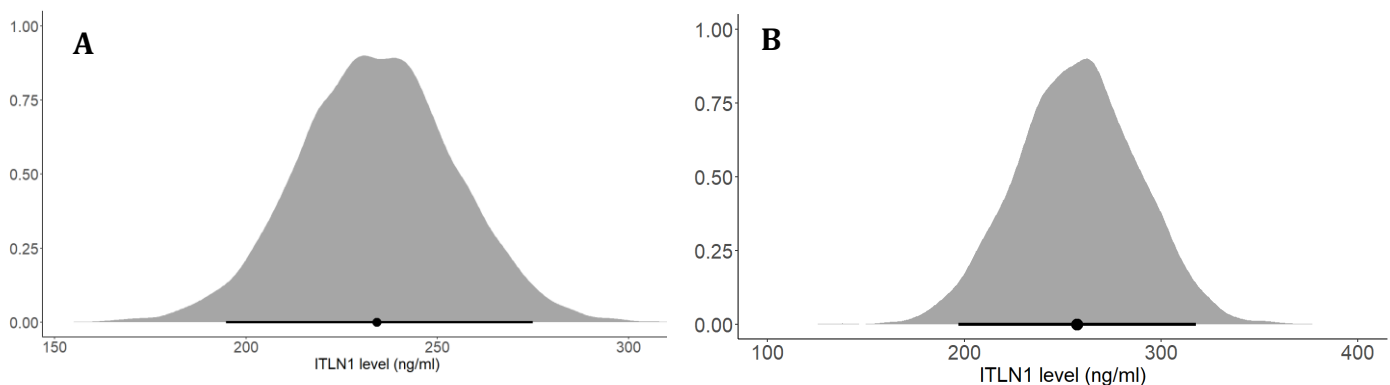
**Figure 4.6.** Forest plot showing meta-analytic results for studies that compared ITLN1 levels in people with breast cancer and healthy individuals. Model-averaged data are presented as SMD and 95% HDI.

### 4.3.3. Determining the level of ITLN1 in healthy individuals

In addition to the previously mentioned 15 studies that compared people with cancer to healthy individuals (Table 4.1), another 148 studies that measured ITLN1 levels in healthy individuals were included in this analysis. The mean age of healthy individuals was  $44 \pm 12$ . Of the 163 included studies, 48 examined only females, 19 measured male participants only, while the remaining 96 evaluated a combination of females and males.

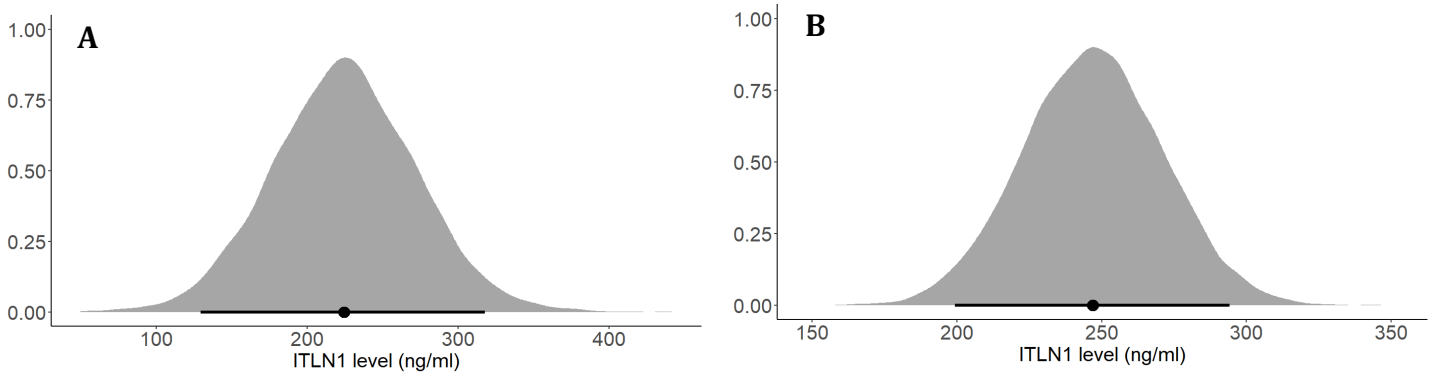
The vast majority of the available literature as well as the results observed by the authors of the present report in clinical samples indicate that ITLN1 values are in the ng/ml range. Consequently, ITLN1 levels smaller than 1 ng/ml (i.e., difficult to measure) or greater than 2000 ng/ml (i.e., likely hyper-physiological) were excluded from the current analysis. After removing the studies containing unreasonable ITLN1 values, a total of 136 studies were subsequently examined. Of these, 52 were considered to provide high-quality data according to the criteria imposed by the quality assessment. Appendix F includes a summary table with all studies that measured ITLN1 in healthy individuals.

The mean ITLN1 level in 10118 healthy individuals (Figure 4.7A) was  $234 \pm 21$  ng/ml (95%HDI: 193 to 275). The average concentration of ITLN1 was similar when examining high-quality studies ( $n=3301$ ) only (Figure 4.7B):  $257 \pm 31$  ng/ml (95%HDI: 195 to 318). Based on the available data, it can be argued that circulating ITLN1 ranges from 195 ng/ml to approximately 318 ng/ml in healthy individuals.



**Figure 4.7.** Posterior distribution of ITLN1 levels in healthy individuals for all studies (A) and high-quality studies only (B). The point represents the mean of the posterior distribution and the line represents the 95% HDI.

Several subgroup analyses were conducted to determine whether the participants' gender or BMI affects ITLN1 concentrations. There was no substantial difference in ITLN1 levels between females ( $249 \pm 34$  ng/ml) and males ( $251 \pm 53$  ng/ml). Additionally, participants were grouped according to their BMI (Figure 4.8). Since none of the participants had a BMI < 20, healthy individuals were divided into two groups: BMI  $\leq 25$  and BMI > 25. Interestingly, ITLN1 concentrations were slightly lower in participants with BMI  $\leq 25$  ( $224 \pm 48$  ng/ml) than in participants with BMI > 25 ( $246 \pm 24$  ng/ml).



**Figure 4.8.** Posterior distribution of ITLN1 levels in healthy individuals with a BMI  $\leq 25$  (A) and a BMI > 25 (B). The point represents the mean of the posterior distribution and the thick line represents the 95% HDI.

## **4.4. Discussion**

### **4.4.1. Main findings**

The present study aimed to evaluate the differences in ITLN1 levels between people with cancer and healthy individuals and to establish the physiological concentration of circulating ITLN1 in healthy individuals. The meta-analysis suggested that circulating ITLN1 concentrations were not different between groups when multiple types of cancer were combined in the analysis (Figure 4.2). The same was observed in the sensitivity analysis (Appendix G, Figure 4.9) as well as in the analysis based on study quality (Figure 4.3). Another systematic review [52] also reported that circulating ITLN1 levels did not differ between groups when all types of cancer were included in the same analysis. Additionally, previous literature that examined the role of ITLN1 [49] reported that distinct types of cancer can differentially influence ITLN1 concentration. To examine this claim, a subgroup analysis was conducted by dividing the included studies based on cancer type. The analysis highlighted that people with gastrointestinal cancer had substantially higher levels of ITLN1 compared to healthy controls (Figure 4.4). Indeed, all studies that examined gastrointestinal cancers (n=6) observed that the healthy controls showed lower ITLN1 concentration and thus, the meta-analytical model suggested the presence of strong evidence in favour of this conclusion. Furthermore, there was weak evidence in favour of elevated ITLN1 levels when healthy individuals were compared to people with urological (Figure 4.5) or breast cancer (Figure 4.6). This confirms the observations we made in our previous narrative review [155] and indicates that the trajectory of circulating ITLN1 is influenced by the cancer type. Other research also emphasised that certain adipokines such as adiponectin [192], resistin [193] or leptin [194] were associated with obesity-related cancers. Therefore, it can be argued that ITLN1 behaves similarly since its levels are increased in patients with gastrointestinal cancer compared to healthy counterparts. An alternative explanation could be that gastrointestinal cancers are found in local proximity to the VAT depots and consequently, the tumour proximity might affect local ITLN1 values. This relationship, as well as the concentration of ITLN1 in urological and breast cancer should be examined by future research since only a limited number of studies have been published to date.

The current systematic review aimed to examine both circulating and tissue concentrations of ITLN1. Due to the low availability of data on tissue levels, only circulating concentrations were meta-analysed. Interesting findings were also reported by studies that measured ITLN1 in different tissues. Recently, our group analysed a cohort of people with upper gastrointestinal cancer [44] and reported that ITLN1 mRNA levels were higher in the VAT of people with cancer compared to healthy controls, but no difference was observed when SAT concentrations were compared between groups. Moreover, this study suggested that ITLN1 was a characteristic of cancer-associated weight loss. Other research [59] noted that ITLN1 protein expression was elevated in gastric cancer tissue compared to the normal gastric mucosa. Interestingly, some studies [57,58] indicated that colorectal cancer patients with higher ITLN1 tumour concentrations had a better prognosis than those with lower levels. Therefore, the increased expression of circulating ITLN1 in gastrointestinal cancer patients compared to the lower levels observed in healthy individuals could be determined by the tumour and/or by the weight-loss specific to these types of cancer. The idea that higher ITLN1 tumour concentrations could have a protective role within cancer cohorts is also intriguing and further research should evaluate this hypothesis. Tissue concentrations of ITLN1 were also analysed in lung [75], ovarian [69] or breast cancer [43], but the available evidence is not sufficient for a comprehensive discussion about the adipokine's effects and roles in these cancer types.

Using a Bayesian approach, the present meta-analysis also estimated HDIs for the mean level of circulating ITLN1 in healthy individuals (Figure 4.7). The mean concentration of ITLN1 was  $243 \pm 21$  ng/ml when all studies were included in the analysis and  $257 \pm 31$  ng/ml when considering high-quality studies only. Surprisingly, it was observed that ITLN1 was considerably variable even in the subgroup of studies that were of high quality (i.e., from 2 to 780 ng/ml). Thus, it can be argued that the adipokine's variable levels were not caused by differences in the blood collection method or by dissimilarities in the assays used to quantify its concentration. A previous review that evaluated the biology and the role of ITLN1 in various diseases [49] also reported a wide range of values for circulating ITLN1 in healthy individuals (i.e., from 2 to 850 ng/ml). However, to date, no study has investigated potential reasons for the observed high degree of variability in circulating ITLN1. The

findings of the current review could represent a starting point for future in-vitro and in-vivo work to better understand ITLN1 behaviour and function. Moreover, the levels of ITLN1 were not different between healthy females and males. Overweight and obese individuals (Figure 4.8B) expressed marginally higher ITLN1 concentrations ( $246\pm 24$  ng/ml) than individuals with a  $BMI\leq 25$  ( $224\pm 48$  ng/ml; Figure 4.8A). Since VAT is the primary source of ITLN1, the elevated levels observed in individuals with  $BMI>25$  could be attributed to excess overall adiposity. This contradicts prior research emphasising that circulating ITLN1 was lower in obese individuals [195,196]. Yet, the systematic review of Arab and colleagues [184] showed a high level of heterogeneity and evidence of publication bias in reports of the relationship between ITLN1 and overweight/obesity and, similar to the present study, also failed to observe any differences between groups when high-quality studies were evaluated. Additionally, despite the high number of participants included in the present review, the differences indicated by the meta-analytical model are minimal given the variable ITLN1 levels observed in healthy individuals. Also, since distinct systematic reviews observed different directions of the relationship between BMI and ITLN1 and given the small differences between groups, it could be argued that the evidence is mixed and there is no substantive relationship between ITLN1 and BMI. Plausibly, there may be other factors influencing the relationships and further work should focus on discovering and understanding these mediators. We previously suggested that metabolic status may be the driver of ITLN1 levels rather than overweight/obesity per se [155]. However, we did not have sufficient data to examine this hypothesis in the current study. Future studies could also examine the use of metabolic syndrome to characterise the role and level of ITLN1.

Five studies that measured ITLN1 in cancer did not include a healthy control group and were consequently excluded from the meta-analysis (Table 4.1). The levels of circulating ITLN1 in the studies that examined people with prostate cancer [62,63,64] were overall higher than the mean ITLN1 concentration of healthy individuals (i.e.,  $257\pm 31$  ng/ml). The studies assessing urological cancer that were included in the meta-analytic model (Figure 4.5) showed divergent results. It would be interesting to observe the extent to which the results of the subgroup analysis change if the previously mentioned studies (i.e., that did not include a control group) would be added to the statistical model. Consequently, the meta-analysis

was updated and these studies [62,63,64] were added to the model (Appendix G, Figure 4.10). Since these studies failed to include a control group, the mean ITLN1 level of healthy individuals that was determined earlier (i.e., 234±21 ng/ml) was used as a reference point. Following this robustness check (Appendix G, Figure 4.10), the overall ITLN1 difference between patients with urological cancer and healthy individuals was still low and not considerably different from the initial model (Figure 4.5). Similarly, one study [43] analysed patients with breast cancer without including a control group and reported values that were lower compared to the mean ITLN1 level of healthy individuals that was established earlier in this review. This observation is in accordance with the meta-analytical model (Appendix G) that evaluated patients with breast cancer as three out of four of the included reported higher ITLN1 concentration in the control group. Furthermore, Tahmasebpour and colleagues [43] also measured gene expression and noticed that ITLN1 was downregulated in breast cancer tissue as opposed to adjacent normal tissue. To conclude, the majority of the studies examining circulating and tissue levels of ITLN1 in breast cancer patients indicate a tendency for higher concentration in healthy individuals and healthy tissues. However, the statistical model suggested that the available evidence is weak, and it is recommended that future studies examining ITLN1 concentration in cancer include suitable controls to allow effective comparisons in meta-analyses.

#### **4.4.2. Limitations and methodological weaknesses**

All meta-analytic models from the present review showed strong evidence in favour of heterogeneity and weak evidence of publication bias. The high degree of heterogeneity could be caused by the wide spread of ITLN1 values (from 1 to 750 ng/ml) or by the methodological variations observed across the literature. Indeed, there were multiple methodological issues observed in the studies included in this review and this is a major limitation of the available literature. Overall, only 33% of the studies in the meta-analysis comparing people with cancer to healthy individuals (Figure 4.2) and 38% of the studies included in the statistical model estimating the mean ITLN1 level (Figure 4.7) were of high quality. For the purpose of the present review, a high-quality study collected blood in the

morning after an overnight fast, described the assay used to quantify ITLN1 and reported values that were in accordance with the characteristics of the assay (e.g., within detection limits). The studies that were not included in the high-quality subgroup failed to meet one, two or all of these standards. It can be argued that the criteria that must be met for inclusion in the high-quality subgroup (i.e., blood collection method, accurate description of the assay and precise methods of reporting results) should be standard practice in modern-day research and thus, the number of low-quality studies was exceptionally high. Similar methodological weaknesses were observed in a recent review conducted by our research group that looked at the relationship between cytokines and cancer cachexia (Chapter 2). Poor data reporting and suboptimal descriptions of the methodology are too often encountered in the literature that evaluates the role of (adipo)cytokines in cancer (cachexia). Authors, reviewers and editors should aim to encourage transparency and promote basic methodological norms that facilitate the interpretation of the results and lead to more reliable conclusions in meta-analyses.

The current systematic review had several limitations. Firstly, only a few studies compared ITLN1 between healthy individuals and people with cancer. The subgroup assessments based on cancer type also included a limited number of studies and the data generated by these meta-analytic models should be interpreted with caution. Secondly, there is not enough research that evaluated the expression of ITLN1 in various tissues. This is particularly relevant in patients with cancer since the characteristics of different tissues could facilitate the understanding of a tumour's malignant behaviour. Lastly, the screening process was conducted independently by one author. Although having only one reviewer is not recommended in systematic reviews, this study's inclusion criteria were not strict, and the screening was conducted with a conservative approach. Since all studies that measured ITLN1 levels were included in the review, having a second reviewer would increase validity but would not improve the effectiveness of the screening.

Overall, it is challenging to conclude if circulating cytokine levels are clinically relevant in cancer or other medical conditions. The circulating ITLN1 concentrations we determined [44] in samples coming from healthy individuals (i.e., 2.3 ng/ml) and cancer patients (i.e., 2.8 ng/ml), using an ELISA kit (Amsbio, EH0564; notably no longer available), are substantially



different from the average ITLN1 values discussed in this study. The range of circulating ITLN1 concentrations we observed in a sample of patients from the REVOLUTION trial (Chapter 3) was also distinct from our previous observations [44] and from what was discussed earlier in the present study. Specifically, circulating ITLN1 was measured in patients with various types of cancer using a different ELISA kit (Abcam, ab269545) and the values ranged from 7 ng/ml to 48 ng/ml, with a mean level of 18 ng/ml. The high degree of variability observed in circulating (adipo)cytokine levels goes beyond ITLN1 as we highlighted in a recent systematic review [197]. The concentrations of multiple cytokines (i.e., IL-6, TNF- $\alpha$ ) were heterogenous in both healthy individuals and cancer patients. Furthermore, previous research indicated that several cytokines (e.g., IL-1 $\beta$ ) are often undetectable [198,199] and this was also observed in our laboratory when we analysed patients from the REVOLUTION trial. Interestingly, multiple studies analyse the detectable levels without discussing the possible reasons for values being below the limit of detection for some samples. The rate of false positives in meta-analyses and systematic reviews will drastically increase if these practices are repeatedly used across the literature. The clinical relevance of circulating (adipo)cytokine levels remains somewhat uncertain and future studies should explore the mechanisms that determine the high degree of variability and whether tissue concentrations (e.g., tumour) are a more relevant measurement.

#### **4.4.3. Conclusions**

Circulating ITLN1 did not show any difference between groups when combined tumour types were considered in the same analysis. Yet, the concentration of the adipokine was considerably higher in patients with gastrointestinal cancer compared to healthy individuals. ITLN1 concentrations may be overall lower in breast cancer patients compared to controls, but the data were not sufficient to draw a strong conclusion. Another key finding of the present systematic review was the estimated mean ITLN1 level in healthy individuals:  $257 \pm 31$  ng/ml. This value could be a useful starting point for studies that aim to examine the role and the behaviour of ITLN1 using in-vitro and in-vivo models. Future research on circulating ITLN1 and other biomarkers should improve methodological quality by adhering

to basic norms. The blood collection method, the assay used to quantify ITLN1 and the complete set of results should be reported and thoroughly described. Efforts should be made to monitor the evolution of ITLN1 during cancer by implementing longitudinal study designs.

## 5. The effect of intelectin-1 on human skeletal muscle cells

### 5.0. Abstract

**Background:** Intelectin-1 (ITLN1) is an adipokine with an apparent role in cancer. Higher ITLN1 levels were observed in gastrointestinal cancer patients compared to controls. Also, ITLN1 mRNA and protein concentrations were higher in the visceral adipose tissue of cachectic patients compared to healthy individuals, indicating its potential involvement in cancer cachexia. This study aims to examine the effects of ITLN1 on skeletal muscle cells.

**Methods:** Human skeletal muscle myotubes sourced from three healthy donors were grown and treated with 0, 10, 100 and 500ng/ml ITLN1 for 6 hours. The glucose uptake assay was used to measure insulin sensitivity, western blotting was conducted to examine Akt phosphorylation and protein synthesis, and the global transcriptome was assessed using microarray analysis. The data were analysed using Bayesian statistical modelling.

**Results:** Increased ITLN1 concentrations contribute to a reduction in skeletal muscle glucose uptake, with 74% more chances of observing higher uptake rates in controls compared to the 500ng/ml group. Akt phosphorylation peaked at 100ng/ml, and statistical modelling suggested a substantial probability of detecting lower phosphorylation values in the 0ng/ml group compared to all other treatments. ITLN1 did not influence protein synthesis, and none of the doses had a considerable effect at the individual gene level. Category enrichment analysis indicated that 122 genes involved in myogenesis were downregulated (FDR=0.008). ARRDC3, a gene that might be involved in skeletal muscle regulation, was increased in the 500ng/ml group compared to all other treatments and showed the highest differential expression level.

**Conclusion:** Elevated ITLN1 levels have a negative effect on glucose uptake and myogenesis. The highest Akt phosphorylation rates were observed in the 100ng/ml group, when ITLN1 was close to its physiological concentration. ITLN1 did not alter protein synthesis. Future research should monitor ARRDC3 behaviour and examine whether the magnitude of the effects observed in the present study is affected by longer exposure to ITLN1.

## 5.1. Introduction

ITLN1 is a type of lectin that binds to microbial carbohydrate chains in a calcium-dependent manner [200]. Human ITLN1, also known as omentin, can be found in the large and small intestine, but it also expressed in extra-intestinal tissues and in VAT [201,202]. Previous studies suggested that ITLN1 is produced in VAT by the stromal vascular fraction cells and not by adipocytes and that SAT expression is minimal [44,47]. Other research indicated that ITLN1 was also found in epicardial fat, renal collecting tubes, lungs and ovaries [46,49]. ITLN1 was proposed to have a role in antimicrobial defence since it can bind to microbial carbohydrate chains, but not to human glycans [46,200]. Furthermore, it was observed that ITLN1 increases insulin-stimulated glucose uptake in adipocytes, enhances Akt phosphorylation and contributes to iron metabolism due to its interaction with lactoferrin [47,203]. More recently, several reviews indicated that ITLN1 might have a role in cancer [49,52,155]. Indeed, our recent meta-analysis (Chapter 4) found considerably higher levels of circulating ITLN1 in gastrointestinal cancer patients compared to healthy controls and a tendency for lower levels in women with breast cancer as opposed to healthy controls. Other research that evaluated ITLN1 suggested that the adipokine was upregulated in gastric cancer tissue compared to normal gastric mucosa [59] and downregulated in breast cancer tissue compared to normal tissue [43]. Interestingly, various molecular pathways were proposed for ITLN1, but its mechanisms of action are still not fully understood. Thus, it can be argued that ITLN1 has a role in cancer, but the adipokine's behaviour at cellular level requires further elucidation,

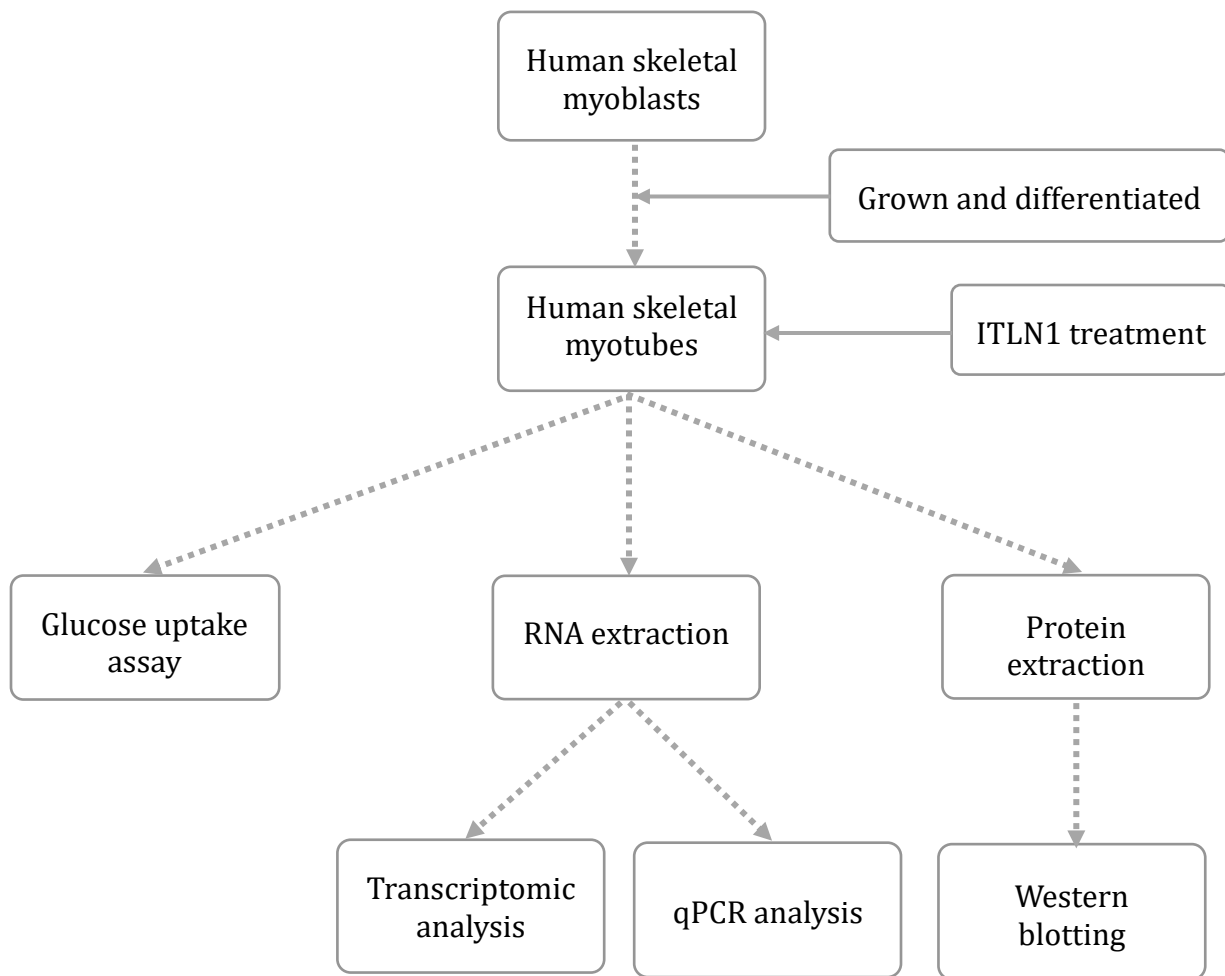
One study conducted by our research group [44] examined 16 patients with upper gastrointestinal cancer and evaluated the global transcriptome of VAT and SAT depots. Patients with cancer-associated cachexia, defined as per the consensus developed by Fearon and colleagues [7], were compared to weight-stable cancer patients and to healthy controls. The study found that ITLN1 mRNA levels were significantly elevated in the VAT of both cancer groups compared to controls and no differences were detected between weight-stable and weight-losing cancer patients. Additionally, VAT ITLN1 protein concentrations were higher in cachectic patients compared controls, with no dissimilarities observed between weight-stable patients and either of the two groups. Interestingly, ITLN1 was

significantly downregulated in SAT compared to VAT in the cancer groups, while the control group did not express any differences. Lastly, ITLN1 mRNA and protein concentrations measured from participants' SAT did not differ between groups. Overall, these findings contribute to the hypothesis that ITLN1 might have a role in cancer and cancer-associated cachexia.

Multiple definitions of cachexia were proposed over the past 20 years [11]. One common perspective of these definitions and a major symptom of this wasting syndrome is the loss of skeletal muscle mass. Yet, the relationship between ITLN1 and skeletal muscle has not yet been reported. Therefore, the present study aims to evaluate ITLN1 effects on skeletal muscle cells. Specifically, this study examined the global transcriptome, insulin sensitivity and protein synthesis in human primary myotubes in response to various ITLN1 concentrations.

## 5.2. Methods

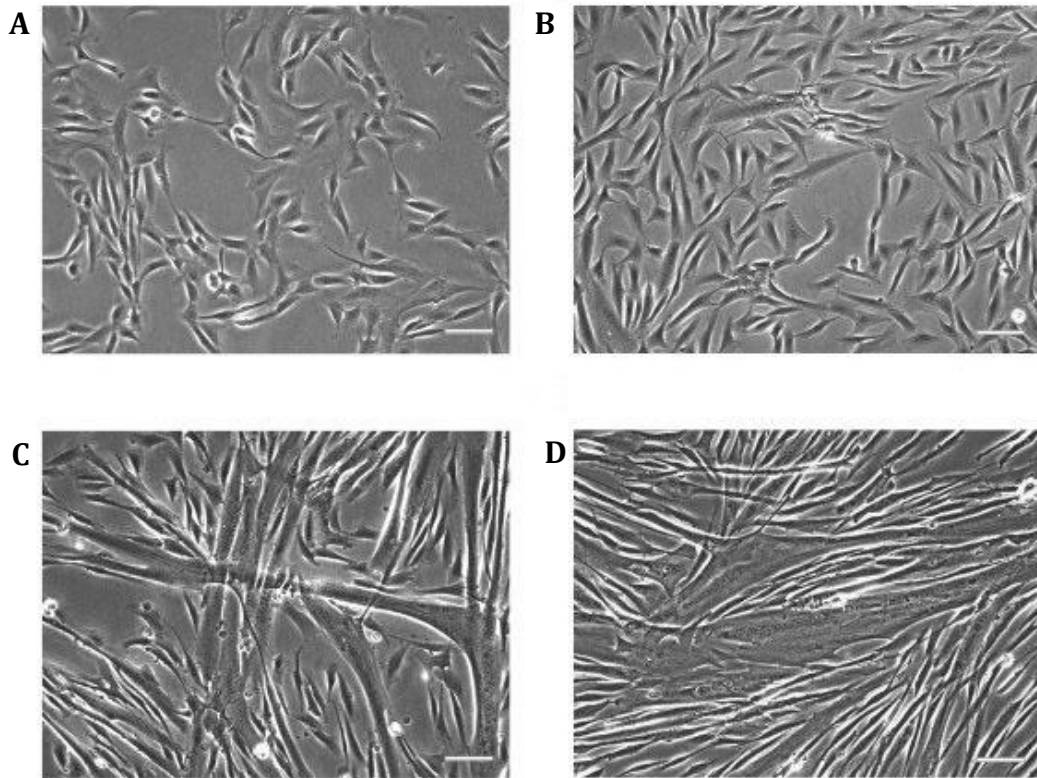
In summary, human skeletal myoblasts were grown, differentiated into myotubes and treated with different concentrations of ITLN1 (i.e., 0, 10, 100 and 500 ng/ml) for six hours. After the treatment, the glucose uptake assay was conducted, and RNA and protein were extracted for subsequent work. The RNA was used for transcriptomics analysis and quantitative polymerase chain reaction (qPCR) analysis, while the protein was required for western blotting. The study flow diagram was highlighted in Figure 5.1 and the protocols of the techniques used to complete this study were subsequently described.



**Figure 5.1.** Study flow diagram.

### 5.2.1. Cells and culture procedures

Primary human skeletal muscle cells (SkMCs) sourced from the quadriceps of three different healthy males (aged 27±4) of distinct ethnicities (i.e., White, Black and Hispanic) were purchased from Lonza (CC-2580, UK) and cultured as per the manufacturer's protocol (lot numbers: 20TL182717, 20TL070666 and 18TL180368). The cells were incubated at 37°C and 5% CO<sub>2</sub> in growth medium (CC-3245, Lonza, UK) and passaged when a confluence of 80% was reached. The cells were washed with Dulbecco's phosphate-buffered saline (DPBS, 14040117, Thermo Fisher, UK) and the culture medium was replaced every 48 hours. After four passages, the myoblasts were harvested using Gibco's trypsin-EDTA (25200056, Thermo Fisher, UK), counted with a Bio-Rad TC20 automated cell counter using cell counting slides (Bio-Rad, 1450016, UK) and transferred to 6-well or 96-well (polystyrene) plates. To induce differentiation, the cells were incubated in Dulbecco's Modified Eagle Medium/Ham's F-12 supplemented with 2% horse serum (DMEM, 12634010, Thermo Fisher, UK) for 3 to 5 days depending on protocol requirements. The development of the myotubes was examined daily using a Motic AE2000 microscope. Although no pictures were taken to highlight the differentiation of the myoblasts into myotubes due to the unavailability of equipment, Figure 5.2 presents the model developed by Trenerry and colleagues [204] that was used as a reference to monitor the evolution of the muscle cells. Before each assay, the differentiated cells were incubated with different doses of ITLN1 (9137-IN-050, Bio-Techne, UK) for 6 hours. ITLN1 was reconstituted in deionised water at 2000ng/ml and subsequently diluted to 10, 100 and 500ng/ml. No vehicle (e.g., dimethyl sulfoxide) was added to the control (i.e., 0ng/ml) treatment.



**Figure 5.2.** Images of developing human skeletal muscle cells [204] that were used as a reference to monitor the cells cultured in the current study. Primary myoblasts were grown (A) differentiated for 1 (B), 5 (C) and 10 (D) days. The scale bar represents 50  $\mu\text{m}$ . Image available via license: CC BY 2.0.

### 5.2.2. Glucose uptake assay

Glucose uptake was measured with Promega's Glucose Uptake-Glo Assay (PRJ1341, Fisher Scientific, UK). As per the protocol, the skeletal muscle cells were differentiated for five days in 96-well plates. One day before the assay, the DMEM supplemented with 2% horse serum was removed, the cells were washed using DPBS and 100 $\mu\text{l}$  DMEM without serum was added to each well. On the day of the assay, the myotubes were treated with 0 (i.e., control), 10, 100 and 500 ng/ml ITLN1 for six hours. All treatments were analysed at least in triplicate. Afterwards, the myotubes were incubated with 100 $\mu\text{l}$  DMEM (no serum) plus 1 $\mu\text{M}$  insulin (11061-68-0, Sigma-Aldrich, UK) and ITLN1 for one hour at 37°C in 5% CO<sub>2</sub>. The medium was removed and 50 $\mu\text{l}$  of 0.1mM 2-deoxyglucose (2DG) in PBS was added for 30 minutes;



2DG is transported across the membrane and phosphorylated in the same manner as glucose. The newly formed analyte, 2DG6P, is membrane-impermeable and accumulates in the cell. Following this step, a low pH buffer solution was added to stop 2DG uptake, lyse the cells and denature cellular NADPH, while a subsequent high-pH neutralisation buffer was used to neutralise the acid. Lastly, the cells were incubated for one hour at 25°C with a 2DG6P detection reagent formed of a luciferase reagent, glucose-6-phosphate dehydrogenase (G6PDH), NADP<sup>+</sup>, reductase and reductase substrate. Following a series of reactions, a luminescent signal proportional to the concentration of 2DG6P was produced and luminescence was recorded on a Synergy LX multi-mode reader (Biotek, USA).

### **5.2.3. RNA extraction**

After five days of differentiation in a 6-well plate, various concentrations of ITLN1 (i.e., 0, 10, 100 and 500ng/ml) were added to the skeletal muscle cells for six hours. Following the treatment, the RNeasy mini kit (Qiagen, 173046459, UK) was used to extract and purify total RNA. Briefly, the myotubes were lysed and homogenised with 350µl of highly denaturing buffer (i.e., Buffer RLT, provided as part of the kit) that inactivates RNAses. An equal volume of a 70% ethanol solution was added to provide suitable binding conditions. The samples were subsequently added to RNeasy spin columns, allowing total RNA to bind to the membrane. Contaminants were washed away from the membrane using several buffers provided by the kit manufacturer (e.g., Buffer RW1, Buffer RPE). After each step, the spin columns were centrifuged at 8000 × g for different durations, as per the protocol. Lastly, total RNA was eluted in 50µl RNase-free water. The concentration and the quality of the RNA were assessed for all samples with a Denovix DS-11 FX+ spectrophotometer (Denovix, UK). A minimum RNA concentration of 45ng/µl was required to perform the subsequent experiments (i.e., transcriptomics and qPCR analysis). The ratios of the spectrophotometric absorbance at 260nm to the values measured at 280nm and 230nm (i.e., 260/280 and 260/230) were used to examine RNA purity. In both instances, a ratio of approximately 2 was considered acceptable and indicated the presence of high-quality RNA (Denovix, UK).

#### **5.2.4. Protein extraction**

The SunSET technique was used to examine myotube protein synthesis [205]. Myoblasts were differentiated for five days in 6-well plates. As per the SunSET protocol, 200mg/ml puromycin was added to the muscle cells 48 hours before protein extraction [205, 206]. At low concentrations, puromycin is incorporated in newly synthesised proteins. Moreover, ITLN1 was added at concentrations of 0, 10, 100 and 500ng/ml for six hours before extracting the proteins. After removing the DMEM, 500µl of ice-cold radioimmunoprecipitation assay (RIPA) buffer with a protease inhibitor was added to each well for one minute to facilitate protein extraction. Subsequently, a cell scraper was used to detach the cells from the surface of the plate. The samples were transferred to 1.5ml microcentrifuge tubes and spun for five minutes at 12000 × g and 4°C. Following centrifugation, the pellet of insoluble proteins was removed and only the supernatant (i.e., soluble protein) was collected and used for subsequent work. The soluble protein concentration was determined using a Qubit Protein Assay Kit (Thermo Fisher, Q33212, UK) on a Qubit 4 fluorometer (Thermo Fisher, UK).

#### **5.2.5. Transcriptomics analysis**

The GeneChip WT Plus Reagent kit (Thermo Fisher, 902280, UK) was used to prepare the RNA collected from human skeletal muscle cells for whole transcriptome expression analysis. In summary, reverse-transcription priming was conducted with 100ng of total RNA to synthesise amplified complementary DNA (cDNA). Following this step, the cDNA was purified, fragmented and labelled with biotin. All reactions (i.e., first-strand cDNA synthesis, second-strand cDNA synthesis, in vitro transcription cRNA synthesis, 2<sup>nd</sup> cycle primers-cRNA annealing, 2<sup>nd</sup> cycle ss-cDNA synthesis, RNA hydrolysis, fragmentation and labelling) were conducted on a Veriti™ 96-Well Fast Thermal Cycler (Thermo Fisher, 4375305, UK) as recommended by the kit manufacturer (Thermo Fisher, 2020). Table 5.1 highlights a detailed overview of the temperature, duration and number of cycles that were used to conduct the reactions. Subsequently, a hybridisation cocktail was prepared (Table 5.1) with reagents provided in the GeneChip WT Plus Kit (Thermo Fisher, 902280, UK) and the samples were

hybridised to Clariom D GeneChip human microarrays (Thermo Fisher, 902922, UK) at 45°C for 16 hours in a GeneChip Hybridisation Oven 640 (Thermo Fisher, 00-0331, UK). Afterwards, the microarrays were washed and stained (Thermo Fisher, 900720, UK) following the manufacturer's directions using the GeneChip Fluidics Station 450 (Thermo Fisher, 00-0079, UK). Lastly, images of the arrays were captured and CEL files were generated using a GeneChip Scanner 3000 (Thermo Fisher, 00-0210, UK). The microarray data were uploaded to Gene Expression Omnibus (accession number GSE225037).

**Table 5.1.** Thermal cycler reaction protocols as recommended by Thermo Fisher (2020).

Reaction	Reaction volume	Lid temperature	Step 1	Step 2	Step 3	Step 4
First-strand cDNA synthesis	10µl	42°C	25°C for 1 hour	42°C for 1 hour	4°C for 2 mins	
Second-strand cDNA synthesis	30µl	Disabled	16°C for 1 hour	65°C for 10 mins	4°C for 2 mins	
In vitro transcription cRNA synthesis	60µl	40°C	40°C for 16 hours	Hold at 4°C		
2 <sup>nd</sup> cycle primers-cRNA annealing	28µl	70°C	70°C for 5 mins	25°C for 5 mins	4°C for 2 mins	
2 <sup>nd</sup> cycle ss-cDNA synthesis	40µl	70°C	25°C for 10 mins	42°C for 90 mins	70°C for 10 mins	Hold at 4°C
RNA hydrolysis	44µl	70°C	37°C for 45 mins	95°C for 5 mins	Hold at 4°C	
Fragmentation	48µl	93°C	37°C for 60 mins	93°C for 2 mins	Hold at 4°C	
Labelling	60µl	70°C	37°C for 60 mins	70°C for 10 mins	Hold at 4°C	
Hybridisation control	Variable	65°C	65°C for 5 mins			
Hybridisation cocktail	Variable	99°C	95°C for 5 mins	45°C for 5 mins		

Mins = minutes; cDNA = complementary deoxyribonucleic acid; cRNA = complementary ribonucleic acid; ss-cDNA = single-stranded cDNA.

### **5.2.6. qPCR analysis**

After extracting the RNA from skeletal muscle cells, cDNA was prepared with the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher, K1622, UK) following the manufacturer's guidelines. Briefly, 1µl of oligo(dT)18 was mixed with the template RNA and ddH<sub>2</sub>O to a total volume of 12µl for each reaction. Then, 1µl RiboLock RNase inhibitor, 1µl of RevertAid reverse transcriptase, 2µl dNTPs (10mM) and 4µl reaction buffer were added to a final volume of 20µl. All samples were analysed in triplicate and 500ng of total RNA were used as a template for the reverse transcription reaction (i.e., 60 minutes at 42°C followed by 5 minutes at 70°C) conducted on a Veriti™ 96-Well Fast Thermal Cycler (Thermo Fisher, 4375305, UK). Subsequently, cDNA was diluted 1:5 and qPCR was carried out with 5µl Luminaris Hi Green Low ROX qPCR Master Mix (Thermo Fisher, K0374, UL), 3.4µl ddH<sub>2</sub>O, 0.3µl reverse primer and 0.3µl forward primer for each reaction. The primers (Appendix J) were selected from the Primer Bank [206] to assay selected genes considered following the microarray data analysis (i.e., ARRDC3, H1-0, H1-2, TM4SF1, TXNIP, OGA, ADNP2, AHNAK2, OGT). Following the MIQUE guidelines [207], several qPCR assays were used (i.e., B2M, GAPDH, YWHAZ, ACTB, HPRT) for data normalisations of target genes. The PCR reaction was completed on a LightCycler 480 instrument (Roche, UK) using the following protocol: 2 minutes at 50°C for uracil-DNA-glycosylase treatment, 10 minutes at 95°C for DNA denaturation, followed by 40 quantification cycles that consist of 15 seconds at 90°C, 30 seconds at 60°C and 30 seconds at 72°C.

### **5.2.7. Western blotting**

Three micrograms of protein were made up in Lammeli sample buffer (Bio-Rad, 1610747, UK) and loaded in a pre-cast 4-20% Criterion TGX 18-well gel (Bio-Rad, 5671094, UK). Electrophoresis was conducted at 150V for one hour and 15 minutes on a PowerPac HC instrument (Bio-Rad, UK) in a tris/glycine/SDS buffer (Bio-Rad, 1610732, UK). Following SDS-PAGE, the proteins were transferred to an Amersham PVDF membrane (Sigma Aldrich, GE10600023, UK) at 100V for one hour in a transfer buffer made of 25mM Tris, 190 mM glycine and 20% methanol. Membranes were blocked with a Pierce protein-free (PBS)

blocking buffer (Thermo Fisher, 37584, UK) for one hour at room temperature, followed by incubation in primary antibodies at 4°C overnight, with gentle agitation (i.e., 40rpm) on a see-saw rocker (Fisher Scientific, 10470655, UK). Subsequently, the membranes underwent 3 five-minute washes in TBST – a tris-buffered saline (Bio-Rad, 1706435, UK) with added Tween 20 (Fisher Scientific, UK). This step was followed by incubation in the appropriate secondary antibodies for one hour on a see-saw rocker at room temperature and by three more washes with TBST. Lastly, the membranes were covered with an enhanced chemiluminescent reagent - Cytiva Amersha EC Prime Western Blotting Detection Reagent (Fisher Scientific, 10308449, UK) - for five minutes at room temperature and protein expression was visualised on a ChemiDoc XRS+ instrument (Bio-Rad, UK).

The aim of this technique was to determine the expression of puromycin, phosphorylated Akt (p-Akt) and total Akt. Puromycin incorporation was measured since it reflects the rate of protein synthesis [205]. Furthermore, Akt phosphorylation can control cellular metabolism and survival [79,209] and previous work indicated that ITLN1 could influence Akt-mediated growth pathways [80,81]. Primary antibodies used were mouse anti-puromycin monoclonal antibody (Merck, MABE343, UK), p-Akt (Thr308) rabbit antibody (Cell Signalling, 9275, UK), pan Akt rabbit antibody (Cell Signalling, 4691S, UK). Secondary antibodies used were goat anti-mouse IgG2a heavy chain (HRP) antibody (Abcam, Ab97245, UK) and anti-rabbit IgG, HRP-linked antibody (Cell Signalling, 7074, UK). After determining p-Akt concentrations, the membrane was washed and antibodies were stripped with a Restore Western Blot stripping buffer (Thermo Fisher, 21059, UK) for 15 minutes. Following this procedure, the membrane was washed once again and probed for total Akt.

### **5.2.8. Data analysis**

Data were analysed using the programming language R, version 4.2.1 and were reported as mean  $\pm$  standard deviation (SD) unless otherwise specified. The current study used a Bayesian approach to interpret the data. The complete dataset and the R script used for the analysis can be found in Appendix H.

The publicly available software ImageJ (version 1.53) was used to quantify western blots and generate optical density values [210]. The Akt phosphorylation data were normalised by analysing p-Akt concentrations relative to total Akt levels. The puromycin incorporation values were not normalised as the aim of this technique was to compare global protein synthesis between groups (i.e., 0, 10, 100 and 500ng/ml). Glucose uptake assay and western blotting data were subsequently assessed using a Bayesian linear model and the R packages *rstanarm*, *emmeans*, *tidybayes* and *bayesplot*. Means and HDIs were used to describe the results. An HDI indicates a range of plausible values contained in a probability interval (e.g., 90%) of the posterior distribution.

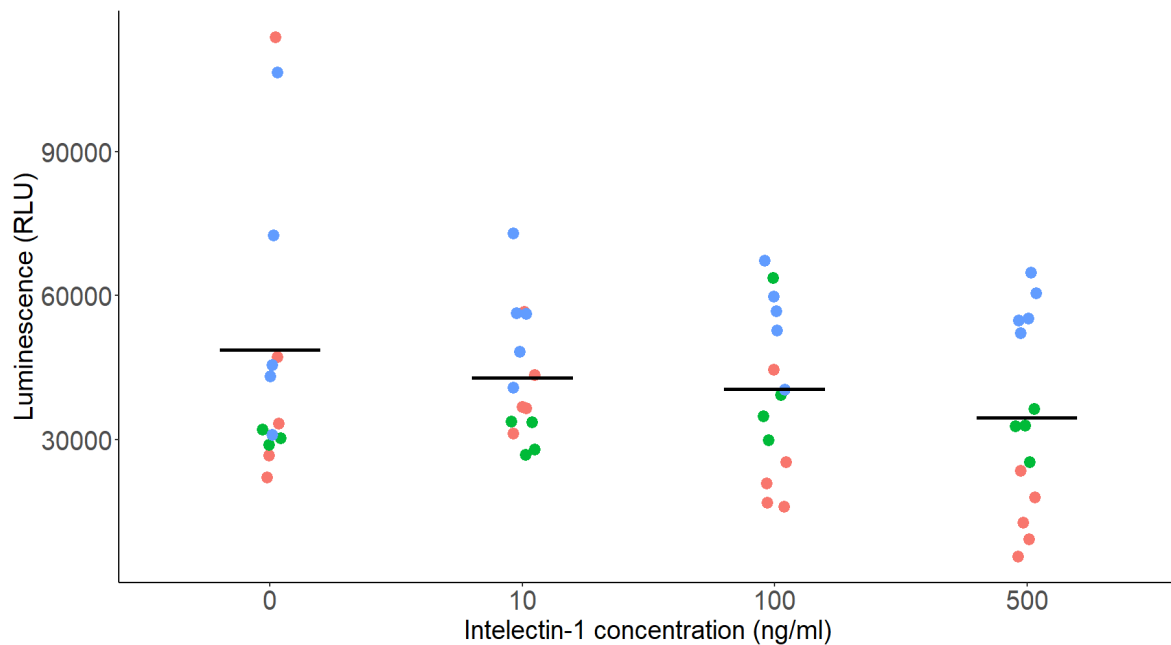
Microarray probes were mapped to specific genes using updated chip definition files (CDF) from the Brainarray project [211]. Robust Multichip Analysis (RMA) was used for background corrections and quantile normalisation in order to adjust microarray signal distributions to be approximately equal. Afterwards, the quality of the raw microarray data was examined and validated by evaluating the relative log expression (RLE) plot and the normalised unscaled error (NUSE) plot [212]. The SCAN-UPC method [213] was used to normalise data and identify expressed probes. In high throughput experiments, genes that are not expressed add noise that can lead to biases in biological interpretation [214] and thus, the universal expression code (UPC) was used to evaluate whether probes are active (i.e., transcribed) or not active. The genes that had an UPC value below 0.5 and a log<sub>2</sub>-scaled expression value below 1 were filtered out. The integrity of the data was verified with principal component analysis and it was observed that the main source of variation was biological. Subsequently, a linear model was used to examine the genes that were differentially expressed in response to different ITLN1 doses. Category enrichment analysis was conducted using the manually curated Hallmark Genesets collection from the Broad Institute [215]. Competitive gene testing was used to compare sets of genes relative to all other genes [216] and statistical significance was accepted when the false discovery rate (FDR) was less than 5%. The following R packages facilitated the exploration and analysis of microarray data: *aroma.affymetrix*, *scan.upc*, *org.hs.eg.db*, *GSEABase*, *pheatmap*, *limma*, *camera* (Appendix H). The quality control plots (i.e., NUSE, RLE) are included in Appendix I.

The R package *NormqPCR* from Bioconductor [217] was used to analyse qPCR data. B2M, GAPDH, YWHAZ, ACTB, HPRT were used as normaliser genes and the geNorm method [218] was implemented to choose the most stable reference genes. Subsequently, the cycle threshold values for the genes of interest (i.e., ARRDC3, H1-0, H1-2, TM4SF1, TXNIP, OGA, ADNP2, AHNAK2, OGT) were normalised using the geometric mean of the selected control genes. Relative gene expression was determined using the delta-delta Cq method [219]. A linear model was fitted to the data using the R package *stats* to check if genes were differently expressed between groups. Moreover, post hoc testing was conducted using the R package *emmeans*.

## 5.3. Results

### 5.3.1. Glucose uptake assay

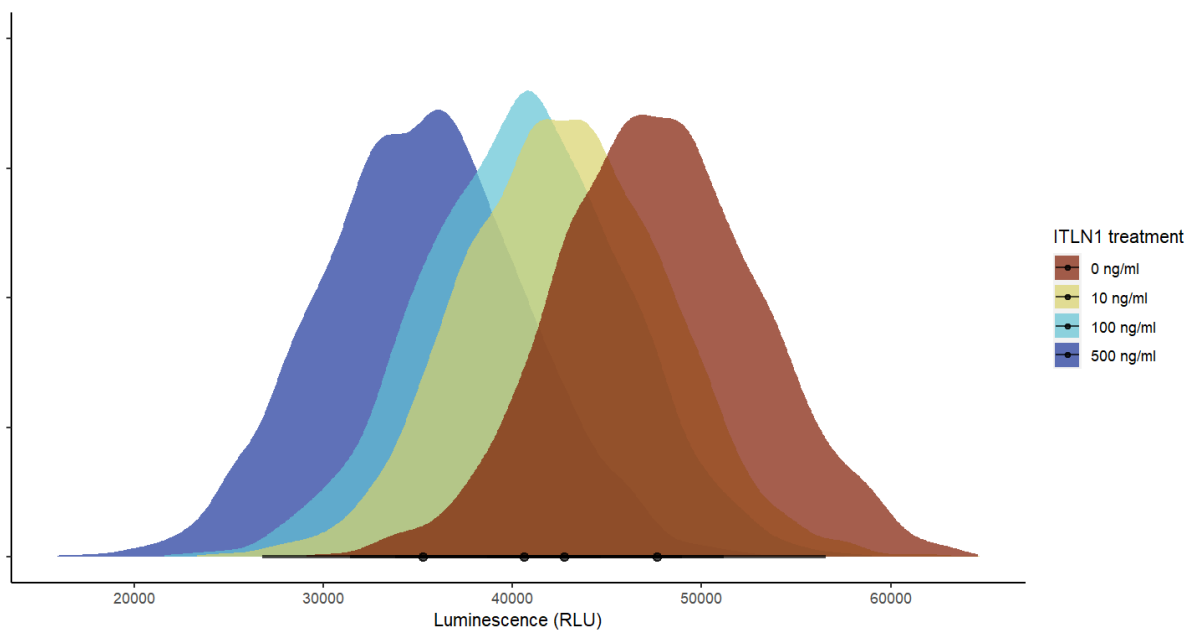
The influence of an acute treatment (i.e., 6 hours) with different ITLN1 concentrations (i.e., 0 ng/ml, 10 ng/ml, 100 ng/ml and 500 ng/ml) on insulin-mediated glucose uptake in human skeletal muscle myotubes was assessed with the Glucose Uptake-Glo Assay (Figure 5.3).



**Figure 5.3.** Glucose uptake rate measured by assessing relative light units (RLU) after treating skeletal muscle myotubes with different ITLN1 concentrations for 6 hours. Each colour represents a different lot of cells (n=3), while the line indicates the mean luminescence value for each ITLN1 dose. The aim was to analyse all treatments at least in triplicate for each lot of cells; however, more measurements were available due to increased cell proliferation levels that consequently led to a higher cell yield.



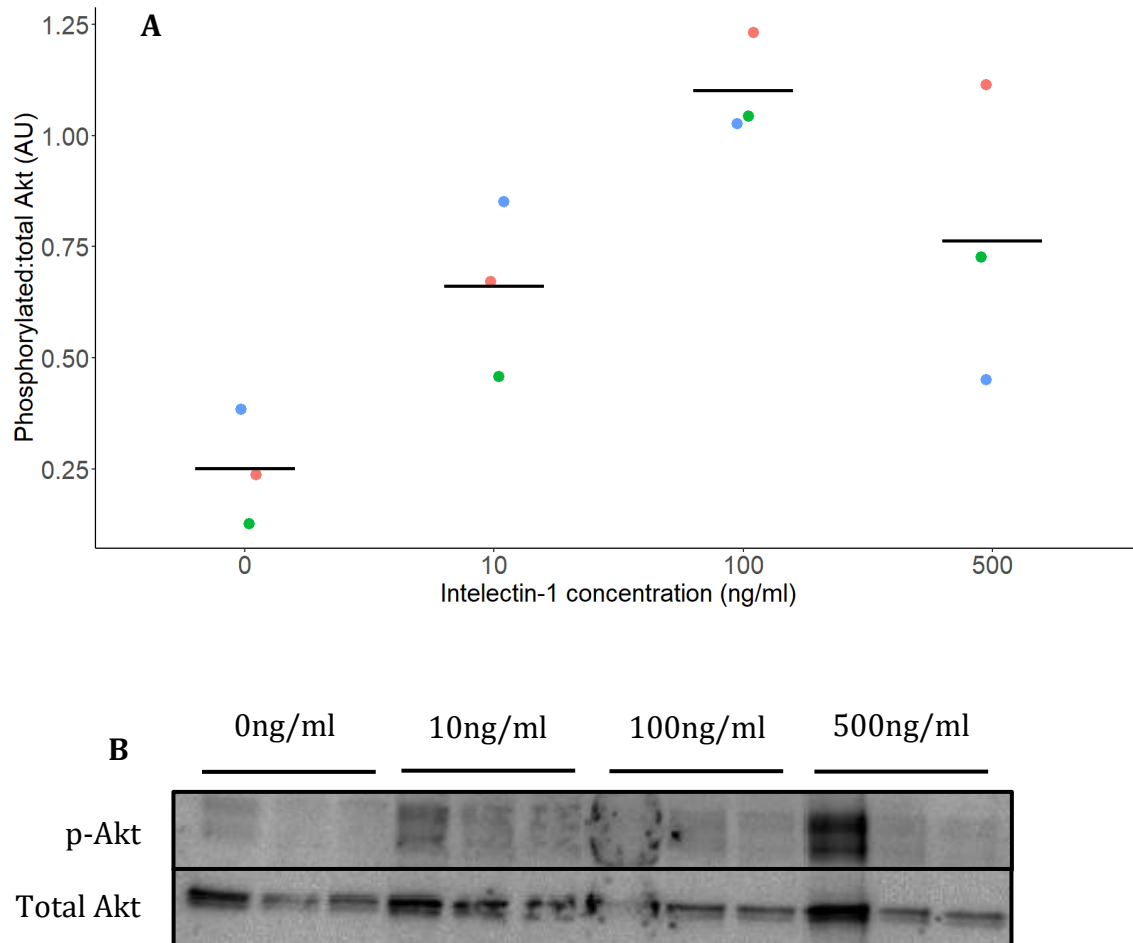
A Bayesian linear model was used to examine whether glucose uptake rates were different between treatments. The mean luminescence values were not considerably different between treatments:  $48653 \pm 30218$  in the 0ng/ml group,  $42916 \pm 13463$  in the 10ng/ml group,  $40539 \pm 17508$  in the 100ng/ml group and  $34495 \pm 20000$  in the 500ng/ml group. The posterior distributions generated by the model (Figure 5.4) suggested a 74% chance of obtaining higher glucose uptake rates in the control treatment (i.e., 0ng/ml) compared to the posterior distribution of the 500ng/ml group. No other relevant differences were observed when the other groups were compared to each other. A detailed description of posterior distributions can be found in Appendix K.



**Figure 5.4.** Posterior distributions of luminescence values generated from a Bayesian linear model that analysed data from the glucose uptake assay. The point represents the mean of the distribution, while the line indicates the 90% HDI.

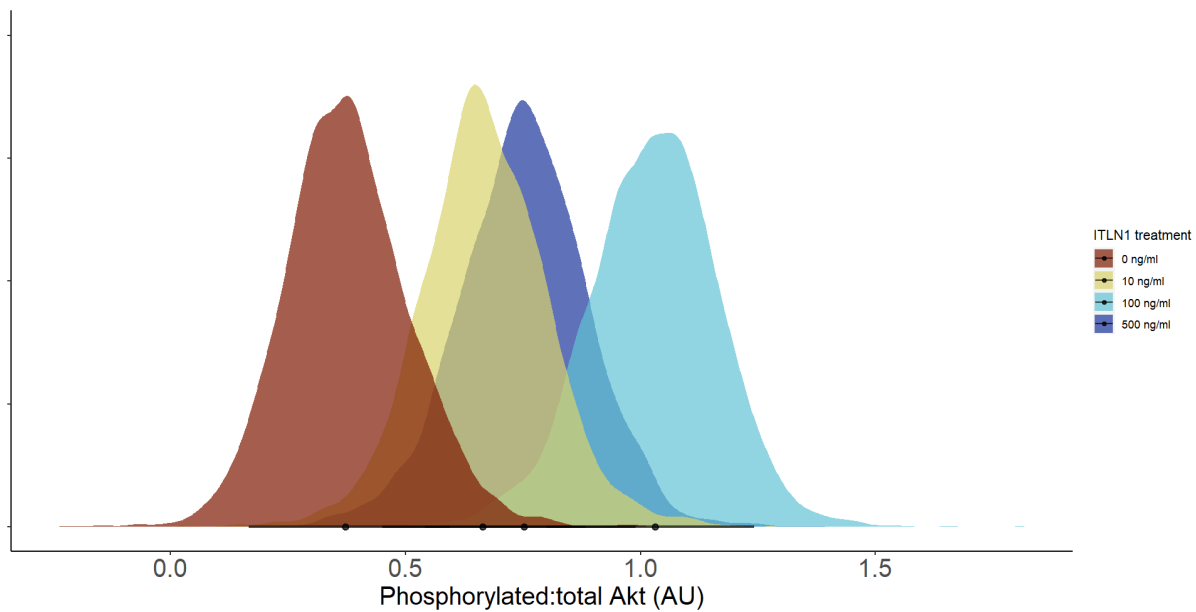
### 5.3.2. Western blotting

The influence of ITLN1 dose on Akt phosphorylation was examined by western blotting (Figure 5.5). Specifically, the ratio between phosphorylated and total Akt was determined (Figure 5.5A) after pAkt and total Akt concentrations were quantified from the same membrane (Figure 5.5B).



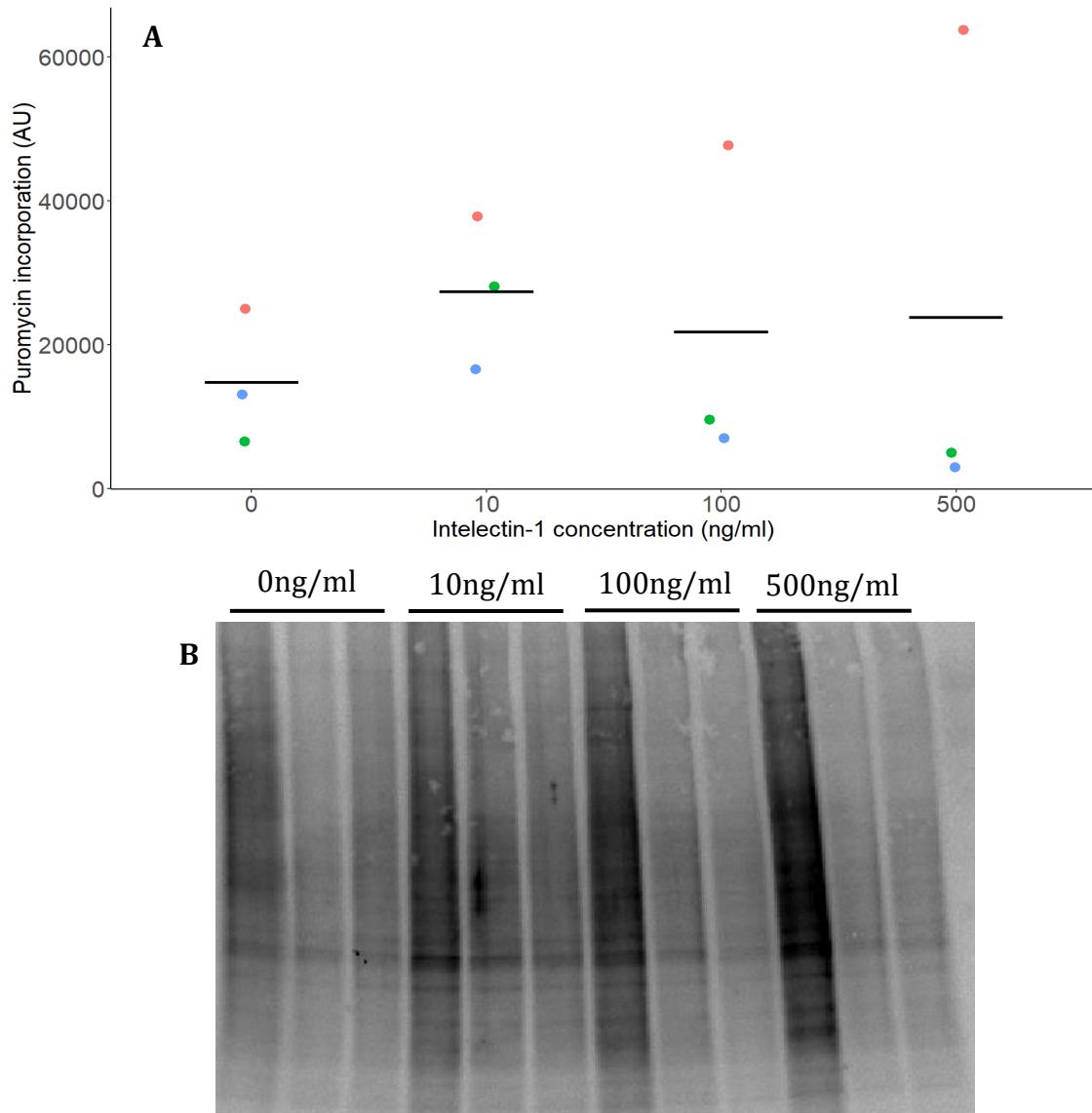
**Figure 5.5.** The ratio between p-Akt and total Akt determined by western blotting following a treatment with different ITLN1 concentrations for 6 hours. Each colour represents a different lot of cells (n=3), while the line indicates the mean optical density values of each group (A). Representative blot images for p-Akt and total Akt. The bands for both analytes were observed at a molecular weight of approximately 60kDa. Each ITLN1 treatment was analysed once per cell line (B).

Overall, the ratio was considerably higher in the 100ng/ml treatment ( $1.1 \pm 0.1$  AU) compared to the control dose ( $0.3 \pm 0.1$ ) and also elevated in the 10ng/ml ( $0.7 \pm 0.2$ ) and 500ng/ml ( $0.8 \pm 0.3$ ) groups as opposed to the control group. The posterior predictive distributions developed by the Bayesian linear model indicated a 99% chance of observing a higher ratio if the cells were treated with 100ng/ml ITLN1 in comparison to the control treatment (Figure 5.6). The same analysis suggested that the probability of detecting a lower ratio in the 0ng/ml group compared to the 10ng/ml and 500ng/ml treatments would be 70% and 86%, respectively. A comprehensive description of posterior distributions can be found in Appendix K.



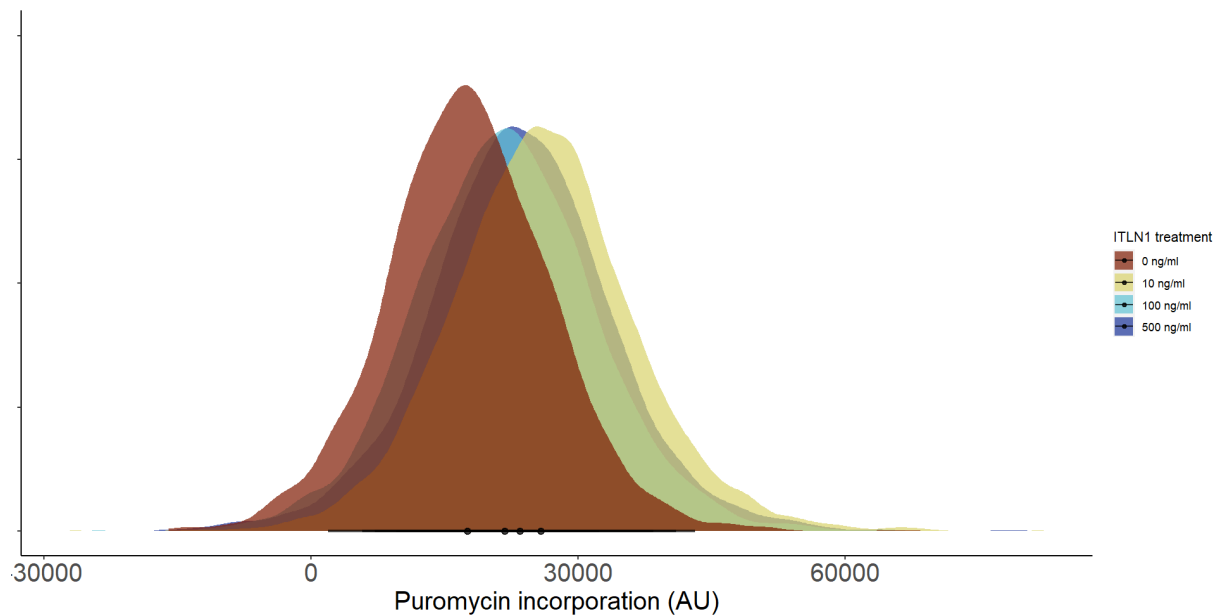
**Figure 5.6.** Posterior distributions of the ratio between p-Akt and total Akt generated from a Bayesian linear model that analysed western blotting data. The point represents the mean of the distribution, while the line indicates the 90% HDI.

The effect of ITLN1 on protein synthesis was estimated by measuring the rate of puromycin incorporation (Figure 5.7).



**Figure 5.7.** Puromycin incorporation determined by western blotting following a treatment with different ITLN1 doses for 6 hours. Each colour represents a different lot of cells (n=3), while the line indicates the mean optical density values of each group (A). Representative blot images for puromycin incorporation, an indicator of total protein synthesis. Puromycin levels were compared between groups without using a loading control. Each ITLN1 treatment was analysed once per cell line. (B).

The mean optical density values (AU) were not substantially different between the 0ng/ml (14770±9666), 10ng/ml (27408±10457), 100ng/ml (21784±22800) and 500ng/ml (23765±34426) treatments (Figure 5.7A). Additionally, the posterior distributions developed through Bayesian linear modelling (Figure 5.8) were not considerably different between groups, indicating that the ITLN1 concentrations used in the experiment did not influence puromycin incorporation. Appendix K includes more details about each posterior distribution.

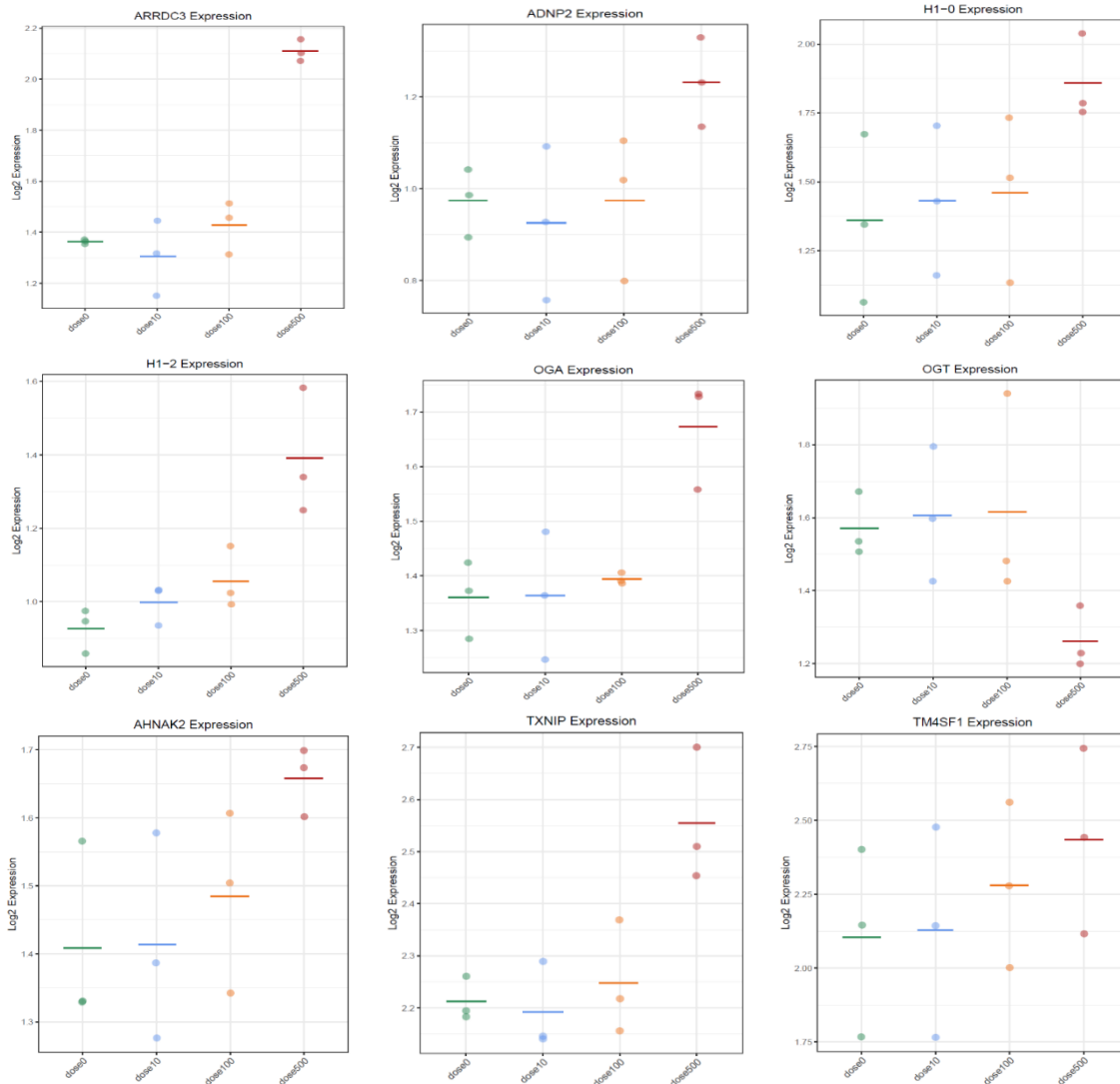


**Figure 5.8.** Posterior distributions of puromycin incorporation generated from a Bayesian linear model that analysed western blotting data. The point represents the mean of the distribution, while the line indicates the 90% HDI.

### 5.3.3. Microarray analysis

Overall, 88% of the probesets were identified as unlikely to be expressed in myotubes and removed after the data were filtered using the SCAN-UPC method [213]. Eliminating these genes from the analysis improved statistical power and data reliability, as indicated by principal component analysis. None of the ITLN1 treatments (i.e., 0ng/ml, 10ng/ml, 100ng/ml and 500ng/ml) had a substantial effect at individual gene level. Furthermore, category enrichment analysis was conducted using the curated Hallmark Genesets collection

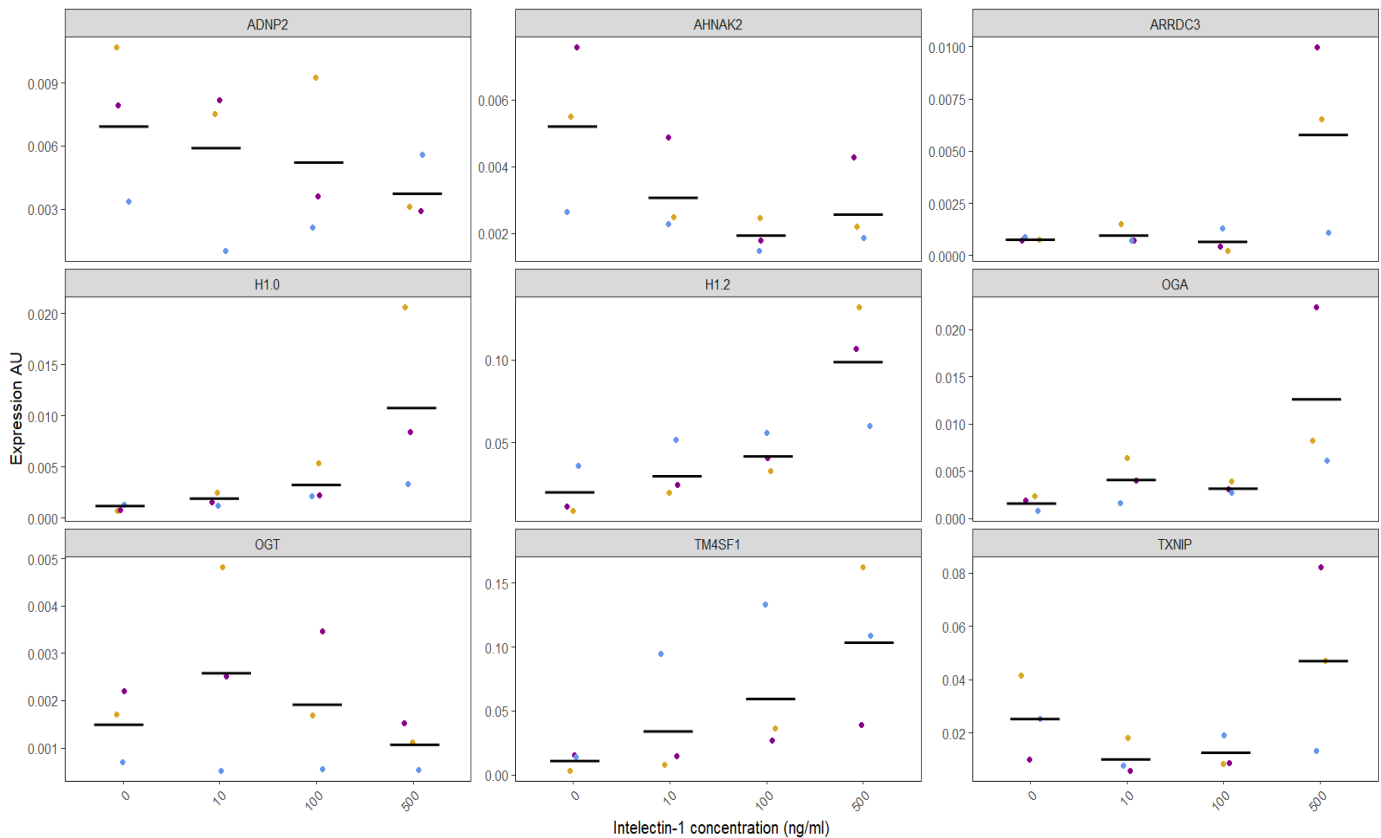
from the Broad Institute [215]. The complete set of results from the category enrichment analysis was included in Appendix I. It was observed that 122 genes involved in myogenesis (Appendix I, Figure 5.12) and 39 genes responsible for cholesterol homeostasis (FDR=0.008) were considerably downregulated. The genes that showed the highest levels of differential expression (i.e., *ARRDC3*, *ADNP2*, *H1-0*, *H1-2*, *OGA*, *OGT*, *AHNAK2*, *TXNIP* and *TM4SF1*) were highlighted in Figure 5.9.



**Figure 5.9.** Genes that showed the highest level of differential expression in the microarray data analysis. The x-axis indicates the ITLN1 treatment (i.e., dose): 0ng/ml, 10ng/ml, 100ng/ml and 500ng/ml. The y-axis indicates the relative expression of the gene. The line represents the mean value of each group.

### 5.3.4. Quantitative polymerase chain reaction gene validation

The genes that showed the highest level of differential expression in the microarray analysis (Figure 5.9) were examined with qPCR. Several housekeeping genes (i.e., B2M, GADPH, YWHAZ, ACTB, HPRT) were used as reference values for qPCR data normalisation. Following statistical analysis of qPCR data, it was observed that the results were mainly in agreement with the microarray results. Specifically, the differences between groups for most of the genes (i.e., AARDC3, H1-0, H1-2, TM4SF1, TXNIP, OGA, OGT) were similar when the microarray analysis (Figure 5.9) was compared to the qPCR data (Figure 5.10). Conversely, ADNP2 and AHNAK2 were genes that failed to confirm the microarray assessment. A linear model and subsequent post-hoc tests indicated a considerable difference in the expression of AARDC3, H1-0, H1-2 and OGA when the 500ng/ml ITLN1 treatment was compared to the control group.



**Figure 5.10.** qPCR analysis of gene expression in response to different ITLN1 concentrations. The line represents the mean value of each group. Each colour represents a different lot of cells (n=3).

## 5.4. Discussion

This is the first study that aimed to investigate the effect of ITLN1 on human skeletal muscle cells. Specifically, the aim of the current study was to examine the effect of an acute treatment with different ITLN1 doses on the metabolic homeostasis and growth of human skeletal myotubes by assessing the global transcriptome, glucose uptake and protein synthesis.

The data collected following the glucose uptake assay was not considerably different between groups (Figure 5.3). Bayesian modelling suggested there is a 74% probability of observing elevated glucose uptake in the control group as opposed to the group treated with 500ng/ml ITLN1 (Figure 5.4). Even though the differences between groups are not substantial, the analysis indicated a trend towards decreased insulin-stimulated glucose uptake rates in skeletal muscle cells treated with higher ITLN1 concentrations. This finding is particularly relevant since glucose is the primary energy source of skeletal muscle cells [220]. Skeletal muscle modulates a great proportion of insulin-stimulated glucose disposal, and insulin-stimulated glucose uptake rates were lower in tumour-bearing mice compared to controls [221]. As observed in our recent Bayesian meta-analysis (Chapter 4), ITLN1 levels are higher in gastrointestinal cancer patients as opposed to healthy individuals. These cancers (e.g., pancreatic, colorectal) are commonly associated with cachexia [222] – a syndrome characterised by insulin resistance, reduced glucose uptake and low energy availability [223,224]. The elevated ITLN1 levels observed in gastrointestinal cancer might contribute to or be associated with the glucose uptake impairment specific to cachexia. However, no other study evaluated the impact of ITLN1 on skeletal muscle glucose uptake, so the present data cannot be directly compared to previous research. In a study by Yang and colleagues [47], different ITLN1 (i.e., 0, 150 and 300 ng/ml) doses were added to adipocytes and it was observed that ITLN1 improves insulin-mediated glucose uptake [47]. Thus, it can be hypothesised that ITLN1 affects glucose uptake in a tissue-specific manner. However, it is important to note that the authors of this study [47] used a combination of SAT and VAT. Using both types of tissue could diminish the validity of the study's findings since there are numerous physiological differences between SAT and VAT [225] and previous research identified ITLN1 as highly expressed in the VAT, but not in the SAT of cancer cachexia patients [44]. To conclude, ITLN1 might negatively affect the rate of glucose uptake in



skeletal muscle. Yet, there is scope for additional research that could elucidate if the effect of ITLN1 on glucose uptake is tissue-specific by comparing the behaviour of distinct tissues (i.e., muscle, SAT and VAT) in response to different ITLN1 concentrations.

Akt is a protein kinase involved in the growth, differentiation and survival of cells, with numerous downstream targets that can modulate cell morphology, act as a tumour suppressor and regulate cell metabolism [226]. The ratio between p-Akt and total Akt was considerably higher in the group treated with 100ng/ml compared to the control condition and elevated in the 10ng/ml and 500ng/ml treatments as opposed to the 0ng/ml group (Figure 4A). Akt phosphorylation increased gradually until a peak was reached at 100ng/ml. This value was the closest to the physiological ITLN1 concentration estimated in our meta-analysis (i.e.,  $257 \pm 31$  ng/ml). It could be argued that the phosphorylation of Akt is optimal when the level of ITLN1 is close to 'normal' and dysregulated when the myotubes are treated with abnormal concentrations. This hypothesis could be tested in a cell culture model that, besides the dosage used in the current study, implements two additional treatments: a 250ng/ml dose that reflects the ITLN1 level of healthy individuals and a 2000ng/ml hyper-physiological concentration. The hypothesis would be confirmed if Akt phosphorylation is maximal when myotubes are treated with a physiological dose and minimal in the hyper-physiological group. Furthermore, Yang and colleagues [47] suggested that ITLN1 can increase Akt phosphorylation in adipocytes and upregulate insulin-stimulated glucose uptake via Akt activation. However, the data from the present study contradict prior beliefs since ITLN1 did not have a linear relationship with Akt phosphorylation. Although previous literature [227, 228] emphasised that Akt mediates insulin-stimulated glucose uptake in skeletal muscle, there was no degree of congruence observed between the glucose uptake results (Figure 5.3) and the Akt phosphorylation measurements (Figure 5.5A). Therefore, ITLN1 levels that are close to physiological concentrations have the biggest influence on Akt phosphorylation in myotubes. The dose-response relationship between ITLN1 and Akt phosphorylation and the adipokine's tissue-specific effects should be further investigated.

Western blotting was also used to determine the rate of puromycin incorporation (Figure 6A), a parameter that reflects the rate of protein synthesis [205]. The Bayesian model indicated that skeletal muscle protein synthesis was not substantially different between

ITLN1 treatments. This finding is particularly relevant since previous research [44] observed higher levels of ITLN1 in cancer cachexia, a syndrome that is characterised by muscle wasting and negative protein balance [229, 230]. It can be argued that ITLN1 affects the cachectic phenotype through other mechanisms (e.g., reduction of skeletal muscle glucose uptake) that have a subsequent impact on protein synthesis. Nevertheless, it is essential to note that individuals with cancer cachexia typically suffer prolonged exposure to aberrant (adipo)cytokine levels. The present study investigated protein synthesis only after an acute treatment with ITLN1 and further research should evaluate the effects of chronic exposure.

The investigation of microarray data at the single gene level revealed that none of the genes was considerably different between groups. Category enrichment analysis was used to examine sets of genes with common biological functions [231] and it was observed that 122 genes involved in myogenesis were substantially downregulated. This finding is relevant in the context of cancer cachexia since an acute treatment with ITLN1 negatively affected the genes responsible for the formation of skeletal muscle tissue. Based on this result, further research can be conducted to determine whether the magnitude of the differences between groups would be increased by extending the period of exposure to ITLN1. Furthermore, the genes that showed the highest levels of differential expression (i.e., ARRDC3, H1-0, H1-2, TM4SF1, TXNIP, OGA, ADNP2, AHNAK2, OGT) were selected for qPCR analysis. The microarray data were confirmed by qPCR analysis for all genes except ADNP2 and AHNAK2. As highlighted in Figure 9, ARRDC3 was considerably higher in the 500ng/ml treatment as opposed to all other groups. Previous research identified ARRDC3 as a tumour suppressor gene [232] and suggested that it might play a role in the regulation of skeletal muscle [233]. In a rodent model developed by Gordon and colleagues [233], ARRDC3 levels were elevated in a fasted state and decreased after mice were refed. However, the available evidence about the role and function of ARRDC3 is limited and future research could explore if this gene is associated with the cachectic phenotype.

The current study has several limitations that should be taken into consideration when interpreting its findings. It is unknown whether the evidence generated by this cell culture model that used skeletal muscle cells from three healthy individuals would be relevant in clinical settings. Although all myotubes were sourced from healthy donors, one cell line

showed a divergent response compared to the others in the western blotting data (highlighted in Figure 5.7A using pink points) and in the microarray analysis (line B from Figure 5.12, Appendix I), indicating the presence of interindividual differences. Repeating the experiments with additional skeletal muscle tissue samples would explore the magnitude of the interindividual variability and increase the validity of the findings. Further insights into the mechanistic action of ITLN1 might also be revealed by analysing the adipokine's behaviour in a rodent model. Moreover, the cells were treated for 6 hours with various ITLN1 concentrations. The dosage strategy was chosen before conducting the meta-analysis that assessed the levels of ITLN1 and this treatment duration (i.e., 6 hours) was considered appropriate for an initial experiment of this type. As previously discussed, future cell culture models should analyse the effect of various ITLN1 concentrations (i.e., 250ng/ml –physiological dose, 2000ng/ml – hyper-physiological dose) and observe whether chronic exposure to ITLN1 affects skeletal muscle cells differently than an acute treatment.

To conclude, elevated ITLN1 concentrations negatively affect insulin-mediated glucose uptake by skeletal muscle cells. Additionally, the highest rates of Akt phosphorylation were observed in the 100ng/ml treatment, when ITLN1 was close to its physiological concentration (i.e., 250ng/ml). There was no apparent effect of ITLN1 on muscle protein synthesis estimated by western blotting, while microarray data analysis suggested that the genes responsible for myogenesis were downregulated. Since there is not enough information about the effect of ITLN1 on skeletal muscle cells and about the relationship between ITLN1 and cancer cachexia, several pathways for further research were proposed. These included the study of ARRDC3, a gene with an apparent role in the regulation of skeletal muscle mass, and the development of in vitro in vivo models that would provide a comprehensive overview of ITLN1 behaviour.

## **6. General discussion**

Cancer cachexia research has been emerging in recent years primarily due to the poor outcomes associated with this syndrome [11]. A search for “cancer cachexia” on PubMed returns a total of 6990 studies, of which 22% were published between 2020 and 2023. Despite the increase in the amount of available evidence, cancer cachexia remains an unmet clinical need. It has been widely accepted that inflammation has a critical role in the loss of skeletal muscle mass specific to this wasting syndrome [107]. Multiple studies indicated that pro-inflammatory cytokines and adipokines could be involved in the pathogenesis and development of cachexia [31,97]. However, this relationship is not completely understood given the complexity of the cytokine network and additional research is still required. Therefore, the overarching objective of this thesis was to characterise the role and behaviour of (adipo)cytokines in cancer cachexia. This was achieved through a series of studies that are subsequently discussed.

### **6.1. Synopsis of findings**

Initially, a systematic review was conducted to examine the relationship between (adipo)cytokines and cachexia in patients with incurable cancer (Chapter 2). Overall, 31 different (adipo)cytokines were identified across the studies (n=17) that were included in the review. Of these, IL-6, TNF- $\alpha$  and IL-8 were associated with weight loss and cachexia. Specifically, IL-6 levels were elevated in cachectic patients compared to healthy controls in all six studies that examined this matter. Five out of eight studies reported that IL-6 concentrations were higher in individuals with cachexia compared to weight-stable cancer patients. Moreover, the levels of TNF- $\alpha$  were significantly increased in people with cancer cachexia as opposed to healthy controls in five out of six studies; the sixth study also reported higher TNF- $\alpha$  concentrations but failed to meet the threshold for traditional statistical significance (i.e.,  $p \leq 0.05$ ). Although it was examined only by a few studies, IL-8 was another cytokine that showed a potential relationship with cancer cachexia. Its levels were elevated in cachectic individuals compared to non-cachectic, pre-cachectic and healthy groups, making it a promising target for future investigations. Besides the previous observations, the

systematic review also noted a considerable degree of methodological heterogeneity. As highlighted in Table 2.2, the criteria according to which participants were grouped are highly variable. Some studies used the Fearon consensus [7] or mGPS [150] to classify individuals, while others used weight loss thresholds (i.e., 5%, 10%) or definitions that were not validated by previous research. Lastly, it is worth mentioning that a meta-analysis could not be conducted due to the lack of consistent grouping criteria and poor standards of data reporting.

The relationship between adipokines and the cachectic phenotype was explored further in Chapter 3 by analysing data from the REVOLUTION trial. This study aimed to examine if adiponectin, leptin, resistin, ITLN1 and visfatin can predict cachexia status and mGPS. Visfatin was excluded from the analysis since it was below the lowest level of detection in all samples. Bayesian ordinal regression suggested that the adipokines cannot be used as predictors of cachexia and mGPS in this group of patients. Interestingly, leptin was negatively associated with mGPS and cachexia status, while a positive relationship was observed between resistin and mGPS. Although statistically meaningful, these relationships were weak and future studies should discover other biomarkers that can be used alongside leptin and resistin to create a more reliable predictive model.

An adipokine that was extensively studied in recent years is ITLN1. Chapter 4 aimed to evaluate the role of ITLN1 in cancer and determine its physiological concentration. A Bayesian meta-analysis was conducted to assess the differences in circulating ITLN1 between cancer patients and healthy individuals. No differences were observed between groups when all 15 studies were included in the same statistical model. However, a subgroup analysis found considerably strong evidence in favour of higher ITLN1 levels in people with gastrointestinal cancer compared to healthy controls. ITLN1 concentrations were not substantially different between groups when individuals with urological or breast cancer were examined. A high degree of heterogeneity was observed in all statistical models that were analysed in this study. Additionally, 148 studies that measured circulating ITLN1 in 10118 healthy individuals were used to determine an interval for the physiological concentration of this adipokine. The mean ITLN1 level was 234ng/ml, with a 95% HDI that ranged from 193 to 275. The results were relatively similar in the analysis that investigated

only high-quality studies (i.e., 95%HDI: 195 to 318). Finally, ITLN1 levels were not different between females and males or between BMI groups (i.e., BMI $\leq$ 25 as opposed to BMI $>$ 25).

Miller and colleagues [44] suggested that ITLN1 might be involved in the development of cancer cachexia. A cell culture model was used to test their hypothesis and examine the effect of various ITLN1 concentrations on human skeletal muscle myotubes (Chapter 5). Specifically, glucose uptake, protein synthesis and the global transcriptome were assessed in response to different ITLN1 doses (i.e., 0, 10, 100 and 500ng/ml). The study found that ITLN1 had a negative effect on insulin-mediated glucose uptake. Statistical modelling indicated a 74% probability of observing lower glucose uptake rates in the 500ng/ml group compared to the control treatment. Furthermore, western blotting was used to measure Akt phosphorylation and protein synthesis. The highest level of Akt phosphorylation was observed in the 100ng/ml treatment and it was proposed that ITLN1 levels close to physiological concentrations have the strongest effect on phosphorylation rates. Remarkably, protein synthesis was not affected in any of the groups. This finding was particularly relevant since previous research [44] suggested that ITLN1 might be involved in cancer cachexia, a syndrome that affects protein synthesis. However, it was hypothesised that ITLN1 could regulate other processes that subsequently contribute to the loss of skeletal muscle mass specific to cachexia. The microarray data indicated that an acute treatment with ITLN1 did not have an effect at individual gene level. Category enrichment analysis suggested that myogenesis was downregulated. Also, the highest level of differential expression was observed in ARRDC3, a gene with an apparent role in skeletal muscle mass regulation. Based on these findings, it could be argued that ITLN1 might influence skeletal muscle biology and future research should focus on elucidating the adipokine's specific mechanisms of action.

## 6.2. Directions for future research

The systematic reviews conducted as part of this thesis observed various methodological limitations that were omnipresent in the literature. Less than a third of the studies that measured (adipo)cytokine concentrations in patients with incurable cancer adequately reported the details of the assay that was used to quantify biomarkers. Similarly, the assay characteristics and the blood collection methods were poorly described in approximately 65% of the studies included in the review that examined ITLN1. Given the variability of (adipo)cytokines, it is essential to establish when the blood samples were collected (i.e., preferably in the morning, after an overnight fast) and the details (i.e., detection limits, sensitivity) of the assay that was used to quantify the biomarkers. This will allow future reviews to identify the (adipo)cytokines with the highest level of reliability (i.e., lowest level of variability) and the optimal methods of assessing them. In the long term, having consistent blood collection and assessment approaches will also rule out the hypothesis that the differences in (adipo)cytokine levels are driven by methodological dissimilarities. Furthermore, several studies did not report data for relationships that were not statistically significant and failed to discuss analytes that were below detection levels. If these practices continue to be used in the literature, the rate of false positive findings in meta-analyses and systematic reviews will increase. There is a clear absence of large human trials that evaluate cancer cachexia. Consequently, aggregating multiple smaller studies in systematic reviews and meta-analyses is the most effective way of generating evidence that could be relevant for clinical practice. Moving forward, this can only be done by improving methodological homogeneity and the standards of data reporting.

Besides the relationships observed between adipokines, mGPS and cachexia, Chapter 3 provided useful insights about the challenges associated with assessing patients with incurable cancer. The REVOLUTION trial [156] was designed to monitor both clinical and biochemical parameters by conducting a series of comprehensive assessments at baseline and every six weeks until the end of life. The amount of data collected in this study should be a reference point for research that aims to analyse individuals with incurable cancer. However, there are several aspects that future trials should aim to improve. Only three individuals survived until T2 (Table 3.1) and thus, this timepoint was excluded from the

analysis. Increasing the initial sample size and the frequency of measurements would have a positive impact on the availability of data. Moreover, blood was collected between 09:00 and 16:00 due to the priorities of patient care. As previously discussed, future studies that analyse (adipo)cytokine levels should aim to standardise blood collection methods by obtaining blood in the morning, after an overnight fast. Lastly, only six individuals did not receive any therapy or medicine at baseline. It would be interesting to observe the effect of different therapies on (adipo)cytokine concentrations. The health status of patients enrolled in studies that evaluate cancer cachexia is inevitably poor and it can be argued that the vast majority of participants will receive a form of therapy. If these treatments affect (adipo)cytokine concentrations, future research should find methods of dealing with this confounding variable (i.e., therapy) in the statistical interpretation of the results.

Chapter 5 included the first study that examined the effect of ITLN1 on skeletal muscle cells. In addition to the findings that were described earlier, there is scope for supplementary research that could further characterise ITLN1 behaviour. Firstly, different dosing strategies should be explored by assessing the effects of physiological (i.e., 250ng/ml) and hyper-physiological ITLN1 concentrations on human myotubes. Also, the impact of prolonged exposure to ITLN1 (i.e., 12, 24 and 48 hours) on glucose uptake, protein synthesis and the global metabolome should be determined. Additionally, it was proposed that ITLN1 could act in a tissue-specific manner. This hypothesis could be tested by culturing subcutaneous adipocytes, visceral adipocytes and myotubes, treating them with similar ITLN1 doses, and comparing the responses. The experiment could be further developed by adding tumour-conditioned media. This would generate a comprehensive overview of the effects of ITLN1 on various tissues in the presence and absence of a tumour-specific environment.



### **6.3. Conclusions**

The findings of this thesis could lead to progress in the field of cancer cachexia. Specifically, a systematic review of the literature identified that IL-6 and TNF- $\alpha$  levels were elevated in cachectic individuals. Leptin was negatively associated with mGPS and cachexia status, while a positive relationship was observed between resistin and mGPS in a sample of patients with incurable cancer. These (adipo)cytokines might be regulators of cancer cachexia and should be further examined in human trials. An index that predicts the onset and progression of cachexia could be initially developed based on IL-6, TNF- $\alpha$ , leptin and resistin levels. Other biomarkers with strong predictive capabilities must also be identified. One adipokine that should be monitored due to its potential role in cancer cachexia is ITLN1. Its levels were considerably higher in individuals with gastrointestinal cancer compared to healthy controls. Moreover, a cell culture model indicated that increased ITLN1 concentrations diminish insulin-mediated glucose uptake in skeletal muscle cells and downregulate the expression of genes involved in myogenesis. Regardless of the pathways that will be followed by future research, it is imperative to increase methodological homogeneity and quality.

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## Appendices

### Appendix A – Detailed search strategy

*Ovid Medline(R) and Epub Ahead of Print, In-process & Other Non-Indexed Citations and Daily*

1. exp cancer/
2. neoplas\*.ab,ti.
3. metastasis\$.ab,ti.
4. 1 or 2 or 3
5. exp cytokines/
6. exp Inflammation/
7. exp interferon/
8. tumo\$r necrosis factor.ab,ti.
9. tnf.ab,ti.
10. exp interleukin/
11. 5 or 6 or 7 or 8 or 9 or 10
12. symptom\*.ab,ti.kw.
13. depress\*.ab,ti.kw.
14. nausea.ab,ti.kw.
15. fatigue.ab,ti.kw.
16. sleep.ab,ti.kw.
17. quality of life.ab,ti.kw.
18. anxiety.ab,ti.kw.
19. pain.ab,ti.kw.
20. cachexia.ab,ti.kw.
21. weight loss.ab,ti.kw.
22. anorexia.ab,ti.kw.
23. 12 or 13 or 14 or 15 or 16 or 17 or 18 or 18 or 20 or 21 or 22
24. 4 and 11 and 23
25. limit 24 to (humans and yr="2004-Current")

*Ovid EMBASE 1980 to 2019 Week 49*

1. exp cancer/
2. neoplasms.ab,ti.
3. metastasis\$.ab,ti.
4. 1 or 2 or 3
5. exp cytokines/
6. exp Inflammation/
7. exp interferon/
8. tumor necrosis factor.ab,ti.
9. tnf.ab,ti.
10. exp interleukin/
11. 5 or 6 or 7 or 8 or 9 or 10
12. symptom\*.ab,ti.kw.
13. depress\*.ab,ti.kw.
14. nausea.ab,ti.kw.
15. fatigue.ab,ti.kw.
16. sleep.ab,ti.kw.
17. quality of life.ab,ti.kw.
18. anxiety.ab,ti.kw.
19. pain.ab,ti.kw.
20. cachexia.ab,ti.kw.
21. weight loss.ab,ti.kw.
22. anorexia.ab,ti.kw.
23. 12 or 13 or 14 or 15 or 16 or 17 or 18 or 18 or 20 or 21 or 22
24. 4 and 11 and 23
25. limit 24 to (humans and yr="2004-Current")



*CINAHL Complete on EBSCO host*

S1 MJ cancer

S2 TI neoplasm OR AB neoplasm

S3 TI metastasis OR AB metastasis

S4 S1 OR S2 OR S3

S5 MH cytokines

S6 MJ interleukin

S7 MH inflammation

S8 MJ interferon

S9 TI tumor necrosis factor OR AB tumor necrosis factor

S10 TI tnf OR AB tnf

S11 S5 OR S6 OR S7 OR S8 OR S9 OR S10

S12 TI symptom OR AB symptom OR SU symptom

S13 TI depression OR AB depression OR SU depression

S14 TI fatigue OR AB fatigue OR SU fatigue

S15 TI sleep OR AB sleep OR SU sleep

S16 TI quality of life OR AB quality of life OR SU quality of life

S17 TI anxiety OR AB anxiety OR SU anxiety

S18 TI pain OR AB pain OR SU pain

S19 TI cachexia OR AB cachexia OR SU cachexia

S20 TI weight loss OR AB weight loss OR SU weight loss

S21 TI anorexia OR AB anorexia OR SU anorexia

S22 TI nausea OR AB nausea OR SU nausea

S23 S12 OR S13 OR S14 OR S15 OR S16 OR S17 OR S18 OR S19 OR S20 OR S21 OR S22

S24 S4 AND S11 AND S23

*PsycINFO on EBSOC host*

S1 MJ cancer

S2 TI neoplasm OR AB neoplasm

S3 TI metastasis OR AB metastasis

S4 S1 OR S2 OR S3

S5 MJ cytokines

S6 MJ interleukin

S7 MJ inflammation

S8 MJ interferon

S9 TI tumor necrosis factor OR AB tumor necrosis factor

S10 TI tnf OR AB tnf

S11 S5 OR S6 OR S7 OR S8 OR S9 OR S10

S12 TI symptom OR AB symptom OR SU symptom

S13 TI depression OR AB depression OR SU depression

S14 TI fatigue OR AB fatigue OR SU fatigue

S15 TI sleep OR AB sleep OR SU sleep

S16 TI quality of life OR AB quality of life OR SU quality of life

S17 TI anxiety OR AB anxiety OR SU anxiety

S18 TI pain OR AB pain OR SU pain

S19 TI cachexia OR AB cachexia OR SU cachexia

S20 TI weight loss OR AB weight loss OR SU weight loss

S21 TI anorexia OR AB anorexia OR SU anorexia

S22 TI nausea OR AB nausea OR SU nausea

S23 S12 OR S13 OR S14 OR S15 OR S16 OR S17 OR S18 OR S19 OR S20 OR S21 OR S22

S24 S4 AND S11 AND S23

*Central – Cochrane Library*

1. MeSH descriptor: [Cytokines] explode all trees
2. MeSH descriptor: [Inflammation] this term only
3. MeSH descriptor: [Interferons] explode all trees
4. MeSH descriptor: [Interleukins] explode all trees
5. MeSH descriptor: [Tumor Necrosis Factors] in all MeSH products
6. MeSH descriptor: [Acute-Phase Proteins] explode all trees
7. (cytokine\*):ti,ab,kw
8. (interleukin\*):ti,ab,kw
9. #1 or #2 or #3 or #4 or #5 or #6 or #7 or #8
10. MeSH descriptor: [Neoplasms] explode all trees
11. ("Cancer"):ti,ab,kw
12. MeSH descriptor: [Neoplasm Metastasis] explode all trees
13. #10 or #11 or #12
14. symptom:ti,ab,kw
15. depression:ti,ab,kw
16. nausea:ti,ab,kw
17. fatigue:ti,ab,kw
18. sleep:ti,ab,kw
19. quality of life:ti,ab,kw
20. anxiety:ti,ab,kw
21. pain:ti,ab,kw
22. cachexia:ti,ab,kw
23. weight loss:ti,ab,kw
24. anorexia:ti,ab,kw
25. #14 or #15 or #16 or #17 or #18 or #19 or #20 or #21 or #22 or #23 or #24
26. #9 and #13 and #25 with Publication Year from 2004 to 2019, in Trials

*Web of Science – Core collection*

1. TS=(cancer)
2. TS=(neoplasm)
3. TS=(metastasis)
4. #1 or #2 or #3
5. TS=(cytokine\*)
6. TS=(inflammation)
7. TS=(interferon)
8. TS=(tumor necrosis factor)
9. TS=(tnf)
10. TS=(interleukin)
11. #5 or #6 or #7 or #8 or #9 or #10
12. TI=(symptom)
13. TI=(depression)
14. TI=(nausea)
15. TI=(fatigue)
16. TI=(sleep)
17. TI=(quality of life)
18. TI=(anxiety)
19. TI=(pain)
20. TI=(cachexia)
21. TI=(weight loss)
22. TI=(anorexia)
23. #12 or #13 or #14 or #15 or #16 or #17 or #18 or #19 or #20 or #21 or #22
24. #4 and #11 and #23

## Appendix B – Quality assessment tool

The following questions were selected from the modified Downs and Black (MDB) checklist [100] and used to determine the quality of studies included in the current review.

Question	Score (yes = 1; no = 0)
<b>Reporting</b>	
Is the hypothesis/aim/objective of the study clearly described?	
Are the main outcomes of the study clearly described?	
Are the characteristics of the patients included in the study clearly described?	
Are the main findings of the study clearly described?	
Does the study provide estimates of the random variability in the data for the main outcomes?	
Have actual probability values been reported (e.g. 0.035 rather than <0.05) for the main outcomes except where the probability value is less than 0.001?	
<b>External validity</b>	
Were the subjects asked to participate in the study representative of the entire population from which they were recruited?	
Were those subjects who were prepared to participate representative of the entire population from which they were recruited?	
<b>Internal validity</b>	
Were the statistical tests used to assess the main outcomes appropriate?	
Were the main outcome measures used accurate (valid and reliable)?	
<b>Total score</b>	

## **Appendix C – R script**

The R script that was used to analyse data can was uploaded to the following GitHub repository: <https://github.com/rpaaval/revolution>.

## Appendix D – Description of posterior distributions

**Table 3.2.** Characteristics of the posterior distributions generated by the Bayesian regression model that analysed the relationship between adipokines and mGPS.

<b>Treatment</b>	<b>Mean</b>	<b>Lower 90% HDI</b>	<b>Upper 90% HDI</b>
Intercept 1	0.560	-1.007	2.240
Intercept 2	1.074	-0.503	2.754
Adiponectin	0.003	-0.001	0.007
Intelectin-1	-0.008	-0.040	0.025
Leptin	-0.019	-0.039	-0.002
Resistin	0.071	0.006	0.141

The data represent model coefficients (reported in arbitrary units).

**Table 3.3.** Characteristics of the posterior distributions generated by the Bayesian regression model that analysed the relationship between adipokines and cachexia.

<b>Treatment</b>	<b>Mean</b>	<b>Lower 90% HDI</b>	<b>Upper 90% HDI</b>
Intercept 1	-0.460	-2.047	1.106
Adiponectin	0.001	-0.002	0.005
Intelectin-1	0.013	-0.020	0.049
Leptin	-0.022	-0.048	-0.003
Resistin	0.045	-0.023	0.125

The data represent model coefficients (reported in arbitrary units).

**Table 3.4.** Characteristics of the posterior distributions generated by the Bayesian generalised linear model that analysed the difference in adiponectin (n=10), intelectin-1 (n=10), leptin (n=7) and resistin (n=0) levels between timepoints (i.e., T0 and T1)

<b>Timepoint</b>	<b>Mean</b>	<b>Lower 90% HDI</b>	<b>Upper 90% HDI</b>
Adiponectin (ng/ml)			
T0	275.2	-516.5	1060.1
T1	254.9	-1106.0	1063.0
Intelectin-1 (ng/ml)			
T0	10.7	-18.8	41.1
T1	11.8	-39.6	42.1
Leptin (ng/ml)			
T0	14.4	-8.3	36.8
T1	14.3	-32.3	31.5
Resistin (ng/ml)			
T0	11.6	-16.4	39.3
T1	11.7	-39.2	37.8



## **Appendix E – Detailed search strategy**

*Ovid MEDLINE– 1946 to date: 759 results*

intelectin\* {including related terms} or omentin\* {including related terms}

*Ovid EMBASE – 1946 to date: 1290 results*

Intelectin\*.mp or omentin\*.mp

*Web of Science – Core collection: 948 results*

TS=intelectin\* or TS=omentin\*

*CINAHL – on EBSCO host: 142 results*

intelectin\* or omentin\*

*CENTRAL – Cochrane Library: 80 results*

(intelectin\*):ti,ab,kw and (omentin\*):ti,ab,kw

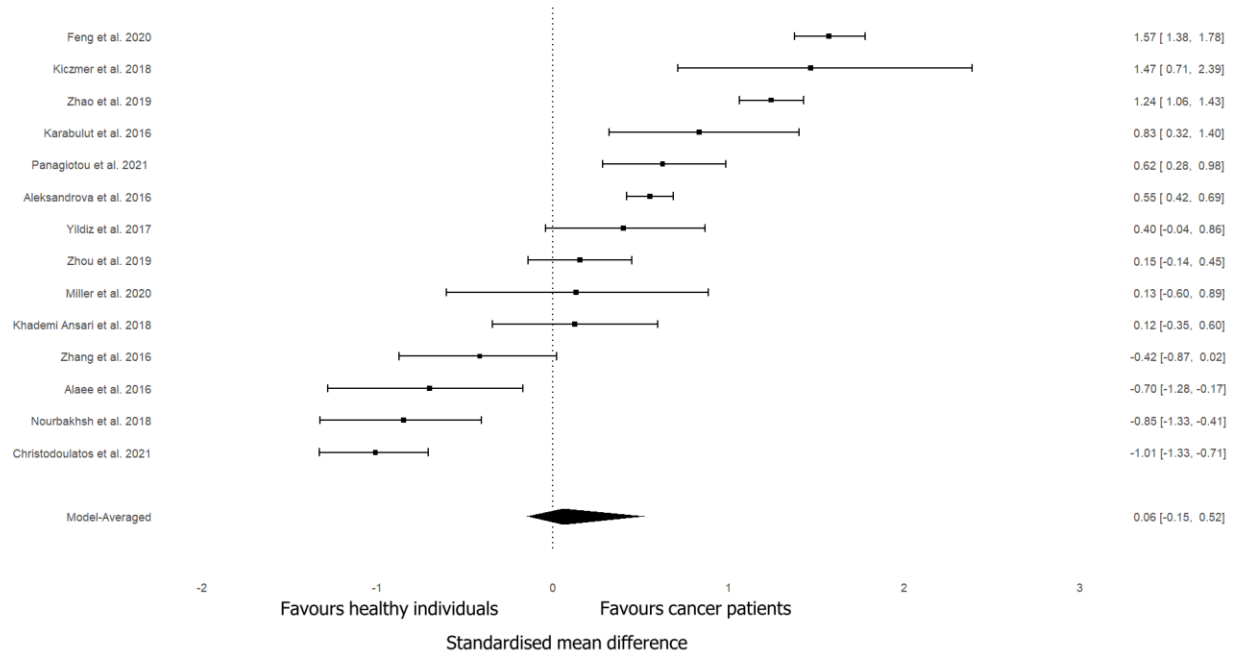
## **Appendix F – R script and complete dataset**

The R script used for data analysis can be found in the following GitHub repository:

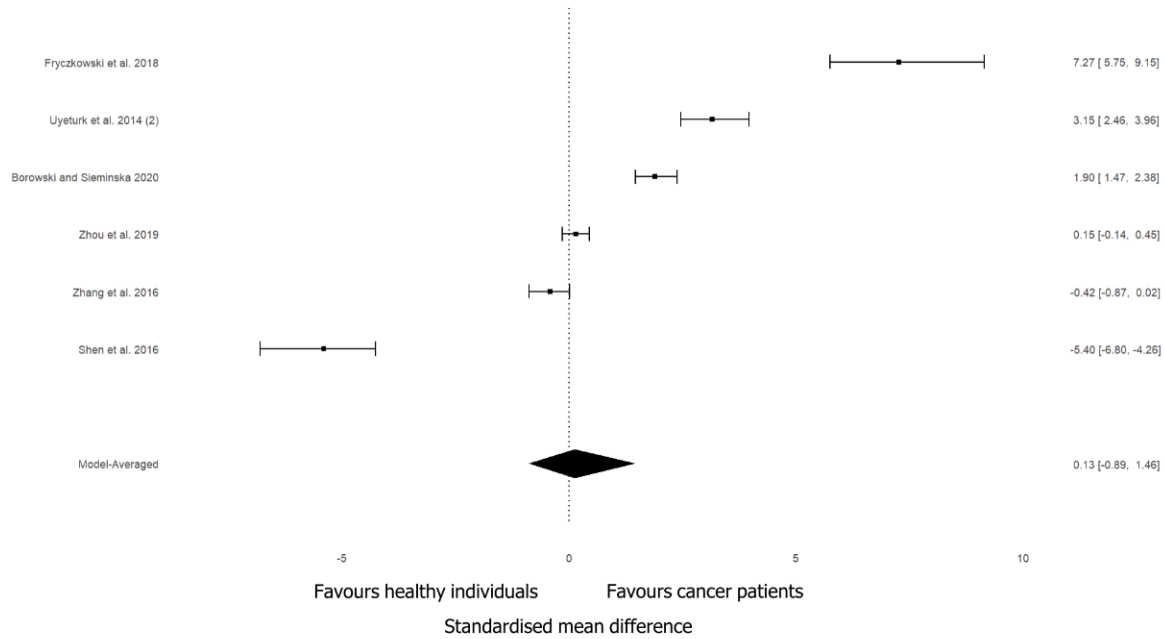
[https://github.com/rpaval/bayesian\\_meta\\_analysis](https://github.com/rpaval/bayesian_meta_analysis)

The repository also includes the dataset that was used to conduct the meta-analyses and the data collection form.

## Appendix G – Additional figures



**Figure 4.9.** Forest plot showing meta-analytic results of the sensitivity analysis for the studies that compared ITLN1 levels in people with cancer and healthy individuals after excluding the outlying study by Shen and colleagues [54]. Model-averaged data are presented as SMD and 95% HDI.



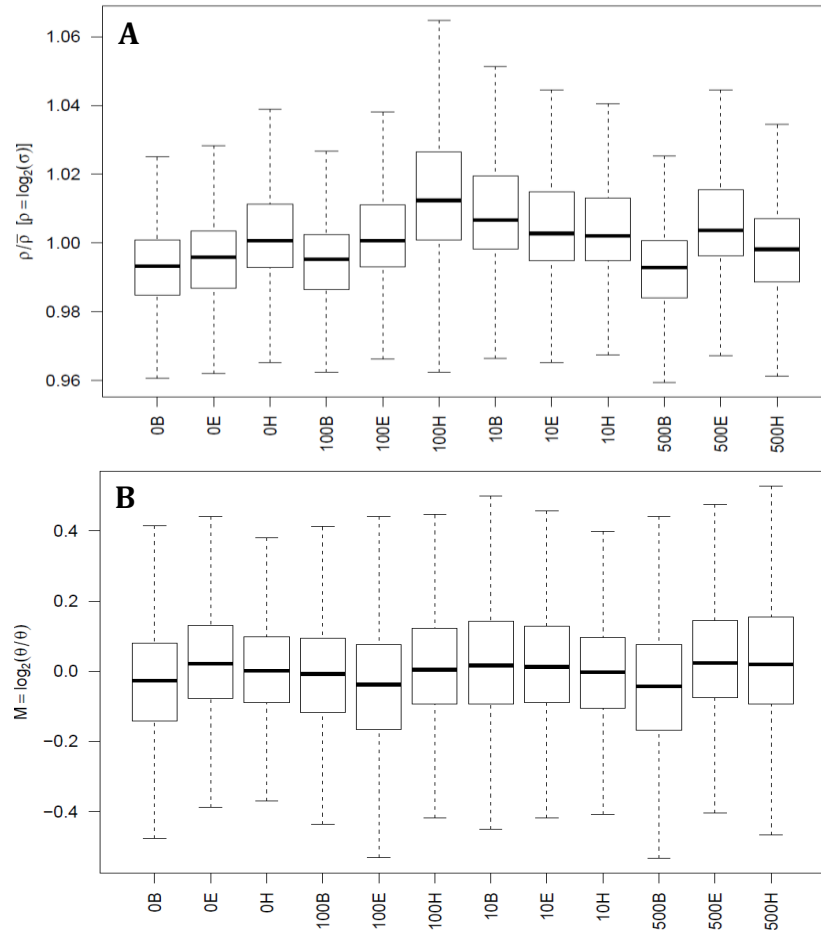
**Figure 4.10.** Forest plot showing meta-analytic results of the robustness check for the studies that compared ITLN1 levels in people with urological cancer and healthy individuals. Three studies (Uyeturk et al., [50]; Fryczkowski et al., [51]; Borowski and Seminska et al., [52]) did not include a control group and the mean ITLN1 of healthy individuals ( $234 \pm 21$  ng/ml) was used as a reference point in these instances. Model-averaged data are presented as SMD and 95% HDI.

## **Appendix H – R script and complete dataset**

The complete dataset that was analysed in the current study, as well as the R script that was used for data analysis, can be found online in the following GitHub repository:

[https://github.com/rpaval/cell\\_culture](https://github.com/rpaval/cell_culture)

## Appendix I – Microarray analysis additional information



**Figure 5.11.** Quality control plots showing the (A) normalised unscaled error (NUSE) and (B) relative log expression (RLE) analysis. The x-axis highlights the cell line (i.e., B, E or H) and the ITLN1 dose (i.e., 0ng/ml, 10ng/ml, 100ng/ml or 500ng/ml). The y-axis shows the quality control metrics. The NUSE analysis indicates the precision of a gene’s expression on an array relative to the other arrays that were examined in the same batch [199]. The values are determined as the standard error of a given gene divided by the median standard error of that gene across all arrays. High-quality arrays should be scattered around 1. The RLE is determined by subtracting the estimated median gene expression across all arrays from the estimated expression of each gene [199]. High-quality arrays should be scattered around 0, indicating that the expression of no array is considerably different from the median of all other arrays.

**Table 5.2.** Category enrichment analysis results.

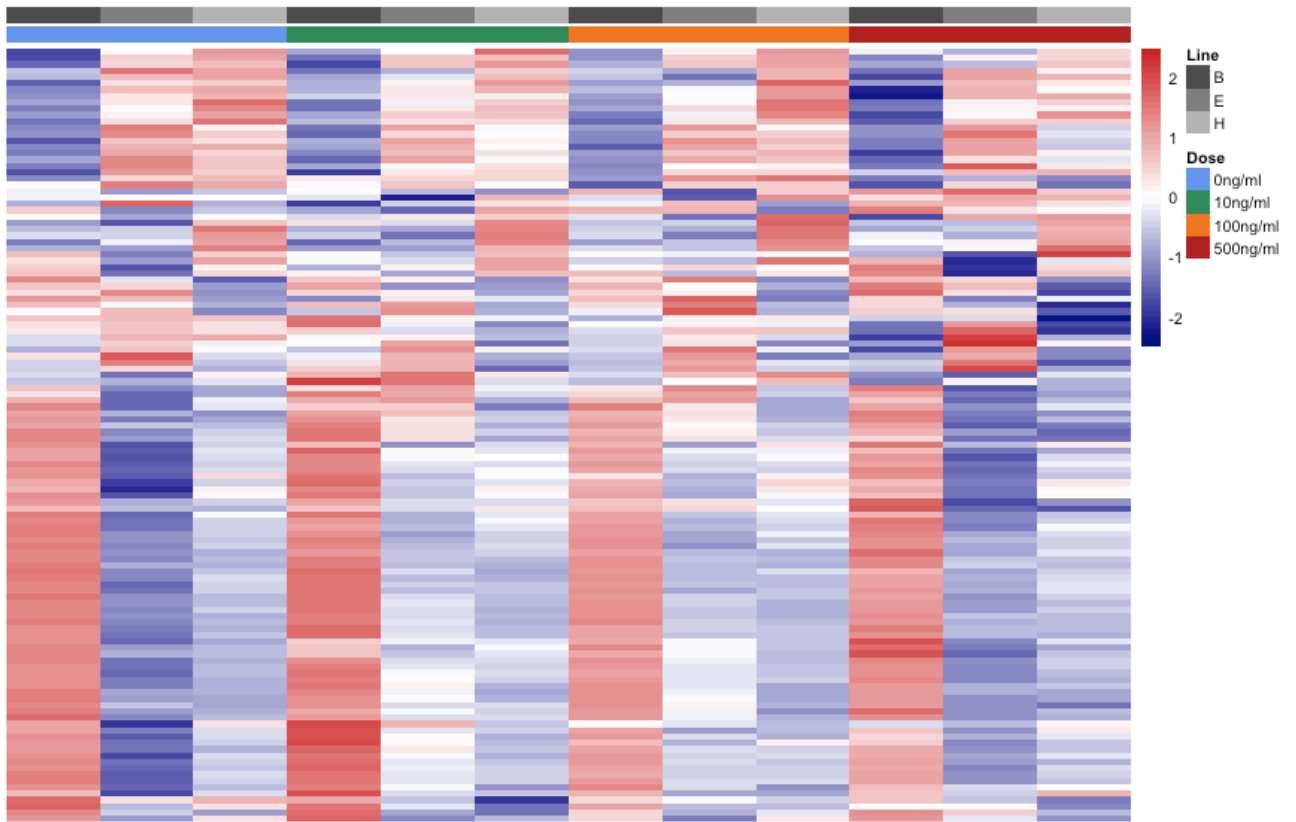
Category	Number of genes	Direction	P value	FDR
MYOGENESIS	122	Down	0.00	0.01
CHOLESTEROL HOMEOSTASIS	39	Down	0.00	0.01
APICAL JUNCTION	78	Down	0.03	0.22
KRAS SIGNALING DN	20	Down	0.03	0.22
XENOBIOTIC METABOLISM	64	Down	0.03	0.22
HYPOXIA	91	Up	0.03	0.22
INTERFERON ALPHA RESPONSE	34	Up	0.04	0.22
MTORC1 SIGNALING	121	Down	0.04	0.22
SPERMATOGENESIS	23	Down	0.04	0.22
NOTCH SIGNALING	12	Down	0.04	0.22
MITOTIC SPINDLE	82	Down	0.05	0.23
TNFA SIGNALING VIA NFKB	86	Up	0.10	0.40
ALLOGRAFT REJECTION	44	Up	0.13	0.49
INTERFERON GAMMA RESPONSE	68	Up	0.14	0.49
UV RESPONSE UP	62	Down	0.15	0.51
PI3K AKT MTOR SIGNALING	40	Down	0.18	0.55
ADIPOGENESIS	95	Down	0.19	0.55
TGF BETA SIGNALING	36	Down	0.20	0.55
IL6 JAK STAT3 SIGNALING	29	Up	0.22	0.55
G2M CHECKPOINT	72	Down	0.22	0.55
COMPLEMENT	74	Up	0.23	0.55
EPITHELIAL MESENCHYMAL TRANSITION	137	Down	0.26	0.57
BILE ACID METABOLISM	30	Down	0.27	0.57
KRAS SIGNALING UP	57	Up	0.28	0.57
OXIDATIVE PHOSPHORYLATION	119	Down	0.29	0.57
WNT BETA CATENIN SIGNALING	16	Down	0.31	0.57
APOPTOSIS	78	Up	0.31	0.57
UV RESPONSE DN	83	Down	0.33	0.58
REACTIVE OXIGEN SPECIES PATHWAY	25	Down	0.34	0.58

PEROXISOME	50	Down	0.35	0.58
APICAL SURFACE	12	Up	0.36	0.58
UNFOLDED PROTEIN RESPONSE	75	Down	0.37	0.58
P53 PATHWAY	79	Up	0.41	0.63
PROTEIN SECRETION	68	Up	0.52	0.77
PANCREAS BETA CELLS	9	Up	0.54	0.77
FATTY ACID METABOLISM	71	Down	0.57	0.78
GLYCOLYSIS	82	Up	0.61	0.82
MYC TARGETS V1	119	Down	0.73	0.90
MYC TARGETS V2	22	Down	0.73	0.90
ESTROGEN RESPON LATE	59	Up	0.75	0.90
E2F TARGETS	61	Down	0.79	0.90
HEME METABOLISM	76	Down	0.81	0.90
COAGULATION	57	Up	0.81	0.90
ANGIOGENESIS	16	Up	0.81	0.90
IL2 STAT5 SIGNALING	71	Down	0.82	0.90
INFLAMMATORY RESPONSE	62	Down	0.83	0.90
ESTROGEN RESPONSE EARLY	63	Down	0.90	0.96
HEDGEHOG SIGNALING	10	Up	0.95	0.98
DNA REPAIR	68	Up	0.96	0.98
ANDROGEN RESPONSE	56	Up	0.99	0.99

---

The first column indicates the geneset that was identified, while the second column suggests the how many genes were downregulated or upregulated (as indicated by the third column). FDR – false discovery rate.





**Figure 5.12.** Heatmap of genes involved in myogenesis, identified following category enrichment analysis. The genes were grouped according to the cell line (labelled as B, E and H) and ITLN1 dose (0, 10, 100 or 500 ng/ml)

## Appendix J – List of primers

**Table 5.3.** Forward and reverse primer sequences as well as accession numbers of the genes of interest measured by qPCR.

<b>Gene</b>	<b>Accession number</b>	<b>Forward primer sequence</b>	<b>Reverse primer sequence</b>
ARRDC3	NM_020901	TGTATTCTAGTGGGGATACCGTC	TCGCATGTCCTCTTGCATGAA
H1-0	NM_005318	CGCGCCAGTCCATTCAGAA	ACAACCTTGATCTGCCGAGTCAG
H1-2	NM_005319	CCGCCTCTAAAGAGCGTAGC	AGACCAAGTTTGATACGGCTG
TM4SF1	NM_014220	TGTGGCAAACGATGTGCGA	TGACACAGTAGCCAGATCCTG
TXNIP	NM_006472	GGTCTTTAACGACCCTGAAAAGG	ACACGAGTAACTTCACACACCT
OGA	NM_012215	CATAGGATGTTTTGGCGAGAGAT	GGTGAGATCGCATAGATGAACTC
ADNP2	NM_014913	AGGACCTTAAAGGCTTTGATCCA	ACAACAGTATGGCTTTGTTTCGAT
AHNAK2	NM_138420	GTGCAGAAACGGAAGATGACC	GCCTCAGTCGTGTATTCGTAGA
OGT	NM_181672	TCCTGATTTGTACTGTGTTTCGC	AAGCTACTGCAAAGTTCGGTT

## Appendix K – Description of posterior distributions

**Table 5.4.** Characteristics of the posterior distributions generated by the Bayesian linear model that analysed data from the glucose uptake assay.

<b>Treatment</b>	<b>Mean</b>	<b>Lower 90% HDI</b>	<b>Upper 90% HDI</b>
0 ng/ml	47624	38652	56587
10 ng/ml	42755	33818	51187
100 ng/ml	40618	31418	48952
500 ng/ml	35267	26803	44670

The data represents luminescence values (relative light units).

**Table 5.5.** Characteristics of posterior distributions generated by the Bayesian linear model that analysed data from western blots measuring p-Akt and total Akt.

<b>Treatment</b>	<b>Mean</b>	<b>Lower 90% HDI</b>	<b>Upper 90% HDI</b>
0 ng/ml	0.372	0.166	0.595
10 ng/ml	0.664	0.451	0.883
100 ng/ml	1.031	0.800	1.241
500 ng/ml	0.752	0.542	0.990

The data represents the ratio between the optical density values of p-Akt and total Akt (arbitrary units).

**Table 5.6.** Characteristics of posterior distributions generated by the Bayesian linear model that analysed data from western blots measuring puromycin incorporation.

<b>Treatment</b>	<b>Mean</b>	<b>Lower 90% HDI</b>	<b>Upper 90% HDI</b>
0 ng/ml	17558	1953	32363
10 ng/ml	25849	9573	43142
100 ng/ml	21795	5736	38486
500 ng/ml	23440	7172	41049

The data represents optical density values (arbitrary units).

## **Appendix L – Published abstracts and studies**

Abstract accepted for oral presentation at the *15<sup>th</sup> international Conference of the Society on Cachexia, Sarcopenia & Muscle Wasting*.

### **The relationship between cachexia and inflammatory biomarkers in patients with cancer; initial findings from the REVOLUTION cachexia characterisation study**

Robert Paval<sup>1</sup>, Rebekah Patton<sup>2</sup>, Judith Sayers<sup>2,3</sup>, Marie Fallon<sup>2</sup>, Richard J E Skipworth<sup>3</sup>, Barry J A Laird<sup>2</sup>, Iain J Gallagher<sup>1</sup> on behalf of the Caledonian Cachexia Collaborative.

<sup>1</sup>Faculty of Health Sciences and Sport, University of Stirling, Stirling UK. <sup>2</sup>Institute of Genetics and Cancer, University of Edinburgh; <sup>3</sup>Department of Clinical Surgery, Royal Infirmary of Edinburgh, Edinburgh,

## **Introduction**

The systemic inflammatory response has a role in the development of cancer cachexia; however, the role of inflammatory biomarkers and their relationship to the clinical phenotype of cachexia needs further elucidation. The REVOLUTION trial<sup>1</sup> is a prospective characterisation of cancer cachexia assessing clinical parameters, patient-reported outcomes, body composition and the biological components of cachexia. Herein we present initial findings relating inflammatory biomarkers to cancer cachexia parameters.

## **Methods**

Using data from the REVOLUTION trial, we assessed C-reactive protein (CRP) in isolation and as part of the modified Glasgow Prognostic Score (mGPS), Body Mass Index (BMI), weight loss (>5% or >2% and BMI<20kg/m<sup>2</sup>) and their relationships to specific inflammatory biomarkers<sup>2,3</sup>. We examined the following: IFN $\gamma$ , IL-1 $\alpha$ , IL-1 $\beta$ , IL-2, IL-4, IL-10, IL-12, MIP-1, MCP-1, visafatin, adiponectin, GDF-15, IL-1RA, IL-6, IL-8, intelectin-1 (ITLN1), leptin, resistin, TNF- $\alpha$  and VEGF. The relationship between these and cancer cachexia parameters was assessed using correlation coefficients and analysis of variance.

## Results

Data were available for 38 patients. CRP was associated with IL-6 ( $r=0.44$ ,  $p=0.02$ ), VEGF ( $r=0.42$ ,  $p=0.02$ ), IL-8 ( $r=0.39$ ,  $p<0.01$ ), resistin ( $r=0.37$ ,  $p=0.04$ ) and ITLN1 ( $r=-0.34$ ,  $p=0.04$ ). In cachectic individuals, resistin ( $r=0.65$ ,  $p<0.01$ ), IL-1RA ( $r=0.45$ ,  $p=0.05$ ), VEGF ( $r=0.48$ ,  $p=0.05$ ) and IL-8 ( $r=0.45$ ,  $p=0.05$ ) were correlated with CRP. Decreasing BMI was associated with GDF-15 ( $r=0.51$ ,  $p<0.01$ ) and IL-1RA ( $r=0.49$ ,  $p<0.01$ ). Increasing inflammation (based on the mGPS) was associated with the ITLN1 ( $p=0.05$ ). Moreover, the levels of ITLN1 were higher at baseline ( $p<0.01$ ) compared to the 6-week follow-up measurement. Weight loss was not associated with any of the inflammatory biomarkers.

## Conclusions

The findings provide further insight into the potential inflammatory drivers of cancer cachexia and how these relate to the clinical phenotype. It is of interest that weight loss, often regarded as a central tenet of cachexia, was not associated with any inflammatory biomarkers.

## References

1. Patton R, Cook J, Haraldsdottir E, et al. REVOLUTION (Routine EVALUatiOn of people Living with caNcer)-Protocol for a prospective characterisation study of patients with incurable cancer. *PLoS One* 2021;16(12):e0261175. doi: 10.1371/journal.pone.0261175
2. Patton R, Paval DR, McDonald JJ, et al. Relationship between cytokines and symptoms in people with incurable cancer: A systematic review. *CROH*, 2021:103222.
3. Paval DR, Patton R, McDonald JJ, et al. A systematic review examining the relationship between cytokines and cachexia in incurable cancer. *J. Cachexia Sarcopenia Muscle* 2022, doi: 10.1002/jcsm.12912.

# A systematic review examining the relationship between cytokines and cachexia in incurable cancer

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## Abstract

Cancer cachexia is an unmet clinical need that affects more than 50% of patients with cancer. The systemic inflammatory response, which is mediated by a network of cytokines, has an established role in the genesis and maintenance of cancer as well as in cachexia; yet, the specific role of the cytokine milieu in cachexia requires elucidation. This systematic review aims to examine the relationship between cytokines and the cachexia syndrome in patients with incurable cancer. The databases MEDLINE, EMBASE, CINAHL, CENTRAL, PsycINFO, and Web of Science were searched for studies published between 01/01/2004 and 06/01/2020. Included studies measured cytokines and their relationship with cachexia and related symptoms/signs in adults with incurable cancer. After title screening ( $n = 5202$ ), the abstracts ( $n = 1264$ ) and the full-text studies ( $n = 322$ ) were reviewed independently by two authors. The quality assessment of the selected papers was conducted using the modified Downs and Black checklist. Overall, 1277 patients with incurable cancer and 155 healthy controls were analysed in the 17 eligible studies. The mean age of the patients was  $64 \pm 15$  (mean  $\pm$  standard deviation). Only 34% of included participants were female. The included studies were assessed as moderate-quality to high-quality evidence (mean quality score: 7.8; range: 5–10). A total of 31 cytokines were examined in this review, of which interleukin-6 (IL-6, 14 studies) and tumour necrosis factor- $\alpha$  (TNF- $\alpha$ , 12 studies) were the most common. The definitions of cachexia and the weight-loss thresholds were highly variable across studies. Although the data could not be meta-analysed due to the high degree of methodological heterogeneity, the findings were discussed in a systematic manner. IL-6, TNF- $\alpha$ , and IL-8 were greater in cachectic patients compared with healthy individuals. Also, IL-6 levels were higher in cachectic participants as opposed to non-cachectic patients. Leptin, interferon- $\gamma$ , IL-1 $\beta$ , IL-10, adiponectin, and ghrelin did not demonstrate any significant difference between groups when individuals with cancer cachexia were compared against non-cachectic patients or healthy participants. These findings suggest that a network of cytokines, commonly IL-6, TNF- $\alpha$ , and IL-8, are associated with the development of cachexia. Yet, this relationship is not proven to be causative and future studies should opt for longitudinal designs with consistent methodological approaches, as well as adequate techniques for analysing and reporting the results.

**Keywords** Cachexia; Cancer; Weight loss; Cytokines

Received: 15 February 2021; Revised: 1 October 2021; Accepted: 6 December 2021

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D. Robert Paval and Rebekah Patton are joint first authors.

Disclaimer: The authors are currently working on the REVOLUTION Trial (NCT04406662).



## Introduction

Cancer cachexia is a complex syndrome characterized by the loss of skeletal muscle mass—with or without loss of fat mass—which cannot be fully reversed using standard nutritional care.<sup>1</sup> This multifactorial syndrome that leads to progressive functional impairment occurs at different rates depending on the type of cancer, affects more than 50% of the patients, and accounts for 20% of cancer-related deaths.<sup>2</sup> Furthermore, it has been established that cachexia diminishes the effectiveness of anti-cancer treatments<sup>3</sup> and negatively affects patients' quality of life.<sup>4</sup> To date, there is no licensed treatment and no standard of care.<sup>5</sup>

Cancer cachexia results from a combination of reduced energy intake, excess energy expenditure, elevated catabolism, and increased systemic inflammation.<sup>6</sup> Previous research suggested that the systemic inflammatory response has a role in the progression of both cancer<sup>7</sup> and cancer-related cachexia.<sup>6,8</sup>

Inflammation is mediated by a network of pro-inflammatory and anti-inflammatory cytokines that are normally in equilibrium. In the cancer state, the equilibrium is disrupted, resulting in a dysfunctional state of simultaneous immune stimulation and suppression.<sup>9</sup> Cytokines operate both within the tumour micro-environment and by interacting with other tissues in the body to generate a systemic response.<sup>10</sup> Indeed, a considerable amount of evidence indicates the contribution of cytokines in cellular events that determine the initiation, promotion, invasion, and metastasis of cancer.<sup>11</sup> Similarly, Fearon and colleagues<sup>12</sup> highlighted that the production rate of several cytokines is associated with the prevalence of cachexia in multiple types of cancer. Even though cytokine levels were correlated with cancer and cachexia in numerous studies, the mechanisms through which these substances act upon the tumour and other body systems are not completely understood.

Multiple systematic reviews<sup>13,14</sup> have evaluated the relationship between cytokines and cancer. Likewise, the role of cytokines in cachexia was previously examined,<sup>12,15,16</sup> but none of the investigations used a systematic approach to appraise the available evidence. Moreover, very few studies<sup>17</sup> assessed the relationship between cytokines and cachexia in individuals suffering from incurable cancer. If the relationship between cytokines and the development of cancer cachexia was elucidated, this may identify key therapeutic targets that could be translated into clinical therapies. To date, no systematic review evaluated the relationship between cytokines and cachexia in patients with cancer. Therefore, this systematic review aimed to explore the relationship between cytokines and the cachexia syndrome (including related symptoms such as weight loss, anorexia, and reduced physical function) in people with incurable cancer.

## Methods

### Search strategy

The following databases were searched for studies published in English between 01/01/2004 and 06/01/2020: MEDLINE, EMBASE, CINAHL, CENTRAL, PsycINFO, and Web of Science. The search strategy was verified by a subject librarian and included (but was not limited to) the following terms: cytokine, interleukin, interferon AND cancer, metastasis, neoplasm AND cachexia, weight loss, anorexia (Supporting Information, Document S1).

### Inclusion and exclusion criteria

Eligible studies met the following criteria: adults (>18 years old); diagnosed with incurable cancer, defined as metastatic cancer or locally advanced cancer treated with palliative intent; measured the level of one or more cytokines; and assessed at least one symptom and/or sign associated with cachexia. Studies examining all primary cancer types were included to ensure that as much information as possible regarding cytokines and cachexia was retrieved. Diagnosis of cachexia was based on the criteria reported by primary authors rather than any specific definition, allowing the inclusion of studies conducted before 2011, the year when the Fearon definition was published.<sup>1</sup> This ensures that as many studies as possible were included, regardless of the definitions or the weight-loss thresholds used to diagnose cachexia.

Studies were excluded if the participants were cancer survivors or being treated with curable intent. Additionally, the studies were not considered for inclusion if patients' symptoms were attributed directly to a form of therapy or medication. Although no criterion regarding the study design was imposed, the current review did not consider case studies, animal models, protocols, or conference abstracts.

### Study selection and quality assessment

Figure 1 highlights the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) flow diagram of study selection. The titles of the studies were screened independently by R. P. using a conservative approach—whenever the title did not provide enough information, the study was included in the next selection phase. Abstract screening was conducted by D. R. P. and R. P. in a similar manner, and the studies identified as relevant were accepted for full-text assessment. Following full-text assessment (D. R. P. and R. P.), the quality of the included studies was appraised by D. R. P., R. P., and J. M. using the modified Downs and Black

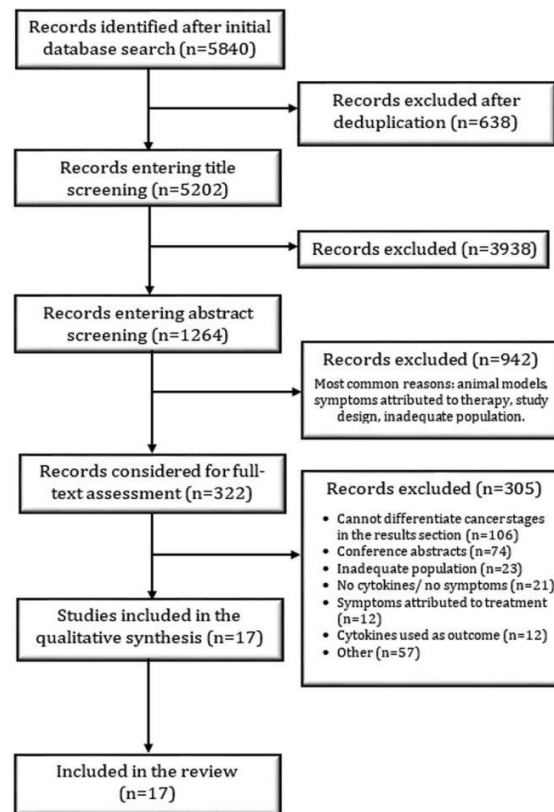


Figure 1 Flow diagram of the study selection protocol.

(MDB) checklist.<sup>18</sup> Studies were rated on a scale from 0 to 10 using standardized criteria, and the quality of the evidence was classified as follows: 0–4 low quality, 5–7 moderate quality, and 8–10 high quality.

#### Data extraction, management, and analysis

A specifically designed collection form was used to systematically capture all the relevant information from the eligible studies. Where studies measured cytokines at multiple time points (2/17 studies), only baseline data were included. No statistical analyses were conducted due to the great level of heterogeneity in study design and data reporting identified between the included studies. Thus, the findings are presented in a descriptive manner, highlighting similarities and discrepancies as well as strengths against weaknesses from the available literature. Lastly, no ethical approval was required for this systematic review.

## Results

### Study characteristics

A total of 5202 studies were identified after removing the duplicates from the database search (Figure 1). After evaluating the titles, 1264 studies were included in the abstract screening phase, of which 322 were selected for full-text screening. At the end of the study selection process, 17 studies met the inclusion criteria of this systematic review. Table 1 summarizes the main characteristics of the eligible studies. Overall, 1277 patients with incurable cancer and 155 healthy controls from 13 different middle-income and high-income countries<sup>36</sup> were recruited from both inpatient and outpatient settings. The mean age of the patients was  $64 \pm 15$  (mean  $\pm$  standard deviation), with female participants making up only a third (34%) of the sample. The most common types of cancers were lung and pancreatic cancer, although various other types such as colorectal, breast, gastric, or oesophageal cancer were evaluated.



Table 1 Characteristics of the included studies

Author (year)	Country	Participants	Cancer type	Cytokines	Blood collection method	Assay method	Sensitivity reported	MDB score
Fortunati et al. (2007) <sup>19</sup>	Italy	33 patients 23 controls	Lung cancer (non-small cell, small cell, and adenocarcinoma)	TNF- $\alpha$ , IL-6	Morning, overnight fast	ELISA	Yes	6
Grim-Stieger et al. (2008) <sup>20</sup>	Austria	61 patients	Breast, colorectal, lung, pancreatic, gastric, and renal cancer	TNF- $\alpha$ , IL-6	Morning, overnight fast	ELISA	No	8
Takahashi et al. (2009) <sup>21</sup>	Japan	16 patients	Oesophageal, gastric, colorectal cancer	TNF- $\alpha$ , IL-6, IFN- $\gamma$ , IL-1Ra, leptin, ghrelin	Morning, overnight fast	ELISA	No	5
Gioulbasanis et al. (2011) <sup>22</sup>	Greece	115 patients	Lung cancer (non-small cell and small cell)	Leptin, adiponectin, ghrelin	N/R	RIA	Yes	8
Scheede-Bergdahl et al. (2012) <sup>23</sup>	Canada	83 patients	Gastrointestinal and non-small cell lung cancer	IL-1 $\beta$ , IL-6, IL-8, TNF- $\alpha$	Morning, overnight fast	Bio-Plex cytokine assay	No	7
Op den Kamp et al. (2013) <sup>24</sup>	Netherlands	26 patients	Non-small cell lung cancer	IL-6, IL-8, IL-10, TNF- $\alpha$ , IFN- $\gamma$	N/R	Multiplex antibody assay	Yes	7
Fujiwara et al. (2014) <sup>25</sup>	Japan	22 controls 21 patients	Pancreatic cancer	IL-6, TNF- $\alpha$ , leptin	Morning, overnight fast	RIA and ELISA	Yes	10
Lu et al. (2014) <sup>26</sup>	China	110 patients	Oesophageal squamous cell carcinoma	MIC-1	Morning, overnight fast	ELISA	No	7
Bilir et al. (2015) <sup>27</sup>	Turkey	46 patients 34 controls	Gastroesophageal, pancreatic, lung, colorectal, ovarian, breast, and laryngeal cancer	IL-1 $\alpha$ , IL-1 $\beta$ , IL-6, TNF- $\alpha$	Morning, overnight fast	ELISA	No	8
Srdic et al. (2016) <sup>28</sup>	Croatia	100 patients	Advanced non-small cell lung cancer	IL-6	N/R	ECLIA	No	8
Penafuerte et al. (2016) <sup>29</sup>	Canada	122 patients	Head, neck, breast, upper gastrointestinal, lung, hepatobiliary, prostate, and colorectal cancer	IL-1 $\alpha$ , IL-1 $\beta$ , IL-3, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, IL-15, IL-18, IFN- $\gamma$ , MCP-1, TNF- $\alpha$ , leptin, ghrelin, adiponectin, TRAIL, TGF- $\beta$ 1	N/R	Bio-Plex cytokine assay	No	9
Lerner et al. (2016) <sup>30</sup>	USA	218 patients	Lung and pancreatic cancer	IL-1, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12, IL-13, IL-17, IFN- $\gamma$ , GDF-15, MCP-1, IP-10	N/R	Bio-Plex cytokine assay	No	9
Bye et al. (2016) <sup>31</sup>	Norway	20 patients 40 controls	Pancreatic cancer	IL-6, IL-10, TNF- $\alpha$ , adiponectin, leptin, IFN- $\gamma$	Non-fasting	ELISA	Yes	8
Fogelman et al. (2017) <sup>32</sup>	USA	89 patients 6 controls	Pancreatic cancer	IL-1 $\beta$ , IL-6, TNF- $\alpha$ , leptin, adiponectin, ghrelin	N/R	N/R	N/R	7
Demiray et al. (2017) <sup>33</sup>	Turkey	67 patients 20 controls	Non-small cell lung cancer	Leptin, resistin	Morning, overnight fast	ELISA	No	8
Murton et al. (2017) <sup>34</sup>	UK	4 patients 4 controls	Advanced non-small cell lung cancer	IL-6, TNF- $\alpha$	Morning, overnight fast	ELISA	No	6
Hou et al. (2018) <sup>35</sup>	Taiwan	146 patients	Pancreatic cancer	IL-1 $\beta$ , IL-6, IL-8, TNF- $\alpha$	N/R	ELISA	No	10

ECLIA, electrochemiluminescence immunoassay; ELISA, enzyme-linked immunosorbent assay; MDB, modified Downs and Black; N/R, not reported; RIA, radioimmunoassay.

The majority of the studies (15/17) measured and reported cytokine levels at a single time point. One study<sup>25</sup> measured intra-day cytokine variation. In this instance, the morning measurements were used in this systematic review as they were taken after an overnight fast. One study<sup>31</sup> measured patients' cytokine levels at enrolment and every 4 weeks until death. This study reported baseline and endpoint data. The baseline measurements were extracted and used in the present review as the endpoint data were not reported separately for cachectic and non-cachectic patients. The mean quality score of the papers from the current review was 7.8 (range 5–10), indicating that the studies incorporated evidence of moderate to high quality (Table 1). Although 6 studies were of moderate quality and 11 were of high quality, several methodological weaknesses were persistent across study reports. The majority of the included studies were marked down as they failed to meet various methodological norms that had an impact on both internal and external validity. Most commonly, the data were not fully reported for all the measured cytokines—some studies specified central tendency values and measures of dispersion only for statistically significant relationships, while other papers only reported *P* values (Table 2). Additionally, several studies did not accurately describe participants' selection criteria and/or the sample collection methodology.

A methodological characteristic that played a pivotal role in the included studies was the timing of blood sampling as previous research<sup>38,39</sup> suggested that cytokine levels show intra-day variation. Only eight studies indicated that blood was collected in the morning after an overnight fast, while the others provided relatively vague information about this matter (i.e. before chemotherapy, using standard methods) or failed to specify the period of the day when blood sampling was performed (Table 1). Furthermore, almost all studies (16/17) reported the assay used to quantify cytokine levels, but only five reported the sensitivity of the assay. The enzyme-linked immunosorbent assay (ELISA) was the most used quantification method, whereas other validated methods such as the electrochemiluminescence immunoassay (ECLIA) and the radioimmunoassay (RIA) were used in some studies.

### Main findings

Table 2 highlights the main findings of the included studies as well as relevant data and grouping criteria. The studies included in the current review analysed the relationship between cytokine levels and cachexia or the degree of weight loss experienced by cancer patients. A great level of variation was observed between the definitions of cachexia and the weight-loss thresholds used across the studies to classify and group participants. A third of the included studies defined cachexia as suggested by Fearon and colleagues,<sup>1</sup> while

several studies referred to cachexia as a syndrome that implies losing more than 5%<sup>19</sup> or 10%<sup>25</sup> body weight. Furthermore, some authors did not use the term 'cachexia' but instead classified the participants according to the amount of weight lost during a period of 3–6 months before the study. The criteria according to which participants were grouped are not homogenous across studies. Although the data could not be subject to a meta-analysis due to the methodological differences, a systematic summary of the findings is subsequently described and discussed.

A total of 31 different (adipo)cytokines were measured across the 17 studies included in the present review (Table 1). The most frequently analysed cytokines were interleukin (IL)-6 (14), tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ) (12), leptin (7), IL-8 (6), IL-1 $\beta$  and interferon- $\gamma$  (IFN- $\gamma$ ) (5), and IL-10, ghrelin, and adiponectin (4).

The majority (11/14) of the studies analysing IL-6 indicated the presence of a relationship between high levels of IL-6 and cachexia or weight loss. Cachectic (weight-losing) patients showed significantly more IL-6 compared with healthy control groups in six out of six studies. When cachectic (weight-losing) individuals were compared with non-cachectic (weight-stable) cancer patients, five out of eight studies indicated that the levels of IL-6 were significantly higher in cachectic participants. A study that compared pre-cachectic patients against those with cancer cachexia observed greater levels of IL-6 in the latter group,<sup>24</sup> while two other studies did not find any differences between pre-cachexia and cachexia.<sup>29,30</sup> Furthermore, two studies<sup>20,23</sup> did not find any statistically significant relationship between IL-6 and weight loss, while Hou *et al.* (2018) indicated the presence of a medium association ( $r = 0.24$ ,  $P = 0.07$ ). Interestingly, Scheede-Bergdahl and colleagues<sup>23</sup> indicated that higher IL-6 levels were positively associated with the presence of sarcopenia. Thus, the evidence suggests higher IL-6 expression in cachectic patients compared with non-cachectic counterparts and healthy individuals.

Another cytokine showing a relationship with the presence of cancer cachexia and weight loss was TNF- $\alpha$ . The levels of TNF- $\alpha$  were significantly higher in cachectic (weight-losing) patients compared with healthy controls in five out of six studies. The sixth study<sup>30</sup> also found a greater concentration of TNF- $\alpha$  in the cachectic group, but the difference was not statistically significant. Only two out of six papers indicated that cachectic (weight-losing) patients expressed more TNF- $\alpha$  than non-cachectic (weight-stable) counterparts, while the other studies did not find any statistically significant differences between groups. Likewise, no difference was observed between pre-cachectic and cachectic patients, while two other studies did not find any significant correlation between TNF- $\alpha$  and weight loss. Therefore, TNF- $\alpha$  levels are elevated in cachectic patients compared with healthy controls, while no significant distinction was noticed between weight-stable and weight-losing cancer patients.

Table 2 Main findings of the included studies

Authors (year)	Variable measured	Grouping criteria	Findings	Data																														
Fortunati et al. (2007) <sup>19</sup>	Cachexia	Cachexia defined as more than 5% weight loss in the previous 6 months.	TNF- $\alpha$ levels were greater in CC patients compared with NC individuals ( $P < 0.05$ ) and controls ( $P < 0.01$ ). IL-6 was greater in CC patients compared with controls ( $P < 0.01$ ) but did not significantly differ from NC patients ( $P > 0.05$ ).	<table border="1"> <thead> <tr> <th></th> <th>CC</th> <th>NC</th> <th>Control</th> </tr> </thead> <tbody> <tr> <td>TNF-<math>\alpha</math></td> <td>4.2<math>\pm</math>0.6</td> <td>2.7<math>\pm</math>0.5</td> <td>1.9<math>\pm</math>0.3</td> </tr> <tr> <td>IL-6</td> <td>11.6<math>\pm</math>1.6</td> <td>8.2<math>\pm</math>1.8</td> <td>0.4<math>\pm</math>0.1</td> </tr> </tbody> </table> <p>Values are shown as mean <math>\pm</math> SD.</p>		CC	NC	Control	TNF- $\alpha$	4.2 $\pm$ 0.6	2.7 $\pm$ 0.5	1.9 $\pm$ 0.3	IL-6	11.6 $\pm$ 1.6	8.2 $\pm$ 1.8	0.4 $\pm$ 0.1																		
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Grim-Stieger et al. (2008) <sup>20</sup>	Weight loss	Evaluated whether participants suffered any weight loss since diagnosis or in the past 3 months.	No significant correlations found between TNF- $\alpha$ and WL since diagnosis ( $P = 0.19$ ) or during the last 3 months ( $P = 0.11$ ). No significant correlation found between IL-6 and WL since diagnosis ( $P = 0.13$ ) or during the last 3 months ( $P = 0.12$ ).	Only $P$ values.																														
Takahashi et al. (2009) <sup>21</sup>	Cachexia	No definition.	CC patients expressed greater levels of TNF- $\alpha$ , IL-6, IL-1Ra ( $P < 0.01$ ), and ghrelin ( $P = 0.04$ ) compared with healthy participants. No difference in IFN- $\gamma$ was observed between groups ( $P = 0.27$ ), while leptin was significantly higher in healthy controls ( $P = 0.02$ ).	Only $P$ values.																														
Gioulbasanis et al. (2011) <sup>22</sup>	Weight loss and nutritional sufficiency	Patients divided into Group A—nutritional sufficiency (15% lost more than 5% body weight), Group B—risk of malnutrition (63% lost weight), and Group C—malnourished (83% lost weight).	The mean levels of leptin were significantly higher ( $P < 0.01$ ) in Group A compared with Group B and Group C. Less adiponectin ( $P = 0.06$ ) was detected in Group A compared with Group B and Group C. Ghrelin levels did not significantly differ ( $P > 0.05$ ) between Groups A, B, and C.	<table border="1"> <thead> <tr> <th></th> <th>A</th> <th>B</th> <th>C</th> </tr> </thead> <tbody> <tr> <td>Leptin</td> <td>5.3<math>\pm</math>4.2</td> <td>3.6<math>\pm</math>3.2</td> <td>1.5<math>\pm</math>1.7</td> </tr> <tr> <td>Adiponectin</td> <td>10.0<math>\pm</math>4.2</td> <td>12.9<math>\pm</math>6.9</td> <td>13.4<math>\pm</math>5.6</td> </tr> <tr> <td>Ghrelin</td> <td>761<math>\pm</math>809</td> <td>818<math>\pm</math>1081</td> <td>817<math>\pm</math>514</td> </tr> </tbody> </table> <p>Values are shown as mean <math>\pm</math> SD.</p>		A	B	C	Leptin	5.3 $\pm$ 4.2	3.6 $\pm$ 3.2	1.5 $\pm$ 1.7	Adiponectin	10.0 $\pm$ 4.2	12.9 $\pm$ 6.9	13.4 $\pm$ 5.6	Ghrelin	761 $\pm$ 809	818 $\pm$ 1081	817 $\pm$ 514														
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Scheede-Bergdahl et al. (2012) <sup>23</sup>	Weight loss and sarcopenia	Participants grouped based on the degree of weight loss 6 months prior enrolment—more or less than 5% weight loss. The presence of sarcopenia assessed by calculating the appendicular lean mass index (Baumgartner, 2000). <sup>37</sup>	The study compared high versus low levels of cytokines. Higher levels of IL-1 $\beta$ and TNF- $\alpha$ were significantly ( $P < 0.01$ ) associated with the presence of more than 5% weight loss. The levels of IL-6 and IL-8 could not significantly predict ( $P > 0.05$ ) weight loss. Similarly, IL-1 $\beta$ and TNF- $\alpha$ were positively associated ( $P < 0.05$ ) with the presence of sarcopenia, while a trend existed for both IL-6 ( $P = 0.06$ ) and IL-8 ( $P = 0.09$ ).	<table border="1"> <thead> <tr> <th></th> <th>OR</th> <th>95%CI</th> </tr> </thead> <tbody> <tr> <td>IL-1<math>\beta</math></td> <td>7.14</td> <td>1.87 to 27.26</td> </tr> <tr> <td>TNF-<math>\alpha</math></td> <td>7.15</td> <td>1.87 to 27.32</td> </tr> <tr> <td>IL-6</td> <td>2.13</td> <td>0.65 to 6.95</td> </tr> <tr> <td>IL-8</td> <td>2.24</td> <td>0.68 to 7.31</td> </tr> <tr> <td></td> <td>OR</td> <td>95%CI</td> </tr> <tr> <td>IL-1<math>\beta</math></td> <td>5.35</td> <td>1.37 to 20.97</td> </tr> <tr> <td>TNF-<math>\alpha</math></td> <td>5.56</td> <td>1.47 to 20.97</td> </tr> <tr> <td>IL-6</td> <td>3.52</td> <td>0.65 to 6.95</td> </tr> <tr> <td>IL-8</td> <td>3.03</td> <td>0.85 to 10.76</td> </tr> </tbody> </table>		OR	95%CI	IL-1 $\beta$	7.14	1.87 to 27.26	TNF- $\alpha$	7.15	1.87 to 27.32	IL-6	2.13	0.65 to 6.95	IL-8	2.24	0.68 to 7.31		OR	95%CI	IL-1 $\beta$	5.35	1.37 to 20.97	TNF- $\alpha$	5.56	1.47 to 20.97	IL-6	3.52	0.65 to 6.95	IL-8	3.03	0.85 to 10.76
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IL-8	2.24	0.68 to 7.31																																
	OR	95%CI																																
IL-1 $\beta$	5.35	1.37 to 20.97																																
TNF- $\alpha$	5.56	1.47 to 20.97																																
IL-6	3.52	0.65 to 6.95																																
IL-8	3.03	0.85 to 10.76																																

(Continues)



Table 2 (continued)

Authors (year)	Variable measured	Grouping criteria	Findings	Data																														
Op den Kamp et al. (2013) <sup>24</sup>	Cachexia	Participants grouped based on the 2011 consensus definition of cancer cachexia.	Significantly ( $P < 0.05$ ) higher levels of IL-6 and IL-8 were observed in the plasma of CC patients compared with individuals with PC and controls. IFN- $\gamma$ was significantly higher ( $P < 0.05$ ) in controls compared with PC patients. The levels of TNF- $\alpha$ and IL-10 did not differ between groups.	Only $P$ values.																														
Fujiwara et al. (2014) <sup>25</sup>	Cachexia	Cachexia defined as ECOG PS 1 to 4, Grade 1 to 4 anorexia, and more than 10% weight loss over the past 6 months.	IL-6 ( $P = 0.35$ ), TNF- $\alpha$ ( $P = 0.27$ ), and leptin ( $P = 0.27$ ) levels did not differ between CC and NC patients.	<table border="1"> <tr> <td></td> <td>CC</td> <td>NC</td> </tr> <tr> <td>IL-6</td> <td>13.8 (2.6-23.4)</td> <td>6.8 (1.4-28.7)</td> </tr> <tr> <td>TNF-<math>\alpha</math></td> <td>7.1 (1.2-30.2)</td> <td>3.3 (1.2-30.0)</td> </tr> <tr> <td>Leptin</td> <td>2.4 (1.0-9.8)</td> <td>4.0 (2.0-9.9)</td> </tr> </table> <p>Values are shown as median (range).</p>		CC	NC	IL-6	13.8 (2.6-23.4)	6.8 (1.4-28.7)	TNF- $\alpha$	7.1 (1.2-30.2)	3.3 (1.2-30.0)	Leptin	2.4 (1.0-9.8)	4.0 (2.0-9.9)																		
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Lu et al. (2014) <sup>26</sup>	Weight loss	Participants divided based on the degree of weight loss before chemotherapy—more or less than 5%.	MIC-1 levels were significantly higher ( $P = 0.01$ ) in patients with >5% weight loss compared with those with $\leq 5\%$ weight loss.	<table border="1"> <tr> <td></td> <td>&gt;5% WL</td> <td><math>\leq 5\%</math> WL</td> </tr> <tr> <td>MIC-1</td> <td>1560 (1090-2141)</td> <td>1124 (776-1560)</td> </tr> </table> <p>Values are shown as median (interquartile range)</p>		>5% WL	$\leq 5\%$ WL	MIC-1	1560 (1090-2141)	1124 (776-1560)																								
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Bilir et al. (2015) <sup>27</sup>	Cachexia	Cachexia—BMI < 20, weight loss during treatment, or weight loss of more than 5% prior to illness in the past 6 months and continuing in the last few months. Refractory cachexia—patients to unresponsive treatment with a life expectancy lower than 3 months and reduced performance status.	IL-1 $\alpha$ ( $P = 0.03$ ), IL-6 ( $P < 0.01$ ), and TNF- $\alpha$ ( $P < 0.01$ ) were higher in people suffering from CC compared with controls. IL-1 $\beta$ ( $P = 0.6$ ) did not differ between groups. IL-1 $\alpha$ was higher in individuals with CC compared with patients with RC ( $P = 0.02$ ). IL-1 $\beta$ was greater in patients with RC compared with individuals with CC ( $P = 0.01$ ). IL-6 ( $P = 0.70$ ) and TNF- $\alpha$ ( $P = 0.12$ ) did not differ between groups.	<table border="1"> <tr> <td></td> <td>CC</td> <td>Control</td> </tr> <tr> <td>IL-1<math>\alpha</math></td> <td>14.0<math>\pm</math>9.9</td> <td>9.9<math>\pm</math>7.5</td> </tr> <tr> <td>IL-1<math>\beta</math></td> <td>3.7<math>\pm</math>0.8</td> <td>3.6<math>\pm</math>0.7</td> </tr> <tr> <td>IL-6</td> <td>16.5<math>\pm</math>4.0</td> <td>5.7<math>\pm</math>3.6</td> </tr> <tr> <td>TNF-<math>\alpha</math></td> <td>15.9<math>\pm</math>7.1</td> <td>12.0<math>\pm</math>5.2</td> </tr> <tr> <td></td> <td>CC</td> <td>RC</td> </tr> <tr> <td>IL-1<math>\alpha</math></td> <td>17.0<math>\pm</math>11.0</td> <td>10.0<math>\pm</math>5.0</td> </tr> <tr> <td>IL-1<math>\beta</math></td> <td>3.4<math>\pm</math>0.4</td> <td>3.9<math>\pm</math>0.9</td> </tr> <tr> <td>IL-6</td> <td>17.4<math>\pm</math>8.0</td> <td>15.4<math>\pm</math>5.4</td> </tr> <tr> <td>TNF-<math>\alpha</math></td> <td>17.0<math>\pm</math>7.0</td> <td>14.0<math>\pm</math>7.0</td> </tr> </table> <p>Values are shown as mean <math>\pm</math> SD.</p>		CC	Control	IL-1 $\alpha$	14.0 $\pm$ 9.9	9.9 $\pm$ 7.5	IL-1 $\beta$	3.7 $\pm$ 0.8	3.6 $\pm$ 0.7	IL-6	16.5 $\pm$ 4.0	5.7 $\pm$ 3.6	TNF- $\alpha$	15.9 $\pm$ 7.1	12.0 $\pm$ 5.2		CC	RC	IL-1 $\alpha$	17.0 $\pm$ 11.0	10.0 $\pm$ 5.0	IL-1 $\beta$	3.4 $\pm$ 0.4	3.9 $\pm$ 0.9	IL-6	17.4 $\pm$ 8.0	15.4 $\pm$ 5.4	TNF- $\alpha$	17.0 $\pm$ 7.0	14.0 $\pm$ 7.0
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Srdic et al. (2016) <sup>28</sup>	Cachexia	Participants grouped based on the 2011 consensus definition of cancer cachexia.	Patients with CC had significantly higher levels of IL-6 compared with patients with NC ( $P = 0.04$ ).	<table border="1"> <tr> <td></td> <td>CC</td> <td>NC</td> </tr> <tr> <td>IL-6</td> <td>15.2</td> <td>7.5</td> </tr> </table> <p>Means with no measure of dispersion.</p>		CC	NC	IL-6	15.2	7.5																								
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(Continues)

Table 2 (continued)

Authors (year)	Variable measured	Grouping criteria	Findings	Data																																																																								
Penafuerte et al. (2016) <sup>29</sup>	Cachexia	Participants grouped based on the 2011 consensus definition of cancer cachexia.	<p>TGF-<math>\beta</math>1: patients with CC (<math>P &lt; 0.01</math>) and PC (<math>P = 0.04</math>) expressed higher levels compared with NC patients; no difference between PC and CC (<math>P &gt; 0.05</math>).</p> <p>IL-8: Patients with CC showed greater levels than individuals with PC (<math>P &lt; 0.01</math>) and NC (<math>P &lt; 0.01</math>); no difference between PC and NC (<math>P &gt; 0.05</math>).</p> <p>IL-6: greater in patients with CC compared with NC (<math>P &lt; 0.01</math>); no difference between PC and CC and between PC and NC (<math>P &gt; 0.05</math>).</p> <p>TRAIL: levels higher in patients with CC compared with NC individuals; no difference between PC and CC and between PC and NC (<math>P &gt; 0.05</math>).</p>	<p>Only <math>P</math> values. Data were not reported for all cytokines.</p>																																																																								
Lerner et al. (2016) <sup>30</sup>	Weight loss	Participants divided based on the degree of weight loss—more than 5%, between 0% and 5%, and no weight loss.	<p>GDF-15 was greater in patients with <math>&gt;5\%</math> WL (<math>P &lt; 0.01</math>) and with <math>\leq 5\%</math> WL (<math>P &lt; 0.01</math>) compared with individuals with no WL.</p> <p>IL-12 levels were greater in individuals with <math>&gt;5\%</math> WL compared with both <math>\leq 5\%</math> WL (<math>P = 0.03</math>) and no WL (<math>P &lt; 0.01</math>).</p> <p>IL-10 levels were higher in patients with <math>&gt;5\%</math> WL compared with both <math>\leq 5\%</math> WL (<math>P = 0.05</math>) and no WL (<math>P = 0.05</math>).</p> <p>IL-7 was greater in participants with <math>&gt;5\%</math> WL compared with both <math>\leq 5\%</math> WL (<math>P = 0.08</math>) and no WL (<math>P = 0.06</math>).</p> <p>Participants with <math>&gt;5\%</math> WL showed greater IL-6 (<math>P = 0.06</math>) and IL-2 (<math>P = 0.07</math>) levels as opposed to patients with no WL.</p> <p>The levels of IL-13 (<math>P = 0.04</math>), IL-8 (<math>P = 0.06</math>), and IL-9 (<math>P = 0.08</math>) were higher in participants with <math>&gt;5\%</math> WL compared with individuals with <math>\leq 5\%</math> WL.</p> <p>All other relationships showed greater, non-significant <math>P</math> values.</p>	<table border="1"> <thead> <tr> <th></th> <th>No WL</th> <th><math>\leq 5\%</math> WL</th> <th><math>&gt;5\%</math> WL</th> </tr> </thead> <tbody> <tr> <td>GDF15</td> <td>1000</td> <td>2000</td> <td>2000</td> </tr> <tr> <td>IL-12</td> <td>43</td> <td>47</td> <td>84</td> </tr> <tr> <td>IL-10</td> <td>29</td> <td>29</td> <td>71</td> </tr> <tr> <td>IL-7</td> <td>19</td> <td>19</td> <td>29</td> </tr> <tr> <td>IL-6</td> <td>23</td> <td>26</td> <td>39</td> </tr> <tr> <td>IL-2</td> <td>36</td> <td>39</td> <td>64</td> </tr> <tr> <td>TNF-<math>\alpha</math></td> <td>99</td> <td>99</td> <td>154</td> </tr> <tr> <td>IL-17</td> <td>143</td> <td>139</td> <td>215</td> </tr> <tr> <td>IL-13</td> <td>28</td> <td>25</td> <td>39</td> </tr> <tr> <td>IL-8</td> <td>79</td> <td>69</td> <td>124</td> </tr> <tr> <td>IFN-<math>\gamma</math></td> <td>199</td> <td>177</td> <td>282</td> </tr> <tr> <td>IL-4</td> <td>8</td> <td>7</td> <td>10</td> </tr> <tr> <td>IL-5</td> <td>24</td> <td>25</td> <td>33</td> </tr> <tr> <td>MIP-1<math>\beta</math></td> <td>139</td> <td>83</td> <td>97</td> </tr> <tr> <td>MCP-1</td> <td>97</td> <td>83</td> <td>115</td> </tr> <tr> <td>IL-9</td> <td>23</td> <td>18</td> <td>30</td> </tr> <tr> <td>IP-10</td> <td>285</td> <td>281</td> <td>312</td> </tr> </tbody> </table> <p>Median values with no measure of dispersion.</p>		No WL	$\leq 5\%$ WL	$>5\%$ WL	GDF15	1000	2000	2000	IL-12	43	47	84	IL-10	29	29	71	IL-7	19	19	29	IL-6	23	26	39	IL-2	36	39	64	TNF- $\alpha$	99	99	154	IL-17	143	139	215	IL-13	28	25	39	IL-8	79	69	124	IFN- $\gamma$	199	177	282	IL-4	8	7	10	IL-5	24	25	33	MIP-1 $\beta$	139	83	97	MCP-1	97	83	115	IL-9	23	18	30	IP-10	285	281	312
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(Continues)

Table 2 (continued)

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Bye et al. (2016) <sup>31</sup>	Cachexia	Participants grouped based on the 2011 consensus definition of cancer cachexia and on the modified Glasgow Prognostic Score (mGPS)	CC and NC were determined according to the 2011 consensus, and no difference in cytokine levels was observed between the groups ( $P > 0.05$ ). IL-6 was greater in CC patients compared with NC individuals, when the disease was assessed according to mGPS ( $P < 0.01$ ). The other cytokines did not differ between groups ( $P > 0.05$ )	<table border="1"> <thead> <tr> <th></th> <th>CC</th> <th>NC</th> </tr> </thead> <tbody> <tr> <td>IL-6</td> <td>4.4 (2.2-34.6)</td> <td>2.2 (0.5-5.3)</td> </tr> <tr> <td>IL-10</td> <td>0.7 (0-3.4)</td> <td>0.9 (0.2-7.7)</td> </tr> <tr> <td>TNF-<math>\alpha</math></td> <td>7.5 (4.1-22.7)</td> <td>8.4 (3.3-11.9)</td> </tr> <tr> <td>IFN-<math>\gamma</math></td> <td>0.13 (0-13.6)</td> <td>0.12 (0.1-0.5)</td> </tr> <tr> <td>Adiponectin</td> <td>7.2 (5.9-15.6)</td> <td>10.1 (4.6-26.0)</td> </tr> <tr> <td>Leptin</td> <td>1.0 (0.3-23.9)</td> <td>2.69 (0.3-9.8)</td> </tr> </tbody> </table> <table border="1"> <thead> <tr> <th></th> <th>CC</th> <th>NC</th> </tr> </thead> <tbody> <tr> <td>IL-6</td> <td>2.2 (0.5-5.3)</td> <td>12.4(3.2-34.6)</td> </tr> <tr> <td>IL-10</td> <td>0.8(0-7.7)</td> <td>0.7(0-3.4)</td> </tr> <tr> <td>TNF-<math>\alpha</math></td> <td>9.2(3.3-22.7)</td> <td>6.3(4.1-17.9)</td> </tr> <tr> <td>IFN-<math>\gamma</math></td> <td>0.1(0.4-0.8)</td> <td>0.(0.1-0.5)</td> </tr> <tr> <td>Adiponectin</td> <td>8.2(3.9-26.0)</td> <td>8.4(0.3-9.8)</td> </tr> <tr> <td>Leptin</td> <td>2.3(0.3-23.9)</td> <td>1.0(0.3-9.8)</td> </tr> </tbody> </table> <p>Values are shown as median (range)</p>		CC	NC	IL-6	4.4 (2.2-34.6)	2.2 (0.5-5.3)	IL-10	0.7 (0-3.4)	0.9 (0.2-7.7)	TNF- $\alpha$	7.5 (4.1-22.7)	8.4 (3.3-11.9)	IFN- $\gamma$	0.13 (0-13.6)	0.12 (0.1-0.5)	Adiponectin	7.2 (5.9-15.6)	10.1 (4.6-26.0)	Leptin	1.0 (0.3-23.9)	2.69 (0.3-9.8)		CC	NC	IL-6	2.2 (0.5-5.3)	12.4(3.2-34.6)	IL-10	0.8(0-7.7)	0.7(0-3.4)	TNF- $\alpha$	9.2(3.3-22.7)	6.3(4.1-17.9)	IFN- $\gamma$	0.1(0.4-0.8)	0.(0.1-0.5)	Adiponectin	8.2(3.9-26.0)	8.4(0.3-9.8)	Leptin	2.3(0.3-23.9)	1.0(0.3-9.8)
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Fogelman et al. (2017) <sup>32</sup>	Weight loss	The participants in the weight loss group had either 10% weight loss or died at 60 days after baseline. The non-weight loss group failed to meet any of the aforementioned criteria.	<p>IL-1<math>\beta</math>: levels were greater in the control group compared with both no WL and WL (<math>P = 0.07</math>); the levels were higher in the no WL group compared with WL (<math>P = 0.03</math>).</p> <p>IL-6: levels were smaller in the control group compared with both WL (<math>P &lt; 0.01</math>) and no WL (<math>P &lt; 0.01</math>); the levels were higher in the WL group compared with no WL (<math>P = 0.03</math>).</p> <p>TNF-<math>\alpha</math>: levels were smaller in the control group compared with both WL (<math>P &lt; 0.01</math>) and no WL (<math>P &lt; 0.01</math>); levels were higher in the WL group compared with no WL (<math>P = 0.03</math>).</p> <p>IL-8: levels were smaller in the control group compared with both WL (<math>P &lt; 0.01</math>) and no WL (<math>P &lt; 0.01</math>); no significant</p>	<table border="1"> <thead> <tr> <th></th> <th>Control</th> <th>No WL</th> <th>WL</th> </tr> </thead> <tbody> <tr> <td>IL-1<math>\beta</math></td> <td>34.3 (0-79.4)</td> <td>4.4 (0-633.3)</td> <td>0.1 (0-299.7)</td> </tr> <tr> <td>IL-6</td> <td>5.8 (2.9-6.3)</td> <td>14.5 (4.7-1593)</td> <td>37 (5.6-524.3)</td> </tr> <tr> <td>TNF-<math>\alpha</math></td> <td>2.1 (1.5-3.3)</td> <td>3.3 (1.5-16.2)</td> <td>4.1 (2.2-18.9)</td> </tr> <tr> <td>IL-8</td> <td>10.8 (4.6-14.4)</td> <td>37.1 (5.2-3185)</td> <td>56.8 (6.7-2204.8)</td> </tr> <tr> <td>Adiponectin</td> <td>15.3 (8.7 - 27)</td> <td>18.4 (7.5-59.4)</td> <td>19.5 (8.9-599.8)</td> </tr> <tr> <td>Leptin</td> <td>8.9 (2.4-91.1)</td> <td>7.9 (0.2-134.3)</td> <td>8.8 (0.7-91.1)</td> </tr> <tr> <td>Ghrelin</td> <td>0 (0-4.6)</td> <td>0 (0-3351.9)</td> <td>2.7 (0-3351.9)</td> </tr> </tbody> </table> <p>Values are shown as median (range).</p>		Control	No WL	WL	IL-1 $\beta$	34.3 (0-79.4)	4.4 (0-633.3)	0.1 (0-299.7)	IL-6	5.8 (2.9-6.3)	14.5 (4.7-1593)	37 (5.6-524.3)	TNF- $\alpha$	2.1 (1.5-3.3)	3.3 (1.5-16.2)	4.1 (2.2-18.9)	IL-8	10.8 (4.6-14.4)	37.1 (5.2-3185)	56.8 (6.7-2204.8)	Adiponectin	15.3 (8.7 - 27)	18.4 (7.5-59.4)	19.5 (8.9-599.8)	Leptin	8.9 (2.4-91.1)	7.9 (0.2-134.3)	8.8 (0.7-91.1)	Ghrelin	0 (0-4.6)	0 (0-3351.9)	2.7 (0-3351.9)										
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Table 2 (continued)

Authors (year)	Variable measured	Grouping criteria	Findings	Data										
Demiray et al. (2017) <sup>33</sup>	Weight loss	Weight loss at the time of diagnosis defined as more than 10% weight loss within the past 6 months.	differences observed between the WL and no WL groups ( $P > 0.05$ ). All other relationships showed greater, non-significant $P$ values. The levels of leptin ( $P = 0.44$ ) and resistin ( $P = 0.54$ ) did not differ between patients with and without WL.	<table border="1"> <thead> <tr> <th></th> <th>No WL</th> <th>WL</th> </tr> </thead> <tbody> <tr> <td>Leptin</td> <td>10.7±2.5</td> <td>7.2±2.1</td> </tr> <tr> <td>Resistin</td> <td>5.6±0.2</td> <td>6.7±0.3</td> </tr> </tbody> </table> <p>Values are shown mean ± SD.</p>		No WL	WL	Leptin	10.7±2.5	7.2±2.1	Resistin	5.6±0.2	6.7±0.3	
	No WL	WL												
Leptin	10.7±2.5	7.2±2.1												
Resistin	5.6±0.2	6.7±0.3												
Murton et al. (2017) <sup>34</sup>	Cachexia	Participants grouped based on the 2011 consensus definition of cancer cachexia.	Cachectic individuals showed higher levels of IL-6 ( $P < 0.05$ ) and TNF- $\alpha$ ( $P = 0.06$ ) compared with healthy controls.	Only $P$ values.										
Hou et al. (2018) <sup>35</sup>	Cachexia	Participants grouped based on the 2011 consensus definition of cancer cachexia.	There was a positive ( $P = 0.03$ ) relationship between IL-8 and WL. Also, a positive relationship ( $P = 0.07$ ) was observed between IL-6 and WL. The other correlations were less strong and showed greater $P$ values. IL-8 levels were greater in CC compared with NC ( $P = 0.01$ ). IL-1 $\beta$ ( $P = 0.95$ ), IL-6 ( $P = 0.16$ ), and TNF- $\alpha$ ( $P = 0.84$ ) levels did not differ between CC and NC patients.	<table border="1"> <thead> <tr> <th></th> <th>Pearson's coefficient (r)</th> </tr> </thead> <tbody> <tr> <td>IL-1<math>\beta</math></td> <td>0.10</td> </tr> <tr> <td>IL-6</td> <td>0.24</td> </tr> <tr> <td>IL-8</td> <td>0.28</td> </tr> <tr> <td>TNF-<math>\alpha</math></td> <td>0.08</td> </tr> </tbody> </table>		Pearson's coefficient (r)	IL-1 $\beta$	0.10	IL-6	0.24	IL-8	0.28	TNF- $\alpha$	0.08
	Pearson's coefficient (r)													
IL-1 $\beta$	0.10													
IL-6	0.24													
IL-8	0.28													
TNF- $\alpha$	0.08													

BMI, body mass index; CC, cancer cachexia; CI, confidence interval; CTR, control; ECOG PS, Eastern Cooperative Oncology Group Performance Status; NC, cancer non-cachexia; OR, odds ratio; PC, pre-cachexia; RC, refractory cachexia; SD, standard deviation; WL, weight loss.



Similar to the aforementioned cytokines, but with fewer studies to support the findings, IL-8 levels were overall higher in cachectic (weight-losing) patients. The studies that compared healthy controls against individuals with cancer cachexia ( $n = 2$ ) reported that the levels of IL-8 were significantly higher in the diseased group. Additionally, two out of three studies that examined IL-8 levels in cachectic (weight-losing) and non-cachectic (weight-stable) cancer patients found that IL-8 was increased in cachectic participants. Lastly, individuals with cancer cachexia had more IL-8 compared with pre-cachectic patients in both studies that examined this comparison. Overall, IL-8 showed increased levels in participants with cancer cachexia and weight loss compared with non-cachectic, pre-cachectic, and healthy groups, but the strength of these observations is limited given the small number of studies analysing this cytokine.

Leptin, IFN- $\gamma$ , IL-1 $\beta$ , IL-10, adiponectin, and ghrelin did not demonstrate any significant difference between groups when cachectic (weight-losing) patients were compared against non-cachectic (weight-stable) counterparts or healthy participants (Table 2). However, a study worth mentioning was conducted by Scheede-Bergdahl and colleagues<sup>23</sup> who observed that higher levels of IL-1 $\beta$ , as opposed to low IL-1 $\beta$  concentrations, were significantly associated with the presence of more than 5% weight loss [odds ratio (OR) = 7.14,  $P < 0.01$ ] and sarcopenia (OR = 5.35,  $P < 0.05$ ). The other cytokines listed in Table 1 are not discussed because they were analysed by two or fewer studies and not enough information was available.

## Discussion

### Main findings

The aim of the current review was to examine the relationship between cytokines and the cachexia syndrome (including related symptoms such as weight loss, anorexia, and reduced physical function) in people with incurable cancer irrespective of tumour type. Overall, IL-6, TNF- $\alpha$ , and IL-8 were present in greater concentrations in patients losing weight as opposed to healthy individuals. Leptin, IFN- $\gamma$ , IL-1 $\beta$ , IL-10, adiponectin, and ghrelin were also evaluated, but no relationship was observed between the cytokines' circulating levels and the degree of weight loss. Moreover, the definitions of cachexia and the weight-loss thresholds used across the studies to categorize participants were heterogeneous and a more consistent approach should be adopted for future studies.

The levels of circulating IL-6 were elevated in weight-losing and cachectic patients compared with healthy controls in all studies that analysed this cytokine. Furthermore, more than half of the studies that compared cachectic and

weight-losing patients with non-cachectic or weight-stable counterparts indicated the presence of higher IL-6 concentrations in cachectic individuals. The direction of these relationships was also observed by other research and it has been previously suggested that IL-6 is a central regulator of the progression of cancer and cancer-associated cachexia.<sup>40–42</sup> Several studies examined the effect of IL-6 inhibitors on cachexia. Clazakizumab, an anti-IL-6 antibody, was tested in patients with non-small cell lung cancer and improved cachexia and anaemia in phase I and II trials.<sup>43</sup> Despite the fact that the drug seemed well tolerated, there is no phase III trial ongoing. Furthermore, various case reports<sup>44,45</sup> and animal models<sup>46</sup> indicated that tocilizumab might ameliorate cancer-associated cachexia. Often used in patients with rheumatoid arthritis, tocilizumab was associated with increased weight and body mass index in a recent systematic review.<sup>47</sup> Although the previously mentioned reports suggest a potential positive effect of tocilizumab, no clinical trials are currently examining its effect on cancer cachexia. To conclude, assessing the circulating levels of IL-6 could be a useful method of monitoring the development of cancer cachexia and future trials should aim to integrate the cytokine in the multifactorial management of this disorder.

Circulating TNF- $\alpha$  was expressed in higher concentrations in cachectic and weight-losing patients as opposed to healthy individuals. There was no difference in TNF- $\alpha$  when cachectic and weight-losing patients were compared with non-cachectic and weight-stable patients. The available literature highlights the role of TNF- $\alpha$  as a key mediator of cachexia given the cytokine's ability to activate nuclear factor- $\kappa$ B, one of the main pathways that determine skeletal muscle atrophy.<sup>48,49</sup> Various studies focused on analysing the effectiveness of TNF- $\alpha$  inhibitors such as etanercept and infliximab.<sup>50–52</sup> In a cohort of patients with incurable cancer, etanercept only produced a small level of weight gain and failed to treat cachexia.<sup>50</sup> Similarly, pancreatic cancer patients receiving infliximab gained an insignificant amount of weight compared with counterparts receiving a placebo. Another trial analysing the effectiveness of OHR/AVR118, an agent targeting both IL-6 and TNF- $\alpha$ , indicated that cancer patients with cachexia patients improved anorexia, strength, and dyspepsia.<sup>53</sup> This finding reinforces the idea that not one, but multiple cytokines could be responsible for the onset and progression of cancer cachexia and a multimodal approach is required in the management of this disorder.

The majority of the studies analysing IL-8 indicated that the cytokine's expression was greater in patients with cancer cachexia and weight loss compared with non-cachectic, weight-losing, pre-cachectic, and healthy individuals. Although the strength of this observation is limited given the small number of papers examining this cytokine, future research might evaluate the direction of the relationship between IL-8 and cachexia because this matter was not thoroughly explained by the available literature. Further-



more, none of the other cytokines analysed in the current review showed any relationship with the amount of weight lost by patients. However, previous research linked cytokines such as IL-1 $\alpha$ ,<sup>54</sup> IL-1 $\beta$ ,<sup>55</sup> and IFN- $\gamma$ <sup>56</sup> with the occurrence and development of weight loss. Overall, there is not enough evidence available regarding the previously mentioned cytokines to reach a definitive conclusion and future studies should aim to explore this knowledge gap.

### *Inconsistencies in grouping criteria*

The studies included in the current review used distinct methods of defining cachexia and various weight-loss thresholds to group participants (Table 2). Some studies used the consensus definition from 2011<sup>1</sup> or the modified Glasgow Prognostic Score<sup>57</sup> to assess and diagnose cachexia. Multiple studies<sup>26,30</sup> used a 5% weight loss limit as the main grouping criterion and only discussed patients' weight without referring to cachexia as a disorder. Interestingly, various papers classified participants using weight-loss thresholds that appeared to be chosen arbitrarily (i.e. 10%), while others used cachexia definitions that were not validated by previous literature (Table 2). Thus, the results could not be meta-analysed due to the lack of a consistent method of grouping participants. The current review presented findings in a descriptive manner, giving a useful indication of the trajectory of the available evidence. However, conducting a meta-analysis would provide a more precise and reliable summary of the included studies and should allow an effective comparison between them.<sup>58</sup> Consequently, practitioners could make well-informed decisions based on high-quality evidence with a lower risk of bias<sup>59</sup> and this would have a positive impact on patients' treatment and quality of life. Future studies should adhere to definitions and thresholds that are already established by the literature in order to promote uniformity and consensus in the field of cancer cachexia. Otherwise, any novel method for defining and assessing cachexia should be accompanied by a thorough rationale.

### *Limitations and directions for future research*

Most of the studies analysed in this review had a cross-sectional design and do not allow the inference of a causal relationship between cytokines and cachexia. A limitation of the present findings is that only two studies reported multiple cytokine measurements and only the baseline data were used in the current review. Future work in this area should assess cytokine levels longitudinally to fully elucidate their effect on the cachexia phenotype. Moreover, the relationship between cytokines and cachexia was examined in all primary tumour types. Although this may be considered a limitation because cachexia is less common in some can-

cers, failing to include all primary tumour types means that minimal data would be available and important studies might be omitted.

Numerous papers were excluded from the present review as the data of patients with early and advanced forms of cancer were combined in the analysis. Although relevant evidence might have been left out of this study, the information about individuals with incurable malignancies could not be differentiated from the data of patients with operable forms of cancer. Additionally, the assay used to measure cytokine levels is an important methodological factor and it was reported in all but one investigation. However, less than a third of the studies indicated the sensitivity of the assay and, thus, the validity of the results that failed to consider this parameter was low.

Several other errors were observed in the statistical analysis of the results and in the methods used to report findings. In the present systematic review, the available body of literature could not be meta-analysed due to the high degree of methodological heterogeneity as well as the lack of transparency and failure to meet basic standards of data reporting. Specifically, five studies<sup>20,21,24,29,34</sup> only reported *P* values, while two studies<sup>28,30</sup> did not report any measure of dispersion (i.e. standard deviation and interquartile range). Several studies examined multiple cytokines and only displayed data for statistically significant relationships. The use of these practices in the literature leads to biased reporting and inflation of type I errors in systematic reviews. One study<sup>35</sup> examined the correlation coefficient between cytokines and weight loss, while the other nine studies used different methods of reporting data (i.e. measure of central tendency, dispersion, or effect sizes). The remaining studies have major inconsistencies in grouping criteria. Only one study<sup>31</sup> used the Fearon definition,<sup>1</sup> while another classified participants based on nutritional sufficiency.<sup>22</sup> Two studies<sup>25,33</sup> grouped participants based on a 10% weight-loss threshold in the last 6 months, while another study<sup>32</sup> used the same threshold but measured at 60 days prior enrolment. The last four studies<sup>19,23,26,27</sup> grouped patients based on a 5% weight loss limit. Yet, not even these studies could be meta-analysed because they measured different cytokines and use dissimilar methods of reporting data (mean and standard deviation, OR and confidence interval, as well as median and interquartile range). To enable meta-analyses in the future, consensus on cachexia definition, detailed reporting, as well as the standardization of cytokines measured and assays used would be optimal.

Although this review provided useful information, it also highlighted areas where research could be optimized. Future studies should be longitudinal, with an extensive characterization of the cachexia phenotype (including loss of lean mass/weight, patient-reported outcomes of anorexia, fatigue, and quality of life, physical activity, and other measures of function), allowing a better understanding of the relationship

between cytokines and the phenotype. Additionally, future studies should incorporate surrogate markers of the inflammatory response such as acute-phase proteins (i.e. C-reactive protein and serum amyloid A) and also cytokine receptors (i.e. sIL-6R, sIL-2R, IL1-R1, IL1-R2, TNF-R1, and TNF-R2). Adding these markers as a complementary measurement would generate a more accurate overview of the inflammatory state and of the cascade of immune events underlying cancer cachexia. As previously mentioned, increasing homogeneity in study design should be a priority for future research. This can be achieved by grouping participants according to established criteria such as the Fearon definition<sup>1</sup> or the modified Glasgow Prognostic Score.<sup>57</sup> Most importantly, regardless of the study design chosen by researchers, it is crucial to describe the methodology and the results in a transparent manner. Specifically, all measured variables should be reported and not only the significant results (complete datasets can be added as supplementary material to increase a manuscript's reliability); authors should go beyond *P* values and must report data using central tendency values or effect sizes alongside measures of dispersion; the blood collection methods, the type of assay used to measure biomarkers, and the sensitivity of the assay should be described in the methods section.

## Conclusions

A relationship between cytokines, cachexia, and weight loss was observed in the current review. The levels of IL-6 and TNF- $\alpha$  were greater in cachectic patients compared with healthy individuals. A similar result was obtained for IL-8, but fewer studies supported the finding. IL-6 was the only

cytokine expressed in higher concentrations in cachectic participants compared with non-cachectic cancer patients. The other cytokines analysed did not show any notable relationship with cachexia or the amount of weight lost by cancer patients. These findings indicate that a network of cytokines including IL-6, TNF- $\alpha$ , and IL-8 are associated with the development of cancer cachexia. An index created from multiple cytokines might serve as a 'biomarker' that could be used to analyse the onset and progression of cancer cachexia. However, this relationship is not causal and future work should opt for longitudinal designs with consistent methodological approaches, as well as adequate mechanisms of analysing and reporting results.

## Acknowledgements

The authors certify that they comply with the ethical guidelines for authorship and publishing of the *Journal of Cachexia, Sarcopenia and Muscle*.<sup>60</sup>

## Online supplementary material

Additional supporting information may be found online in the Supporting Information section at the end of the article.

## Conflict of interest

None declared.

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## Relationship between cytokines and symptoms in people with incurable cancer: A systematic review

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## ARTICLE INFO

## Keywords:

Cytokines  
Palliative medicine  
Symptoms  
Systemic inflammatory response  
Cancer

## ABSTRACT

**Background:** Development and spread of cancer is linked to the inflammatory response, in which cytokines serve a key role. The inflammatory response may also form the basis for symptoms of cancer. This systematic review examines the relationship between cytokines and symptoms in incurable cancer.

**Methods:** MEDLINE, EMBASE, Cochrane Library, CINAHL, Web of Science and PsycINFO databases were searched for studies from January 2004 to January 2020.

**Results:** Twenty studies were selected (n = 1806 patients, 119 controls). Symptoms studied included depression, fatigue, pain, and loss of appetite. Nine studies examined patients with a specified tumour type, the remainder included patients with a mix of tumour types. Thirty-one cytokines were examined; multiple associations between cytokines and symptoms were described, supporting the hypothesis that cytokines may have a key role in symptom generation.

**Conclusion:** Symptoms of incurable cancer are associated with circulating cytokines. Further study is required to characterise these relationships, and to explore their therapeutic potential.

### 1. Introduction

The systemic inflammatory response (SIR) is inextricably linked to cancer and its progression (Mantovani et al., 2008). Many cancers arise from sites where there is chronic inflammation or irritation. Once cancer is established changes in the tumour microenvironment which are largely promoted by immune and inflammatory cells, facilitate tumour survival and spread (Coussens and Werb, 2002). The SIR is regulated through a complex and vast network of cytokine messengers (Wang et al., 2017). Cytokines are small proteins which are secreted by many cell types. In health, pro-inflammatory and anti-inflammatory cytokines are closely balanced and work to promote wound healing and tissue homeostasis. As cancer progresses this equilibrium is disrupted resulting in a dysfunctional state of both immune stimulation and immune

suppression (Lippitz and Harris, 2016). As the understanding of this process has advanced there now exists growing evidence that the same altered immune state which drives cancer progression also influences the development of symptoms such as fatigue, depression and pain (Laird et al., 2013).

This relationship has been explored by Laird and co-workers who reported a link between routine biomarkers of the SIR such as C-reactive protein (CRP) and patient reported outcome measures of quality of life in people with cancer (Laird et al., 2016; Miller et al., 2019). This would suggest that inflammation is responsible for multiple symptoms. It must be noted however, that biomarkers assessed to date including CRP are largely surrogates of the inflammatory process.

The hypothesis that cytokine networks may be involved in the aetiology of cancer related symptoms has its foundation in work examining

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<https://doi.org/10.1016/j.critrevonc.2021.103222>

Received 28 July 2020; Received in revised form 24 November 2020; Accepted 16 January 2021

Available online 19 January 2021

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'sickness behaviour' in non-malignant conditions. Sickness behaviour is a set of adaptive physiological and behavioural responses to peripheral infection or injury which include fever, pain, fatigue, reduced physical activity and cognitive impairment (Shattuck and Muehlenbein, 2015). Three main cytokines have been linked with sickness behaviour in diseases which involve systemic inflammation namely IL-6, IL-1 $\beta$  and TNF- $\alpha$  (D'Mello and Swain, 2017). Accordingly, use of anti-TNF therapies in inflammatory diseases such as rheumatoid arthritis and psoriasis have shown significant rapid improvements in health related quality of life on initiation of therapy (Ban et al., 2010). These results provide a basis for further exploration of the role of cytokines in cancer symptoms and potential therapeutic targets.

Multiple cytokines have been found in raised levels in the serum of patients with cancer including the interleukins – (notably IL-2, 6, 10, 12 and 18), TNF-  $\alpha$  and Transforming Growth Factor-  $\beta$  (TGF-  $\beta$ ) (Lippitz, 2013). Several studies have assessed the association between one or more of these cytokines and symptoms in specific cancer sites for example: increased levels of IL-6, IL-8 and TNF- $\alpha$  have been associated with sickness symptoms such as pain, nausea and lack of energy in women with breast cancer and with anxiety and depression in patients with colorectal cancer (Oliveira Miranda et al., 2014; Reed et al., 2016). In patients with ovarian cancer, levels of IL-6 have also been linked to sleep disturbance and fatigue (Clevenger et al., 2012). When it comes to incurable cancer the associations between cytokines and symptoms are inconsistently reported. This may be due to methodological differences in symptom recording, sensitivity of cytokine assay used and cross-sectional design of studies (Saligan et al., 2015).

Symptom management and control is imperative in people with incurable cancer where multiple co-occurring symptoms have a significant effect on quality of life and daily function (Ferreira et al., 2008; Gilbertson-White et al., 2011). Maintaining and improving quality of life in these patients is a therapeutic priority where potential for prolongation of survival is reduced. There is an urgent need to explore the inflammatory model of symptom generation and propagation in this group in order to identify specific targets which may have therapeutic utility. To date, a comprehensive critique of studies linking the SIR and symptoms of incurable cancer has not been undertaken.

Therefore, the aim of this systematic review was to examine the relationship between circulating levels of cytokines and symptoms in people living with incurable cancer.

## 2. Methods

The principal aim of this systematic review was to describe the associations between symptoms and levels of circulating cytokine proteins in patients with incurable cancer. This was carried out by an extensive literature search to identify studies dated from January 2004 to January 2020. The last literature search was conducted on the 6th of January 2020. The search was carried out using key terms which were selected based upon preliminary searches, scanning of existing studies and advice from a specialist librarian. Search terms included individual symptoms – i.e. 'depression', 'fatigue', 'pain' and common cytokine classes – i.e. 'interleukin', 'interferon.' Detailed search strategies are outlined in appendix A.

The following databases were comprehensively searched: MEDLINE, EMBASE, Cochrane Library, CINAHL, Web of Science and PsycINFO. The PROSPERO database (international database of prospectively registered systematic reviews in health and social care) was searched beforehand to ensure a previous systematic review in this area had not been performed. Ethical approval was not required for this systematic review. Please note that in this current review we have not examined the potential role of cytokines in cancer cachexia and anorexia, however a review examining these specifically is in preparation.

### 2.1. Eligibility criteria

Eligible studies met the following criteria: written in English, studies which examined adults with incurable cancer (defined as metastatic cancer [histological, radiological or cytological evidence] or locally advanced cancer being treated with palliative intent), studies which assessed symptoms using a validated symptom measure (defined as a patient-reported tool which has been validated in a population with cancer) and studies which measured one or more circulating cytokine protein (defined as per paper reporting and corroborated by RP).

Exclusion criteria included: studies in which the symptom being examined was attributed directly to cancer treatment (i.e. oxalipatin-induced neuropathy, bone pain due to anti-cancer therapy or radiotherapy-related fatigue), studies which included patients who were cancer survivors or healthy caregivers, studies which included patients with both curable and incurable cancer, studies that reported symptom measures and cytokine levels as outcome measures for a clinical trial (with no association made between the two) and studies which were reviews, protocols, case reports and conference abstracts.

### 2.2. Appraisal

Titles of retrieved studies were screened for relevance by RP. Studies which were not available in English, animal studies, and studies which did not include patients with cancer were excluded.

Studies which were selected on the basis of title then underwent independent abstract review by RP and DRP. Abstract review was conducted using the exclusion criteria detailed above.

Studies which were identified as eligible during abstract screening were then independently reviewed in full by RP and DRP using the criteria detailed above. Any disagreements on final inclusion in the review were settled by an independent third party.

Quality assessment of eligible studies was carried out using a modified Downs and Black Checklist (MDBC) (Downs and Black, 1998). The Downs and Black Checklist is a 27-item tool which can be used to evaluate the quality of both randomised and non-randomised studies. For the purposes of this review, the checklist was modified to produce a ten-item quality assessment guide with three sections: reporting, internal validity/bias, and external validity. Studies were reviewed by RP, DRP and JM. Each study was assigned a final quality rating ranging from 0–10. Scores 0–4 were considered low quality, 5–7 moderate quality and 8–10 high quality. A consort diagram was performed in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (Moher et al., 2009).

## 3. Results

The literature review process is shown in Fig. 1. The following numbers of studies were retrieved from the electronic databases: Medline = 1302; EMBASE = 2089; Cochrane = 418; CINAHL = 597; Web of Science = 1324; PsycINFO = 110. Following removal of duplicates 5202 studies were screened for eligibility by title. Of these, 1264 were eligible for abstract screening. Nine hundred and forty-two were then excluded on review of the abstract, leaving 322 studies for full text review, with 20 studies meeting the eligibility criteria for this review.

At the abstract screening stage, the most common reasons for exclusion were: studies including animals, studies including symptoms that were directly attributed to anti-cancer treatment, and studies which were reviews or conference abstracts. At the full text screening stage the most common reason for exclusion was studies that included patients with both curable and incurable cancer. Such studies had no subgroup analysis in the results section making it impossible to draw conclusions regarding the patients with incurable cancer.

A summary of eligible studies is presented in Table 1. In total 1806 patients with incurable cancer and 119 controls were included. Most studies (16) employed a cross-sectional design.

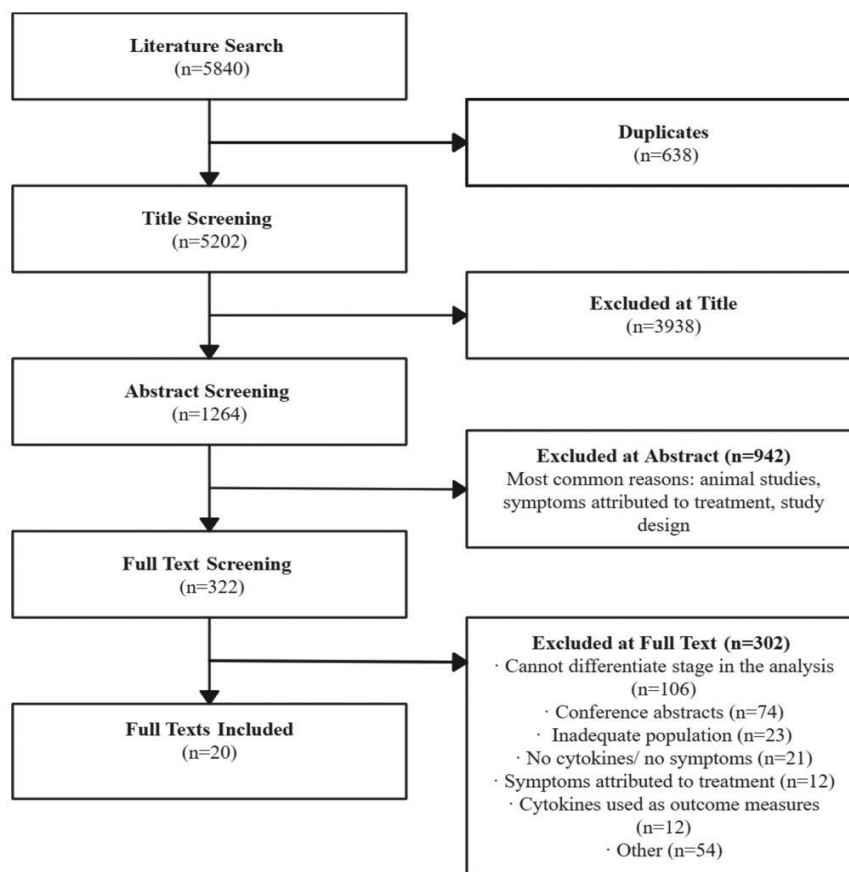


Fig. 1. Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) diagram of literature search process.

### 3.1. Quality assessment

Thirteen studies were considered to be of high quality (Scoring 8–10 on the MDBC) while the remainder were considered to be of moderate quality (scoring 5–7 on the MDBC). The most common reason that studies were awarded a lower score for quality was lack of external validity. Indeed, few studies explained how the population was selected from the source population.

When examining the quality of the included studies, several factors were considered. One of these factors was the use of medications which are known moderators of inflammation inclusive of steroids, non-steroidal anti-inflammatory medications, anti-depressants and opioids. These medications were variably included in this set of studies- nine studies did not exclude any of these medications, 11 studies excluded one or more. Another confounding factor was patients receiving chemotherapy or palliative radiotherapy- the inclusion of these patients was also variable, information on which studies included patients currently receiving cancer treatment is available in [Table 1](#). Another factor was patients with co-existent inflammatory or auto-immune illnesses which may influence cytokine measures- only two studies explicitly excluded people with these conditions. It has also been well established that cytokine levels can show a diurnal variation ([Altara et al., 2015](#)). Therefore, the time of day of blood sampling was important to consider when comparing results. The window for blood sampling was specified in 12 studies. Nine studies had a morning sampling window. Seven of these specified a one to four hour window and two had an

unspecified ‘morning’ window. Two studies had a seven hour sampling window between 10:30 and 17:30; one study had a 14:00 to 17:00 sampling window; and eight studies did not specify when samples were taken. Only two studies specified that samples had been taken when participants were fasted. Further information regarding sample time, assay method used, and sample type are available in [Table 2](#).

### 3.2. Paper characteristics

Symptoms which were most frequently examined were depression, fatigue, pain, and loss of appetite. Ten studies recruited patients from oncology settings. Nine studies examined people with a single specified tumour type (i.e. pancreatic cancer, mesothelioma, astrocytoma, non-small cell lung cancer (NSCLC) while the remainder included people who had a mix of tumour types. In total, 31 different cytokines were evaluated with IL-6, TNF- $\alpha$ , IL-1 $\beta$ , IL-8 and IL-10 the most commonly assessed (20, 12, 8, 6 and 6 studies respectively). A total of 31 different symptom evaluation methods were employed; only six methods appeared in more than one study, including the Structured Clinical Interview for DSM-IV Axis I Disorders (SCID-I) (five studies), Hospital Anxiety and Depression Scale (HADS) (five studies) Brief Fatigue Inventory (BFI) (four studies), European Organisation for the Research and Treatment of Cancer – Quality of Life Questionnaire – C30 (EORTC-QLQ-C30) (four studies) and the Brief Pain Inventory (BPI) (three studies). Key primary findings for each eligible study can be found in [Table 3](#).



**Table 1**  
Summary of Eligible Studies in Alphabetical Order of First Author Surname.

Reference	Setting	Country	Type of Cancer	Number of Participants	Current Treatment	Modified Downs and Black Score
Breitbart et al. (2014)	Outpatient Oncology	USA	Advanced Pancreatic Cancer	Cancer = 43Controls = 32	Gemcitabine Based Chemotherapy	7
de Raaf et al. (2012)	Inpatient/Outpatient Palliative Care	Netherlands	Metastatic Cancer- Multiple Sites	Cancer = 45Controls = 47	Nil	8
Heitzer et al. (2012)	Inpatient Oncology	Austria	Metastatic Cancer- Multiple Sites	Cancer = 38Controls = 20	Nil	7
Inagaki et al. (2008)	Outpatient Palliative Care	Japan	Metastatic Cancer- Multiple Sites	46	Nil	10
Inagaki et al. (2013)	Outpatient Palliative Care	Japan	Metastatic Cancer- Multiple Sites	112	Nil	10
Jacobs et al. (2017)	Outpatient Oncology	USA	Advanced NSCLC	50	Nil	8
Jacobson et al. (2008)	Inpatient Palliative care	USA	Metastatic Cancer- Multiple Sites	73	NR	8
Jehn et al. (2010)	Inpatient Oncology	Germany	Stage IV Cancer- Multiple Sites	114	Chemotherapy	8
Jehn et al. (2012)	Outpatient Chemotherapy	Germany	Metastatic Breast Cancer	70	Chemotherapy	8
Jehn et al. (2015)	Inpatient Oncology	Germany	Metastatic Cancer – Multiple Sites	59	Chemotherapy	8
Kao et al. (2013)	NR	Australia	Inoperable Pleural Mesothelioma	63	Nil	7
Kwak et al. (2012)	Palliative Care	Korea	Metastatic Cancer- Multiple Sites	114	Nil	8
Kwekkeboom et al. (2018)	Outpatient Oncology	USA	Metastatic Cancer- Multiple Sites	155	Nil/Cytotoxic Targeted Therapy/ Chemotherapy	8
Liu et al. (2018)	NR	China	Advanced Lung Cancer- Mixed Histology	Cancer = 92Controls = 20	NR	5
Paulsen et al. (2017)	NR	Norway	Metastatic Cancer- Multiple Sites	49	Nil/Chemotherapy/ Hormonal Therapy	6
Rich et al. (2005)	Outpatient Chemotherapy	France	Metastatic Colorectal Cancer	80	Nil- Pre Chemotherapy	10
Rodrigues et al. (2016)	NR	Brazil	Metastatic Cancer- Multiple Sites	51	Nil/ Palliative Radiotherapy	8
Scheede-Bergdahl et al. (2012)	Outpatient and Inpatient Oncology	Canada	Inoperable Gastrointestinal or NSCLC	83	Palliative Radiotherapy/ Chemotherapy	7
Starkweather et al. (2014)	Inpatient Surgery	USA	Grade III/IV Astrocytoma	22	Nil- Pre Surgery	6
Thomsen et al. (2017)	Outpatient Chemotherapy	Norway	Metastatic Colorectal Cancer	447	Nil- Pre Phase III Trial	9

**Table 2**  
Details of Assay Methods and Nature of Samples Used in Eligible Studies.

References	Nature of Blood Sample	Time of Day Sample Acquired	Fasted	Assay Method	Assay Sensitivity Reported	Lowest Detection Limit Reported
Breitbart et al. (2014)	Serum	14:00–17:00	NR	Multiplex Electro-Chemiluminescent Immunoassay	No	No
de Raaf et al. (2012)	Plasma	08:00–12:00	NR	Enzyme Linked Immunosorbent Assay (ELISA) (IL-1ra)Cytometric Bead Arrays (IL-6, IL-8-)	No	Yes
Heitzer et al. (2012)	Serum	NR	NR	ELISA	No	No
Inagaki et al. (2008)	Plasma	10:30–17:30	NR	ELISA	Yes	No
Inagaki et al. (2013)	Serum	10:30–17:30	NR	Multiplex Electro-Chemiluminescent Immunoassay	No	Yes IL-6 only
Jacobs et al. (2017)	Serum	NR	NR	Multiplex Electro-Chemiluminescent Immunoassay (IL-1β, IL-6, TNF-α) ELISA (TGF-α)	No	Yes
Jacobson et al. (2008)	Plasma	07:30–08:30	NR	ELISA	No	No
Jehn et al. (2010)	Plasma	08:00	NR	Solid-Phase Chemiluminescent Immunoassay	Yes	No
Jehn et al. (2012)	Plasma	10:00	NR	Solid-Phase Chemiluminescent Immunoassay	Yes	No
Jehn et al. (2015)	Plasma	10:00	NR	Solid-Phase Chemiluminescent Immunoassay	Yes	Yes
Kao et al. (2013)	Serum	NR	NR	ELISA	No	No
Kwak et al. (2012)	Plasma	09:00–11:00	NR	Multiplex Bead Array Assay	Yes	No
Kwekkeboom et al. (2018)	Plasma	NR	NR	Multiplex Electro-Chemiluminescent Immunoassay	No	Yes
Liu et al. (2018)	Serum	‘morning’	Yes	ELISA	No	No
Paulsen et al. (2017)	Serum	NR	NR	Multiplex Bead Array Assay	No	No
Rich et al. (2005)	Serum	08:00	NR	ELISA	No	No
Rodrigues et al. (2016)	NS	‘morning’	NR	NR	No	No
Scheede-Bergdahl et al. (2012)	Plasma	NR	Yes	Multiplex Bead Array Assay	No	No
Starkweather et al. (2014)	Serum	NR	NR	ELISA	Yes	No
Thomsen et al. (2017)	Serum	NR	NR	ELISA	No	No



**Table 3**  
Eligible Studies Key Primary Findings.

References	Cytokines Studied	Main Symptom Studied	Symptom Measure	Key Primary Finding(s)	P=	Key Secondary Finding (s)	P=
Breitbart et al. (2014)	IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, IL-12p70, TNF-α, TGF-β, IFN-γ	Depression	SCID-I HAM-D BHS BAI HARS PSQ BPI BFI	Severity of depressive symptoms was associated with IL-6	0.03	Poor sleep was associated with IL-4 Pain severity was associated with IL-12p70 Fatigue intensity was associated with TGF-β	<0.05<0.05 <0.05 <0.05
de Raaf et al. (2012)	IL-6, IL-1ra, IL-8	Fatigue	MFI – physical and mental subscales	IL-6levels significantly associated with physical fatigue IL-1ra levels significantly associated with physical fatigue	0.003 0.03	No inflammatory markers related to mental fatigue	NR
Heitzer et al. (2012)	IL-1β, IL-1α, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IL-18, TNF-α, TNF-β, IFN-γ, MCP-1, MIP-1β, MIP-1α, OPG	Pain	NRS BPI	Decrease in levels of IL-7MPQMCP-1MIP-1βand OPG associated with pain relief MCP-1 MIP-1β and OPG associated with pain relief	0.045 0.016 <0.001 <0.001	OPG levels significantly elevated in nociceptive pain compared to visceral or bone pain	0.028 visceral 0.019 bone
Inagaki et al. (2008)	IL-6	Fatigue	SCID-I CFS	IL-6 levels significantly elevated in clinical fatigue	0.02	IL-6 levels associated with the physical subscale of the CFS IL-6 not associated with affective symptoms of depression (i.e. depressed mood, loss of interest)	0.02 0.910.34
Inagaki et al. (2013)	IL-6, GM-CSF, IL-1β, IL-2, IL-8, IL-10, IL-12p70, TNF-α, IFN-γ	Depression	HADS Cavanaugh Criteria	IL-6 levels associated with physical symptoms of depression (appetite loss/ insomnia/ fatigue) insomnia or without a diagnosis of depression	0.04 fatiguewithout a diagnosis of depression< or without a diagnosis of depression	No significant difference in IL-6 levels between those with or without a diagnosis of depression<0.001	0.34 0.14 0.67
Jacobs et al. (2017)	IL-1β, IL-6, TNF-α, TGF-α	Depression	PHQ-9 M.I.N.I	TNF-α levels significantly elevated in depression	0.005	No significant difference in levels of TGF-α IL-1β IL-6	0.35 0.17 0.19
Jacobson et al. (2008)	IL-6	Depression	HAM-D	There was no association between IL-6 and depression for the whole cohort	>0.05	For those participants where blood was taken 48 h after the initial interview IL-6 was significantly associated with depression IL-6 levels were also associated with depression severity score	<0.05
Jehn et al. (2010)	IL-6	Depression	HADS-D HADS	IL-6 values significantly elevated in depression	<0.001	IL-6 strongly associated with severity of symptoms of depressionIL-6 associated with severity of symptoms of anxiety IL-6 levels associated with severity of depressive symptomsShort term memory was negatively associated with IL-6	<0.001 0.02
Jehn et al. (2012)	IL-6	Anxiety and Depression	SCID-I HADS-D	IL-6 values significantly elevated in depression, but not in anxiety	<0.001IL-6 associated with severity of symptoms of anxiety		<0.0010.41
Jehn et al. (2015)	IL-6	Depression, Cognitive Function	SCID-I VLMT	IL-6 levels significantly elevated in depression	<0.001	Short term memory was negatively associated with IL-6	0.02
Kao et al. (2013)	IL-6, IL-6 Soluble Receptor, VEGF	Health Related Quality of Life	LCSS	Anorexia, Fatigue, overall symptomatic distress, interference with normal	<0.05 anorexia, fatigue, OSD <0.05(INA, GQL)	Fatigue, interference with normal activity, global QOL all associated with IL-6	<0.001fatigue <0.001 INA, GQL

(continued on next page)

Table 3 (continued)

References	Cytokines Studied	Main Symptom Studied	Symptom Measure	Key Primary Finding(s)	P=	Key Secondary Finding (s)	P=
Kwak et al. (2012)	IL-6, TNF-α	Fatigue	BFI-K Structured Interview	activity and global QOL all associated with VEGF IL-6 and TNF-α levels were not significantly different between mild, moderate and severe fatigue	0.94 IL-6 0.68 TNF-α	IL-6 and TNF-α levels not significantly correlated with BFI-K subscale scores	0.29
Kwekkeboom et al. (2018)	IL-1β, IL-6, TNF-α	Pain, Fatigue, Sleep Disturbance	BPI BFI MDASI MSAS FACIT-PAL	TNF-α was associated with symptom cluster distress	0.00	Psychological stress was not related to any inflammatory biomarkers	
Liu et al. (2018)	IL-10, IL-6, IL-8, TNF-α	Depression	SDS	IL-6, IL-10, TNF-α levels were associated with degree of depression (mild, moderate) IL-6 was associated with loss of appetite	<0.01 <0.001<0.01	There was no association between degree of depression and IL-8	>0.136
Paulsen et al. (2017)	IL-1β, IL-1ra, IL-2, IL-4, IL-6, IL-7, IL-8, IL-10, IL-12p70, IL-13, IL-18, TNF-α, MCP-1, MIP-1α, OPG, IFN-γ, sTNF-r1, TGF-β1, MIF	Pain, Appetite, Fatigue	EORTC-QLQ-C30	Fatigue was associated with IL-1ra	<0.01	Pain was not associated with any biomarkers	NR
Rich et al. (2005)	IL-6, TNF-α, TGF-α	Fatigue, Appetite Loss, Nausea	EORTC-QLQ-C30	IL-6, TGF-α levels were significantly elevated in appetite loss	0.012	IL-6 was associated with nausea and vomiting TGF-α levels were associated with fatigue	0.0160.018 0.018
Rodrigues et al. (2016)	IL-1, IL-6, TNF-α	Fatigue	EORTC-QLQ-C30 HADS CFQ FACIT-F PSQI-BR	TNF-α levels were negatively associated with mental fatigue	0.007	IL-6 levels were negatively associated with physical wellbeing	0.017
Scheede-Bergdahl et al. (2012)	IL-1β, IL-6, IL-8, TNF-α	Weakness, loss of appetite, fatigue, quality of life	ESAS-Weakness scale	IL-1β, IL-6, IL-8 were associated with weakness and lack of appetite MQL<0.05	<0.05 (weakness) <0.01 (appetite) ESAS-loss of appetite scale BFI<0.05BFI	IL-6, IL-8 were associated with quality of life Nil cytokines were associated with fatigue	<0.05<0.01
Starkweather et al. (2014)	IL-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12p70, IL-13, IL-15, IL-17, VEGF, TNF-α	Depression	BDI-II	IL-8 levels in serum negatively associated with depressive symptoms	<0.0004		
Thomsen et al. (2017)	IL-6	Health related quality of life	EORTC-QLQ-C30	Higher levels of IL-6 were associated with poorer global QOL, fatigue, nausea, vomiting, pain dyspnoea, appetite loss and constipation	NR		

Glossary.

BAI Beck Anxiety Inventory, BDI-II Beck Depression Inventory-II, BFI Brief Fatigue Inventory, BHS Beck Hopelessness Scale, BPI Brief Pain Inventory, CFQ Chalder Fatigue Questionnaire, CFS Cancer Fatigue Scale, EORTC-QLQ-C30- European Organisation for the Research and Treatment Of Cancer Quality of Life Questionnaire C30, ESAS Edmonton Symptom Assessment System, FACIT-F Functional Assessment for Chronic Illness Therapy-Fatigue, GM-CSF Granulocyte Macrophage Colony Stimulating Factor, GQL Global Quality of Life, HADS-D/A Hospital Anxiety and Depression Scale-Depression /Anxiety Subscale, HAM-D- Hamilton Depression Scale, HARS Hamilton Anxiety Rating Scale, IFN-γ Interferon-γ, IL Interleukin, INA Interference with Normal Activities, LCSS Lung Cancer Symptoms Scale, M.I.N.I Mini International Neuropsychiatric Interview, MCP-1 Monocyte Chemoattractant Protein-1, MFI Multi-Dimensional Fatigue Inventory, MIP Macrophage Inflammatory Protein, MPQ McGill Pain Questionnaire, MQL McGill Quality of Life, NR Not Reported, NRS Numerical Rating Scale, OPG Osteoprotegerin, OSD Overall Symptomatic Distress, PHQ-9 Patient Health Questionnaire-9, PSQI-BR Pittsburgh Sleep Quality Index- Brazilian Portuguese, PSS Perceived Stress Scale, QOL Quality of Life, SCID-I Structured Clinical Interview for DSM-IV Axis I Disorders, SDS Self Rating Depression Scale, TGF Transforming Growth Factor, TNF Tumour Necrosis Factor, VEGF Vascular Endothelial Growth Factor, VLMT Verbal Learning and Memory Test.

3.3. Depression

Clinical depression/depressive symptoms were examined in nine studies (n = 633, controls = 72). Five studies included patients with specific tumour types (lung (Jacobs et al., 2017; Liu et al., 2018), breast (Jehn et al., 2012), pancreas (Breitbart et al., 2014), and astrocytoma (Starkweather et al., 2014)) and four included patients with a mix of tumour types (Inagaki et al., 2013; Jehn et al., 2015; 2010; Rodrigues

et al., 2016). Clinical depression was most frequently diagnosed using the criteria set out in the Structured Clinical Interview for DSM-IV Axis I Disorders (SCID-I). Depressive symptoms were assessed most commonly using HADS; however, many other scales were used to examine individual symptoms related to depression, such as hopelessness (Beck Hopelessness Scale), poor sleep (Pittsburgh Sleep Quality Index) and poor short-term memory (Verbal Learning and Memory Test).

The relationship between clinical depression/depressive symptoms



and IL-6 was the most consistently studied. Jehn et al. (n = 243) found that IL-6 levels were significantly higher in patients with a diagnosis of clinical depression (Jehn et al., 2015, 2012; Jehn et al., 2010). Jehn, Liu and Breitbart (n = 378) also found that the severity of depressive symptoms was associated with IL-6 level (Breitbart et al., 2014; Jehn et al., 2015, 2012; Jehn et al., 2010; Liu et al., 2018). When symptoms of depression were divided into physical (appetite loss/insomnia/fatigue) or affective (depressed mood, anhedonia) symptoms, Inagaki found that IL-6 was only associated with physical symptoms in 112 patients (Inagaki et al., 2013).

As expected, sampling window had an important role in interpretation of results- Jacobson found no association between IL-6 and depression in 73 patients with a mix of tumour types. However, in a sub-analysis where results were separated according to blood sampling time there was a significant association between IL-6 and depression in the nine patients who had had blood taken 48 h after the initial interview; they also found that as time increased between the initial interview and blood draw, the magnitude of the correlation decreased (Jacobson et al., 2008).

In contrast to the results linking IL-6 and depression, both Jacobs and Starkweather (n = 72) found no association between diagnosis/ symptoms of depression and IL-6 (Jacobs et al., 2017; Starkweather et al., 2014). In fact, Starkweather found that in 22 patients with astrocytoma depressive symptoms were negatively associated with IL-8 in serum (Starkweather et al., 2014).

Breitbart and Jehn studied anxiety alongside depression. Breitbart found no association between anxiety symptoms and cytokines in 43 patients with pancreatic cancer (Breitbart et al., 2014). While Jehn observed that whilst IL-6 levels were not associated with a diagnosis of anxiety, they were correlated with severity of anxiety symptoms in 59 patients with breast cancer (Jehn et al., 2015).

Sleep disturbance was examined in the context of depression by Breitbart and Inagaki. Breitbart et al. found an association between overall sleep quality and IL-4 in patients with pancreatic cancer (Breitbart et al., 2014). They also found that insomnia was associated with IL-6 in patients with depression and a variety of tumour types (Inagaki et al., 2013). Short term memory dysfunction was also studied as a symptom of depression by Jehn et al. they found that memory was negatively associated with IL-6 (Jehn et al., 2015).

There was significant methodological heterogeneity in these studies. Five studies excluded participants who were taking anti-depressant medication (Breitbart et al., 2014; Jehn et al., 2015, 2012; Jehn et al., 2010; Starkweather et al., 2014), while three studies included them (Inagaki et al., 2013; Jacobs et al., 2017; Jacobson et al., 2008). One study made no mention of medication (Liu et al., 2018). Time of blood sampling ranged from 08:00 to 17:30 and two studies did not record when samples were taken (Jacobs et al., 2017; Starkweather et al., 2014). Although HADS was the most common method of measuring symptoms of depression, other measures were also used. These included the Hamilton Depression Rating Scale, Patient Health Questionnaire-9, Beck Depression Inventory and the Self Rating Depression Scale.

### 3.4. Fatigue

Fatigue was examined in 12 studies (n = 1288; controls = 79) with four using this as their primary focus (de Raaf et al., 2012; Inagaki et al., 2008; Kwak et al., 2012; Rodrigues et al., 2016). The remainder examined fatigue alongside a variety of other symptoms (Breitbart et al., 2014; Inagaki et al., 2013; Kao et al., 2013; Kwekkeboom et al., 2018; Paulsen et al., 2017; Rich et al., 2005; Scheede-Bergdahl et al., 2012; Thomsen et al., 2017). The most frequently studied cytokine in relation to fatigue was IL-6. IL-6 levels were significantly correlated with fatigue in patients with mesothelioma (n = 63) (Kao et al., 2013) and in patients with mixed tumour types (n = 112) (Inagaki et al., 2013). Elevated levels of IL-6 were found in patients with fatigue and colorectal cancer (n = 447) (Thomsen et al., 2017), and various tumour types (n = 46)

(Inagaki et al., 2008). De Raff and Inagaki (n = 101) found that when fatigue was divided into physical and mental fatigue, there was an association between IL-6 and physical fatigue (de Raaf et al., 2012; Inagaki et al., 2008). Rodrigues and Kwekkeboom also found that TNF- $\alpha$  was linked to different aspects of fatigue. Rodrigues found that TNF- $\alpha$  had an association with mental fatigue in 51 patients while Kwekkeboom found an association with distress from a pain/fatigue/sleep disturbance symptom cluster in 155 patients (Kwekkeboom et al., 2018; Rodrigues et al., 2016).

In contrast to these findings, Kwak, Paulsen, Rich and Scheede-Bergdahl (n = 326) all found no association between fatigue and IL-6 or TNF- $\alpha$ . These studies were performed in patients with a mix of tumour types, colorectal cancer and gastrointestinal (GI) cancer/ non-small cell lung cancer (NSCLC) (Kwak et al., 2012; Paulsen et al., 2017; Rich et al., 2005; Scheede-Bergdahl et al., 2012). Paulsen and De Raff (n = 104) found that IL-1 $\alpha$  levels correlated with physical fatigue and overall fatigue respectively (de Raaf et al., 2012; Paulsen et al., 2017). Vascular Endothelial Growth Factor (VEGF), Transforming Growth Factor Alpha (TGF- $\alpha$ ), and Transforming Growth Factor Beta (TGF- $\beta$ ) were associated with fatigue in one study each; including patients with mesothelioma (n = 63) (Kao et al., 2013), colorectal cancer (n = 80) (Rich et al., 2005), and pancreatic cancer (n = 43) (Breitbart et al., 2014), respectively.

In this set of studies fatigue was evaluated using eight different measurement scales with some studies using multiple scales to capture different aspects of fatigue – the fatigue subscale of the EORTC QLQ-C30 and the Brief Fatigue Inventory were the most commonly used (four studies each). Blood sampling times ranged from 08:00 to 17:00, with five studies giving no indication of when samples were taken (Kao et al., 2013; Kwekkeboom et al., 2018; Paulsen et al., 2017; Scheede-Bergdahl et al., 2012; Thomsen et al., 2017).

### 3.5. Pain

Pain was examined in seven studies in total (n = 803; controls = 20). One study was focused on pain alone (Heitzer et al., 2012) while the remaining six studies examined pain alongside other symptoms (Kao et al., 2013; Kwekkeboom et al., 2018; Paulsen et al., 2017; Rodrigues et al., 2016; Thomsen et al., 2017). Rodrigues, Kao and Paulsen (n = 163) each found no association found between any measured cytokine and pain (Kao et al., 2013; Paulsen et al., 2017; Rodrigues et al., 2016).

Heitzer measured pain via a numerical rating scale in 38 patients with mixed tumour types. This was assessed in the context of a trial of opioid analgesia. Pain was categorised within five separate entities - nociceptive, visceral, bone, neuropathic, and mixed pain. Osteoprotegerin (OPG) was found to be significantly elevated in nociceptive pain as compared with others. The study observed that a decrease in serum levels of IL-7, IL-18, Monocyte Chemoattractant Protein-1 (MCP-1), Macrophage Inflammatory Protein-1 $\beta$  (MIP-1 $\beta$ ) and OPG were all associated with targeted pain relief from opiate analgesia (Heitzer et al., 2012).

Thomsen found that higher levels of IL-6 were associated with pain in 447 patients with colorectal cancer, as measured by the pain subscale of the EORTC-QLQ-C30 (Thomsen et al., 2017). Breitbart found associations between average pain intensity, and IL-1 $\beta$ , IL-4 and IL-12p70 in 43 patients with pancreatic cancer, as measured by the Brief Pain Inventory (Breitbart et al., 2014).

### 3.6. Appetite loss

Appetite loss was measured in five studies (n = 771) including patients with a mix of tumour types (Inagaki et al., 2013; Paulsen et al., 2017), patients with colorectal cancer (Rich et al., 2005; Thomsen et al., 2017) and patients with either gastrointestinal cancer or NSCLC (Scheede-Bergdahl et al., 2012). In all five studies, there was either a higher level of IL-6 or a significant correlation between IL-6 and appetite



loss scores. In addition to IL-6, Scheede-Bergdahl also found an association between appetite loss and IL-8, IL-1 $\beta$  and TNF- $\alpha$  in 83 patients with either gastrointestinal cancer or NSCLC (Scheede-Bergdahl et al., 2012).

### 3.7. Other symptoms

Several other symptoms were analysed in a small number of studies. These included nausea and vomiting (three studies,  $n = 576$ ) (Paulsen et al., 2017; Rich et al., 2005; Thomsen et al., 2017), dyspnoea (three studies,  $n = 559$ ) (Kao et al., 2013; Paulsen et al., 2017; Thomsen et al., 2017), constipation (two studies  $n = 496$ ) (Paulsen et al., 2017; Thomsen et al., 2017), diarrhoea (two studies,  $n = 496$ ) (Paulsen et al., 2017; Thomsen et al., 2017), and weakness (one study,  $n = 83$ ) (Scheede-Bergdahl et al., 2012). Paulsen examined all the above symptoms (aside from weakness) in 49 patients with a mix of tumour type and found no associations with any measured cytokine (Paulsen et al., 2017). Thomsen found that the nausea and vomiting, constipation, and dyspnoea subscales of the EORTC-QLQ-C30 were all associated with IL-6 in 447 patients with colorectal cancer (Thomsen et al., 2017). Rich also found an association between IL-6 and nausea and vomiting in 80 patients with colorectal cancer (Rich et al., 2005). Kao found no association between any measured cytokines and dyspnoea/ cough in 63 patients with pleural mesothelioma (Kao et al., 2013). Scheede-Bergdahl found that weakness assessed using the Edmonton Symptom Assessment System was associated with IL-1 $\beta$ , IL-6 and IL-8 in 83 patients with GI/NSCLC (Scheede-Bergdahl et al., 2012).

## 4. Discussion

The aim of the present systematic review was to examine the association between cytokines and symptoms in people with incurable cancer. We identified clear associations between certain symptoms and various cytokines in the eligible studies.

To illustrate, IL-6 was linked to depression (Breitbart et al., 2014; Inagaki et al., 2013; Jehn et al., 2015, 2012; Jehn et al., 2010; Liu et al., 2018), fatigue (de Raaf et al., 2012; Inagaki et al., 2013, 2008; Kao et al., 2013; Thomsen et al., 2017), appetite loss (Inagaki et al., 2008; Paulsen et al., 2017; Rich et al., 2005; Scheede-Bergdahl et al., 2012; Thomsen et al., 2017) and pain (Thomsen et al., 2017). This link between IL-6 and symptoms is unsurprising, given its key role in the acute phase immune response and as a key mediator in the evolution of chronic inflammatory states (Uciechowski and Dempke, 2020). Our observations are in keeping with the role of IL-6 in cancer per se including promotion of metastasis, angiogenesis, apoptosis, and invasiveness (Kumari et al., 2016; Masjedi et al., 2018; Pop et al., 2017). TNF- $\alpha$  was linked to fatigue (Kwekkeboom et al., 2018; Rodrigues et al., 2016) and appetite loss (Scheede-Bergdahl et al., 2012) which is consistent with its role in the NF- $\kappa$ B pathway and maintenance of local and systemic inflammation (Bazzichetto et al., 2019; Hunter and Jones, 2015; Lippitz, 2013). IL-1 was associated with pain, weakness, appetite loss and fatigue, again consistent with its general cancer role (Setrerrahmane and Xu, 2017).

Despite the observations reported herein, disentangling the associations between the magnitude of cytokine levels and symptoms is challenging. Paradigms with sickness behaviour are useful in understanding this and have demonstrated that communication between the periphery and the brain is key in symptom generation; and that circulating cytokines are crucial mediators of this communication. There are several proposed mechanisms for this communication including direct access to the brain via blood brain barrier deficient areas such as the circumventricular organs and ascending communication via cytokine receptors on vagal afferents (D'Mello and Swain, 2017). Indeed it is widely known that pain, appetite and fatigue are centrally controlled and it seems rational that symptom genesis in cancer is at least partly mediated through cerebral mechanisms (McDonald et al., 2018).

Further supporting evidence of the role of cytokines in symptom generation in cancer can be gleaned by comparisons with non-malignant

inflammatory illness. Kappelmann, in a meta-analysis of anti-cytokine medications in trials for chronic inflammatory conditions, found that depression symptoms improved independently of improvement in physical manifestations of disease (Kappelmann et al., 2018). These observations are now being seen in trials of anti-cytokine drugs, including mono-clonal antibodies, in patients with cancer. Clazakizumab, a mono-clonal antibody against IL-6, demonstrated significant improvements in lung cancer symptom score and fatigue scores in a phase II trial in a population with advanced NSCLC (Bayliss et al., 2011). Additionally a phase III trial of MABp1, an antibody that targets IL-1 $\alpha$ , in patients with advanced colorectal cancer, showed improvements in quality of life (Hickish et al., 2017). These studies provide grounds for optimism that cancer symptoms such as pain and fatigue may be targeted via pro-inflammatory cytokine pathways.

The present review has however raised several areas where further elucidation of the role of pro-inflammatory cytokines in both symptom genesis and treatment, will present challenges. Inflammatory signalling patterns are dynamic and are likely to change as cancer progresses (Coussens and Werb, 2002) whilst studies did not control for factors known to affect cytokine levels; such as concomitant medications, diurnal variation, and co-morbid illness. There was also little consensus on the methods of measuring symptom severity. For example, eight different scales were used to assess fatigue.

Integration of the findings of these studies is also affected by variation in methods for measuring cytokine levels. Researchers used a variety of methods, including ELISA, bead-based assays, and electrochemiluminescent assays and there was little consistency in the way in which cytokines were selected for measurement. In earlier studies, single-plex measurement of one cytokine was more common while more recent studies more often employed multiplex technology to measure a wide range of pro- and anti-inflammatory cytokines. Sensitivity of assays and lower limits of quantification were poorly reported. Data regarding selected assay types is recorded in Table 2.

### 4.1. Future perspectives

Cytokine profiles, in combination with subjective patient reported outcome measures, have the potential to act as objective measures of symptom severity, and indicators of improvement with treatment. Through this, the precise biological and clinical profiles of symptoms could be identified and form the basis of personalised symptomatic management strategies. Cytokine profiles could be used alongside current oncological strategies to identify groups of people who are more susceptible to increased symptom severity and allow the initiation of symptom management programs at the beginning of treatment which may help to avoid long term complications. This potential may be realised with strict adherence to consensus measurement tools and consistency in longitudinal measurement of cytokines in well-defined populations.

### 4.2. Limitations

This review has several limitations. Relevant articles may have been excluded due to close adherence to exclusion criteria. Articles which examined other markers of the SIR including acute phase proteins were excluded as this review focussed solely on cytokines. A common reason for studies to be excluded from this review was that people with early and advanced stages of disease had been included together in analysis and the results which pertained only to people with incurable cancer were impossible to separate. There was also a risk of bias in the selection of patients which was acknowledged by several of the authors. For example, patients included in these studies were all well enough to attend for blood testing and to complete multiple symptom measure questionnaires. Patients who were confined to their home or unable to efficiently access medical care due to poorly controlled symptoms were by nature, excluded. The majority of studies had a cross-sectional design

and in order to build a true characterisation of contributing factors studies must be longitudinal with cytokines and symptom severity measured at multiple time points. It is also important to acknowledge that in many studies in this review there were patients who, despite not having increased levels of one cytokine, still suffered from symptoms. This observation contributes to our understanding that cytokines are likely to act as part of a multifactorial process and are not solely implicated in the onset of specific symptoms. It is clear that further work is required to disentangle the relationships between the complex networks which link cytokines and symptoms in incurable cancer. Future work in this area should build on research to date and where possible address limitations.

**5. Conclusions**

Cancer symptoms such as pain, fatigue and appetite loss are associated with levels of several circulating cytokine proteins, notably IL-6, IL-1 and TNF- $\alpha$ . The observations provide a sound rationale for further work to characterise these relationships more fully. Through identifying key cytokine pathways in symptom development, this may represent targets for therapies. Such studies are already in progress (NCT04406662) and findings are awaited with interest.

**Disclaimers**

The authors are presently working on the REVOLUTION Trial (NCT04406662)

**APPENDIX A – SEARCH STRATEGY**

Database	Search Strategy
MEDLINE	<ol style="list-style-type: none"> <li>1. exp cancer/</li> <li>2. neoplasm.ab,ti.</li> <li>3. metastasis\$.ab,ti.</li> <li>4. 1 or 2 or 3</li> <li>5. exp cytokines/</li> <li>6. exp Inflammation/</li> <li>7. exp interferon/</li> <li>8. tumo\$r necrosis factor.ab,ti.</li> <li>9. tfn.ab,ti.</li> <li>10. exp interleukin/</li> <li>11. 5 or 6 or 7 or 8 or 9 or 10</li> <li>12. symptom*.ab,ti.kw.</li> <li>13. depress*.ab,ti.kw.</li> <li>14. nausea.ab,ti.kw.</li> <li>15. fatigue.ab,ti.kw.</li> <li>16. sleep.ab,ti.kw.</li> <li>17. quality of life.ab,ti.kw.</li> <li>18. anxiety.ab,ti.kw.</li> <li>19. pain.ab,ti.kw.</li> <li>20. cachexia.ab,ti.kw.</li> <li>21. weight loss.ab,ti.kw.</li> <li>22. anorexia.ab,ti.kw.</li> <li>23. 12 or 13 or 14 or 15 or 16 or 17 or 18 or 18 or 20 or 21 or 22</li> <li>24. 4 and 11 and 23</li> <li>25. limit 24 to (humans and yr="2004-Current")</li> </ol>
EMBASE	<ol style="list-style-type: none"> <li>1. exp cancer/</li> <li>2. neoplasm.ab,ti.</li> <li>3. metastasis\$.ab,ti.</li> <li>4. 1 or 2 or 3</li> <li>5. exp cytokines/</li> <li>6. exp Inflammation/</li> <li>7. exp interferon/</li> <li>8. tumo\$r necrosis factor.ab,ti.</li> <li>9. tfn.ab,ti.</li> <li>10. exp interleukin/</li> <li>11. 5 or 6 or 7 or 8 or 9 or 10</li> <li>12. symptom*.ab,ti.kw.</li> <li>13. depress*.ab,ti.kw.</li> </ol>

(continued on next page)

**CRedit authorship contribution statement**

**Rebekah Patton:** Conceptualization, Methodology, Formal analysis, Writing - original draft, Writing - review & editing. **D. Robert Paval:** Conceptualization, Methodology, Formal analysis, Writing - original draft, Writing - review & editing. **James J. McDonald:** Formal analysis, Writing - original draft, Writing - review & editing. **Duncan Brown:** Writing - review & editing. **Iain J. Gallagher:** Conceptualization, Writing - review & editing, Supervision. **Richard J.E. Skipworth:** Conceptualization, Writing - review & editing, Supervision. **Donald C. McMillan:** Conceptualization, Writing - review & editing, Supervision. **Ross D. Dolan:** Writing - review & editing. **Marie Fallon:** Conceptualization, Writing - review & editing, Supervision. **Barry J.A. Laird:** Conceptualization, Writing - original draft, Writing - review & editing, Supervision.

**Declaration of Competing Interest**

The authors report no declarations of interest

**Acknowledgements**

This research did not receive any specific grant from funding agencies in the public, commercial or not-for-profit sectors.





(continued)

Database	Search Strategy
	14. TI=(nausea)
	15. TI=(fatigue)
	16. TI=(sleep)
	17. TI=(quality of life)
	18. TI=(anxiety)
	19. TI=(pain)
	20. TI=(cachexia)
	21. TI=(weight loss)
	22. TI=(anorexia)
	23. #12 or #13 or #14 or #15 or #16 or #17 or #18 or #19 or #20 or #21 or #22
	24. #4 and #11 and #23
	S1 MJ cancer
	S2 TI neoplasm OR AB neoplasm
	S3 TI metastasis OR AB metastasis
	S4 S1 OR S2 OR S3
	S5 MJ cytokines
	S6 MJ interleukin
	S7 MJ inflammation
	S8 MJ interferon
	S9 TI tumour necrosis factor OR AB tumour necrosis factor
	S10 TI tnf OR AB tnf
	S11 S5 OR S6 OR S7 OR S8 OR S9 OR S10
	S12 TI symptom OR AB symptom OR SU symptom
	S13 TI depression OR AB depression OR SU depression
	S14 TI fatigue OR AB fatigue OR SU fatigue
	S15 TI sleep OR AB sleep OR SU sleep
	S16 TI quality of life OR AB quality of life OR SU quality of life
	S17 TI anxiety OR AB anxiety OR SU anxiety
	S18 TI pain OR AB pain OR SU pain
	S19 TI cachexia OR AB cachexia OR SU cachexia
	S20 TI weight loss OR AB weight loss OR SU weight loss
	S21 TI anorexia OR AB anorexia OR SU anorexia
	S22 TI nausea OR AB nausea OR SU nausea
	S23 S12 OR S13 OR S14 OR S15 OR S16 OR S17 OR S18 OR S19 OR S20 OR S21 OR S22
	S24 S4 AND S11 AND S23
PSYCHINFO	

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## The Emerging Role of Interleukin 1 $\beta$ (IL-1 $\beta$ ) in Cancer Cachexia

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Received December 4, 2020; accepted January 27, 2021

**Abstract**— Treatment of cancer cachexia remains an unmet need. The host-tumour interface and the resulting sequestration of the pro-inflammatory cytokine IL-1 $\beta$  is critical in cachexia development. Neuroinflammation mediated *via* IL-1 $\beta$  through the hypothalamic pituitary axis results in increased muscle proteolysis and adipose lipolysis, thus creating a prolonged stress-like environment with loss of appetite and increased resting energy expenditure. Recent trials using a monoclonal antibody targeting IL-1 $\beta$ , canakinumab, have shown a potential role in lung cancer; however, a potential role of targeting IL-1 $\beta$  to treat cachexia in patients with lung cancer is unclear, yet the underlying pathophysiology provides a sound rationale that this may be a viable therapeutic approach.

**KEY WORDS:** Inflammation; Cachexia; Interleukin-1 beta.

### INTRODUCTION

Conservative estimates suggest cancer cachexia is attributable to over half of the cancer deaths worldwide [1]. Yet despite its prevalence, adverse impact on quality of life [2], and efficacy of anti-cancer therapy [3], there remains no standard of care and no licenced therapy [4]. To date, there has been a failure to progress the research

agenda in cancer cachexia and recently tested novel therapies studies have failed to meet trial endpoints and thus regulatory approval [5, 6].

The starting point for much of the work in the last decade has been the international consensus publication of 2011 [7]. In this, cancer cachexia was defined as “a multifactorial syndrome defined by an ongoing loss of skeletal muscle mass (with or without loss of fat mass) that cannot be fully reversed by conventional nutritional support and leads to progressive functional impairment. The pathophysiology is characterised by a negative protein and energy balance driven by a variable combination of reduced food intake and abnormal metabolism.” This definition acknowledged the role of the systemic inflammatory response in cachexia and this has been supported in the intervening years by work showing the importance of systemic inflammatory responses in the progressive nutritional and functional decline of patients with cancer; indeed, the systemic inflammatory response can be regarded as a central tenet of

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cancer cachexia [4, 8–10]. Indeed, the European Society for Clinical Nutrition and Metabolism (ESPEN) suggest that cancer cachexia is synonymous with disease-related malnutrition in combination with inflammation [11, 12].

With reference to previous randomized trials in cachexia, few have characterized the systemic inflammatory response as an entry criterion or as a therapeutic target [5, 6, 13]. In contrast, there are increasing numbers of oncological trials incorporating measures of the systemic inflammatory response as stratification variables [14]. Indeed the presence of the systemic inflammatory response has been firmly established as being associated with weight loss and loss of lean mass [15–17], reduced functional status [18, 19], anorexia [20, 21], quality of life [2, 18, 22–24], and reduced survival [19, 25, 26].

It therefore follows that, with the systemic inflammatory response playing a key role in the development of cachexia, there is a need to target this response. Yet, to date, few studies have investigated anti-inflammatory treatment in patients with cancer cachexia [4, 9, 10].

## INFLAMMATION AND CANCER CACHEXIA

The cancer-associated systemic inflammatory response is recognized to be mediated by a network of inflammatory cytokines, eicosanoids, and other factors as part the tumour-host response. In patients with advanced cancer, pro-inflammatory cytokines predominate leading to an upregulation of Interleukin 1 (IL-1) and increased downstream production of IL-6 [27–31]. Therefore, downregulation of IL-1 is a logical therapeutic target for moderation of the systemic inflammatory response. Indeed, IL-1 inhibitors are established in the treatment of inflammatory joint disease.

### Role of IL-1 in Cachexia

In cachexia, the IL-1 pathway is overactive and contributes to cachexia *via* several mechanisms [32]. It influences tryptophan secretion resulting in increased concentrations and subsequently excess hypothalamic derived serotonin, causing satiety, and appetite suppression [33]. Whilst the actions of these cells normally represent an acute physiological response to stress, they are exacerbated by cancer where an inflammatory environment causes prolonged stress, resulting in overstimulation causing muscle wasting [9]. The impact of IL-1 on this mechanism has

been demonstrated by pre-clinical models in which IL-1 triggered the release of  $\alpha$ -melanocyte stimulating hormone ( $\alpha$ -MSH) from pro-opiomelanocortin (POMC) neurones. Then,  $\alpha$ -MSH stimulates Melanocortin-4 (MC4-R) neurones to induce appetite suppression [34]. Furthermore, IL-1 exhibits an inhibitory effect on Neuropeptide Y (NPY) neurones which also physiologically inhibits MC4-R neurones [35, 36].

Studies have also shown that IL-1 stimulates hypothalamic neurones which release corticotrophin releasing hormone (CRH) [37]. This causes secretion of adrenocorticotrophic hormone and cortisol, which may in turn mediate the catabolic effects of cachexia. This link between IL-1, the hypothalamic pituitary axis (HPA) and muscle wasting was explored further by in a study by Braun et al. where inflammation was induced in mouse models *via* intracerebroventricular injections of IL-1 [38]. The mice exhibited an increase in muscle-specific E3 ubiquitin ligases, a key requirement for the catabolism of muscle. The same result was then also achieved when inflammation was induced peripherally with LPS. Interestingly however, this study showed that muscle catabolism was ameliorated in mice which had undergone an adrenalectomy. Such results infer that activation of the HPA axis is central to the catabolic effects of IL-1.

Two IL-1 genes, IL1A and IL1B, encode IL-1 $\alpha$  and IL-1 $\beta$  and binding of either IL-1 $\alpha$  or IL-1 $\beta$  to the IL-1R1 receptor. Although similarities exist between IL-1 $\alpha$  and  $\beta$ , clear differences are also present with the latter having a greater pro-inflammatory effect, influencing carcinogenesis and invasiveness of cells [39]. The potential role of IL-1 $\alpha$  has been recently reviewed [40].

### Role of IL-1 $\beta$ in Cancer

IL-1 $\beta$  is produced in response to inflammation by monocytes, macrophages, and neutrophils and induces production of stimulation of IL-6 [41]. IL-1 $\beta$  is released from macrophages and along with its potent inflammatory effect it has been widely established that IL-1 $\beta$  production is upregulated in ovarian, lung, and GI cancers and in general these cancers are associated with bad prognoses [42]. IL-1 $\beta$  gene is located on chromosome 2q14 within a 360-kb region and it has been shown that key polymorphisms, IL-1beta +3954, are a risk factor for the development of cachexia in patients with gastrointestinal cancers [42]. This finding has been supported by other work demonstrating the central role of IL-1 $\beta$  in cachexia [43–46].



### Role of IL-1 $\beta$ in Cachexia

IL-1 $\beta$  also plays a role in cachexia development through the CNS. Stimulated *via* peripheral inflammation, central inflammation (specifically the hypothalamus) results in release of multiple inflammatory factors and alteration in neural mechanisms that result in proteolysis and lipolysis. Further, this HPA-mediated abnormal regulation causes muscle breakdown with the resulting supply of amino acid precursors for superfluous hepatic gluconeogenesis. The resulting dysregulation of POMC and AGRP [47], both of which control lean muscle proteolysis and adipose lipolysis, and MC4-R which regulates appetite and energy expenditure [48] causes muscle wasting [9]. IL-1 $\beta$  has been demonstrated as being a critical factor in this process and thus a key player in the loss of lean mass, appetite, and inflammatory upregulation, seen in cancer cachexia [38, 43–46].

### Targeting IL-1 $\beta$ in Cancer

It follows that as the pathophysiological mechanisms of cancer cachexia are mediated by IL-1 $\beta$ , targeting this is a logical approach. Canakinumab is a human monoclonal antibody to IL-1 $\beta$  with a half-life of 26 days. Binding directly to circulating IL-1 $\beta$ , it neutralizes activity between IL-1 $\beta$  and its receptor, making it well suited to a therapeutic role. Although this role has been established for over a decade in rheumatological indications and other immunological diseases, it has recently been shown to be efficacious in prevention of cardiac disease, in comparison to placebo (CANTOS trial) [49]. Of particular relevance was a subsequent analysis of the CANTOS cohort where the incidence of lung malignancy was noted to be reduced in those patients taking higher doses (150 mg or 300 mg) of canakinumab [50]. This finding is perhaps not surprising as the clear role of the inflammatory response in the development of lung cancer is well established. It therefore follows that attenuation of this targeting the potent pro-inflammatory mediator IL-1 $\beta$  *via* canakinumab may yield benefit. Ridker and co-workers also observed that the beneficial effect was most likely in those with raised CRP. Again, this observation echoes recent work by Dolan and co-workers highlighting the role of CRP in influencing outcomes in clinical trials of anti-cancer therapies [14, 51].

The role of Canakinumab in the inflammatory process in malignant disease is being examined; however, the pathophysiological mechanisms are plausible. Currently, the CANOPY suite of clinical trials in lung cancer is underway and findings awaited with interest. However,

the potential role of canakinumab in the prevention or treatment of cancer cachexia is unclear. Survival endpoints are predominant in current trials and whilst quality of life and other patient reported outcomes are examined, key endpoints focussing on cancer cachexia are not within the remit of the current trials.

### Potential Role of Targeting IL-1 $\beta$ in Cancer Cachexia

Several studies further support the potential role of canakinumab in the treatment of cancer cachexia. Djamil and colleagues demonstrated that patients with cancer-related anorexia had significantly higher levels of IL-1 $\beta$  than comparators and that this was correlated with severity of anorexia [52]. In patients with pancreatic cancer, Fogelman and co-workers showed that IL-1 $\beta$  levels were predictive of development of cancer cachexia and was superior in this regard to other cytokines [53]. Another prospective study demonstrated the superiority of IL-1 $\beta$  over IL-6 in terms of correlation with the clinical phenotype of the cachexia syndrome [46].

Other work has echoed these findings further substantiating the clear influence of IL-1 $\beta$  in cancer cachexia genesis [54, 55].

### CONCLUSION

IL-1 $\beta$  represents a cytokine involved in the pathophysiology of cancer, with direct targeting a viable avenue to treat cancer cachexia as well as tumour load *per se*. Work to date highlights the key role of IL-1 $\beta$  in the central mechanisms of cancer cachexia supported by emerging clinical trials. Yet to fully explore this, trials are needed which include patients at risk of cancer cachexia and that use endpoints sensitive enough to examine the multiple clinical sequelae of cancer cachexia. Further, cancer cachexia is a complex phenomenon and targeting IL-1 $\beta$  in isolation may be insufficient but combined with a background standard of cachexia care, it may have its most optimal effect. Canakinumab has been shown to have excellent tolerability in multiple completed and ongoing clinical trials, as well as real world experience [56]. The next logical step is to undertake exploratory trials examining canakinumab with robust cachexia endpoints as key outcomes. Such trials are eagerly awaited.

### ACKNOWLEDGEMENTS

None.

**Availability of Data and Material.** Not applicable.

**Code Availability.** Not applicable.

#### AUTHOR CONTRIBUTIONS

BL conceived the idea and led the manuscript writing. DM, RS, MF, RP, IM, and IG all contributed equally to the manuscript.

#### FUNDING

No funding support was received for this work.

#### DECLARATIONS

**Ethics Approval.** Not applicable.

**Consent to Participate.** Not applicable.

**Consent for Publication.** Approved by all authors.

**Conflict of Interest.** The authors declare no conflicts of interest.

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# The Emerging Role of Intelectin-1 in Cancer

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**Background:** Intelectin (ITLN) is an adipokine with two homologs—ITLN1 and ITLN2—that has various physiological functions. Studies analyzing the relationship between ITLN and cancer are focused on ITLN1; the available literature on ITLN2 and cancer is limited. This review aims to evaluate the role of ITLN1 in cancer without imposing any inclusion criteria, to examine pro- and anticancer roles for ITLN1 and to discuss whether the relationship between ITLN and cancer is mediated by obesity.

**Findings:** Overall, ITLN1 level was highly variable in cancer patients but different from healthy individuals. Compared with control groups, patients with gastrointestinal and prostate cancer showed increased concentrations of circulating ITLN1, while patients with gynecological, breast, bladder, and renal cancer had lower ITLN1 levels. Several studies also evaluated tissue and tumor expression of ITLN1. In gastrointestinal cancer, ITLN1 was increased in tumor tissue compared with adjacent healthy tissue and elevated in the visceral adipose tissue of patients compared with controls. Consequently, the high levels of circulating ITLN1 might be determined by the tumor and by the cancer-associated weight loss in gastrointestinal cancer. ITLN1 can activate the phosphoinositide-3-kinase-protein kinase B/Akt (PI3K/Akt) pathway. The improper regulation of this pathway may contribute to a series of cellular events that favor tumor development and progression. Obesity has been linked with an increased risk of developing some cancers. Indeed, low circulating ITLN1 levels may be a marker of the metabolic effects of obesity, rather than obesity per se, and might contribute to a deregulation of the PI3K/Akt pathway.

**Conclusions:** ITLN1 could be associated with cancer formation and progression. Since circulating ITLN1 levels are highly variable and differ between cancer types, the local tumor production of ITLN1 could be more relevant in determining malignant behavior. Future research should aim to identify the source of ITLN1 variability, to understand the differences in ITLN1 between distinct tumor types, and to further explore the signaling pathways through which this adipokine influences cancer biology.

**Keywords:** omentin, adipokine, cancer, tumor, intelectin, gastrointestinal

## OPEN ACCESS

### Edited by:

Vivek M. Rangnekar,  
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### Specialty section:

This article was submitted to  
Cancer Molecular Targets  
and Therapeutics,  
a section of the journal  
Frontiers in Oncology

**Received:** 01 September 2021

**Accepted:** 07 January 2022

**Published:** 03 February 2022

### Citation:

Paval DR, Di Virgilio TG, Skipworth RJE  
and Gallagher IJ (2022) The Emerging  
Role of Intelectin-1 in Cancer.  
Front. Oncol. 12:767859.  
doi: 10.3389/fonc.2022.767859

## INTRODUCTION

Intelectin (ITLN), also known as omentin, is a 34-kDa lectin that contains a fibrinogen-like domain and a unique intelectin-specific region that makes it distinct from other immune lectins (1, 2). There are two homologs with 83% amino acid identity termed intelectin-1 (omentin-1) and intelectin-2 (omentin-2). ITLN is mainly produced by stromal vascular fraction cells, but not by adipocytes, in visceral adipose tissue, while its levels are very low in subcutaneous adipose tissue (3). Moreover, ITLN1 expression has also been found in epicardial fat, the small intestine, colon, ovary, lungs, and renal collecting tubes, whereas ITLN2 is expressed in intestinal Paneth cells (2, 4, 5). Both ITLN homologs can bind microbial glycan chains but not human glycans, and thus, the adipokine may have a role in antimicrobial defense (2, 6). Previous work has also identified the possible roles for ITLN1 in polyspermy prevention and in iron metabolism *via* interaction with lactoferrin (2).

The interest in the role of ITLN in cancer has been driven by the observation that ITLN1 levels differ between healthy individuals and patients with various types of cancer (7–10). We recently identified ITLN1 and ITLN2 mRNA as increased in the visceral adipose tissue of gastrointestinal cancer patients and found that local but not circulating ITLN1 protein demonstrated a relationship with cancer cachexia (11). It has also been suggested that the relationship between ITLN1 and cancer might be influenced by overweight and obesity (12). Collectively, these findings indicate that ITLN might have a role in cancer biology and that it could be used as a biomarker for cancer itself or cancer progression. Yet, the exact mechanisms through which this adipokine induces physiological changes are not completely understood, and the variable levels of ITLN described in the literature have not been fully explained by previous research. Therefore, we will review the available literature on ITLN in cancer without imposing any inclusion criteria, discuss the proposed pro- and anticancer roles for ITLN, and finally, analyze whether the relationship between ITLN and cancer is mediated by body mass index (BMI). Furthermore, this review will identify current knowledge gaps and propose pathways for future work. Most of the studies analyzing the relationship between ITLN and cancer focus on ITLN1. Although ITLN2 is likely to influence various physiological processes, there are little published data on this topic, and therefore, the present review will focus on ITLN1.

## DISCUSSION

### ITLN1 and Gastrointestinal Cancers

A recent systematic review examining circulating ITLN1 levels in cancer found that ITLN1 was often increased in individuals with colorectal cancer compared with healthy controls (12). Another study evaluating a cohort of Chinese patients identified increased ITLN1 levels as a potential risk factor for colorectal cancer (13). Higher levels of circulating ITLN1 were also found to increase the probability of recurrence or death in colorectal cancer after

surgery; yet, the difference in circulating ITLN1 between the groups was very small (14). A study by Ummugul Uyeturk and colleagues (15) examined circulating ITLN1 levels after surgery and chemotherapy in colorectal cancer and found that the levels remained elevated compared with a healthy control group who did not have surgery or chemotherapy. However, the reported range of results in this study was very low (pg/ml). Circulating ITLN1 levels also seem to be increased in pancreatic cancer patients compared with healthy controls (8, 16). In comparison, two studies have found that higher tumor expression (rather than circulating levels) of ITLN1 was associated with a good prognosis in colorectal cancer (17, 18). Unfortunately, circulating ITLN1 was not assessed in these studies. Zheng and colleagues (19) emphasized that ITLN1 levels were greater in gastric cancer tissue compared with normal gastric mucosa. We previously identified higher ITLN1 expression in the visceral adipose tissue of patients with upper gastrointestinal cancer compared with healthy controls, while the expression of ITLN1 mRNA in subcutaneous adipose tissue and circulating ITLN1 levels did not differ between groups (11).

Although the evidence is not strong enough to generate a definitive conclusion, it can be argued that ITLN1 expression is increased locally and perhaps systemically in gastrointestinal cancers. The elevated circulating levels of this adipokine might be partly determined by the tumor (20) and the degree of cancer-associated weight loss (11, 21). While the function of ITLN1 in these cancers is unknown, some of the evidence presented above would suggest that higher local tumor levels may be a good prognostic indicator. However, the reported circulating levels of ITLN1 are extremely variable. Thus, while ITLN1 may have potential as a biomarker for the diagnosis or progression of gastrointestinal cancers, circulating levels may be too variable to be useful. This question should be investigated with appropriately powered studies.

### ITLN1 and Urological Cancers

The levels of ITLN1 are also dysregulated in prostate cancer. Arjmand and colleagues (12) suggested that prostate cancer patients had significantly higher circulating ITLN1 levels compared with control groups in all the studies included in their review. Although the stated aim of their review was to compare cancer patients with healthy controls, not all of the included studies respected this criterion. In the prostate cancer subgroup analysis, two studies compared cancer patients against individuals with benign prostatic hyperplasia (BPH). In both studies, circulating ITLN1 was higher in prostate cancer than in BPH (22, 23). Similarly, a more recent study observed that prostate cancer patients had greater concentrations of circulating ITLN1 compared with individuals with BPH (24). Zhang et al. (25) examined circulating ITLN1 levels in bladder cancer and found reduced levels in patients compared with healthy controls. However, the reported ITLN1 levels were outside the assay range indicated in the methodology of the study. Circulating ITLN1 was also lower in patients with renal cell carcinoma than in matched controls (26). In summary, the available literature suggests that circulating ITLN1 is elevated in



prostate cancer and reduced in bladder and renal cancer. Further evidence is required to strengthen these interim conclusions.

### ITLN1 and Breast and Gynecological Cancers

Three reports examined the circulating levels of ITLN1 in breast cancer patients compared with healthy controls. Patients with breast cancer had lower circulating ITLN1 levels in all three studies (10, 27, 28). Tahmasebpour and colleagues (10) also observed that ITLN1 gene expression was significantly downregulated in breast cancer tissue compared with adjacent normal tissue. Several studies assessed the relationship between ITLN1 and gynecological cancers. In a subgroup meta-analysis by Arjmand et al. (12), circulating ITLN1 did not significantly differ between women with ovarian cancer and healthy controls. Another study evaluating the same topic (29) indicated that circulating ITLN1 was lower in patients with high-grade ovarian cancer as opposed to healthy women and women with benign gynecological disease. Moreover, these authors observed that ITLN1 mRNA was expressed at a lower level in the omental adipose tissue of patients with high-grade ovarian cancer compared with women with benign disease (29). Interestingly, this finding contradicts our study in upper gastrointestinal cancer (11) where disease severity increased omental ITLN1 expression. Holman et al. (30) and Cymbaluk-Ploska et al. (31) also observed lower circulating ITLN1 in women with endometrial cancer. Although both studies were analyzed in the systematic review by Arjmand and colleagues (12), only Holman et al. (30) compared cancer patients with healthy controls. The comparison group from the other study (31) included women with endometrial polyps (benign endometrial changes). In summary, despite the fact that only a limited amount of data were available, a tendency for lower circulating ITLN1 levels in breast and gynecological cancers was observed. Future studies should aim to explore this relationship and to develop a better understanding of the role of ITLN1 in these types of cancer.

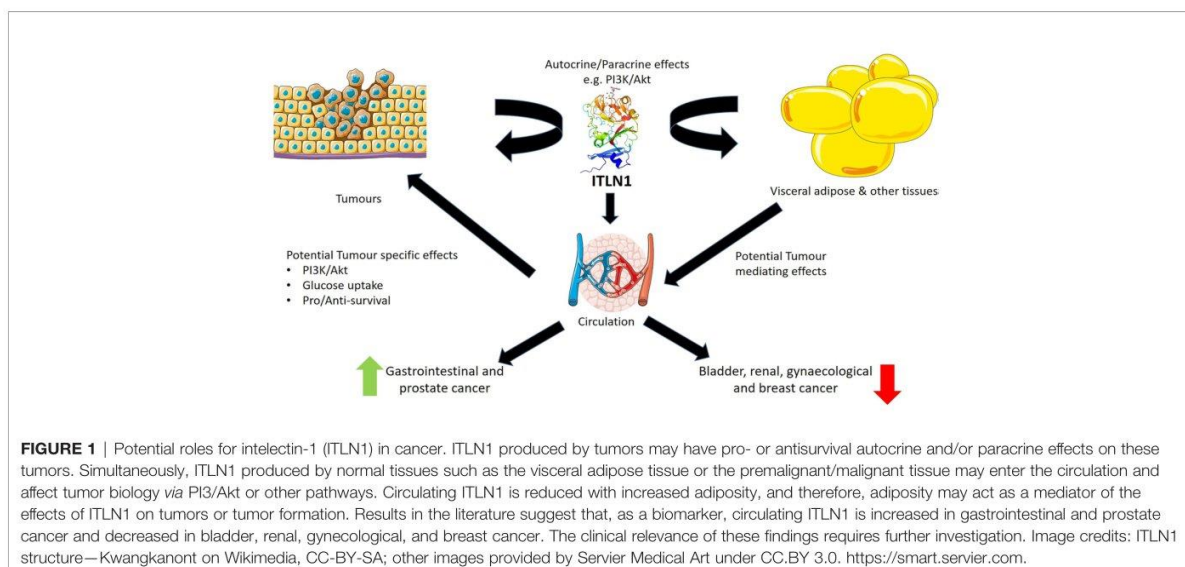
### Other Cancers

In an examination of 42 neuroblastoma tumors, Li et al. (32) found that ITLN1 protein was expressed in 33% of the tumor specimens with variable degrees of staining, from weak to intense. Higher levels of ITLN1 staining tended to occur in tumors with more favorable features (32). Furthermore, data mining of the R2 microarray genomics and visualization resource suggested that higher ITLN1 mRNA expression was associated with a greater probability of survival (32). Another study noted a lower level of circulating ITLN1 in Iranian male smokers with lung cancer compared with apparently healthy smokers. Notably, circulating ITLN1 in smokers with lung cancer was not different from that of healthy non-smokers (33). An *in-silico* meta-analysis of RNA-Seq data noted that ITLN1 mRNA expression was consistently lower in lung tumors compared with healthy lung tissue across seven data sets and that higher ITLN1 mRNA levels in tumors predicted better survival (34). In malignant pleural mesothelioma (MPM), the early work by Wali et al. (35) found that ITLN1 mRNA and protein were highly expressed in tumors as opposed to normal tissue. Subsequently, increased expression of ITLN1 was observed

in mesothelioma cell lines and epithelioid mesotheliomas (36–38). ITLN1 levels were elevated in the pleural fluid of MPM patients compared with the levels seen in lung cancer, tuberculosis, or pneumonia (36). The mean differences were driven by a few individuals with very high levels of pleural ITLN1 (>3,000 ng/ml), while plasma ITLN1 concentrations did not differ between MPM patients and healthy controls (36). Although immunoreactivity for ITLN1 in epithelioid mesothelioma tumors was very strong, ITLN1 seemed to be absent from other tumors (both mesothelioma and non-mesothelioma) unless they were mucus-producing (36–38). Overall, the available data on the previously discussed types of cancer are limited but generate pathways for further research. The direction of the difference in circulating ITLN1 between cancer patients and control groups depends on cancer type. Similarly, different tumors were found to express distinct concentrations of ITLN1. **Table S1** in the **Supplementary Materials** section includes a summary of the studies from this review that measured ITLN1 in human participants diagnosed with cancer. The source of ITLN1 variability between different types of cancer should be one of the main areas explored by future studies.

### Mechanisms for Intelectin Effects in Cancer

**Figure 1** highlights the potential roles of ITLN1 in cancer and its probable mechanisms of action. Previous work has indicated that ITLN1 can influence Akt-mediated growth pathways. The phosphoinositide-3-kinase-protein kinase B/Akt (PI3K/Akt) signal transduction pathway has a crucial role in various cellular functions that contribute to cancer, including metabolism, growth, motility, proliferation, and angiogenesis (39). ITLN1 increases Akt phosphorylation in adipocytes, osteoblasts, and mesenchymal cells (3, 40, 41). Wu and colleagues (40) also indicated that the inhibition of Akt prevents the proliferative effect of ITLN1 in osteoblasts, suggesting that ITLN1 signals through the Akt pathway in these cells. In mesenchymal stem cells, ITLN1 also leads to Akt-mediated proliferation, resistance to oxidative stress, and secretion of proangiogenic factors (41). These actions may be particularly important in tumors as mesenchymal stem cells are part of the tumor microenvironment and can favor tumor growth (42). Activation of mesenchymal gene programs is also a characteristic of advanced tumors (43). Overall, these findings suggest that ITLN1 could locally potentiate PI3K/Akt signaling and glucose uptake in cancer cells to promote survival. The ITLN1/TMEM207 axis is another pathway that may influence carcinogenesis (44). TMEM207 is a transmembrane protein that has a role in ITLN1 processing. It was suggested that low levels of TMEM207 contribute to a decrease in ITLN1 concentrations and, consequently, promote colorectal carcinogenesis (44, 45). The available research on this pathway is still limited and future studies are needed to confirm the relationship between TMEM207 and ITLN1. Given that circulating ITLN1 varies between different types of cancer, the local level or the tumor production of ITLN1 may be more important than the circulating level in contributing to malignant behavior.



Conversely, other groups have found that ITLN1 may play an antitumor role. In colorectal cancer cell lines, the reduction of ITLN1 expression by short-hairpin RNA increased cell growth and proliferation as well as Akt, extracellular signal-regulated kinase (ERK), and epidermal growth factor receptor (EGFR) phosphorylation (18). ITLN1 treatment reduced the proliferation and the migratory ability of gastric cancer cells by upregulating hepatocyte nuclear factor 4 $\alpha$  (HNF4 $\alpha$ ) via increased inhibition of nuclear factor kappa B (NF $\kappa$ B) (46). Furthermore, a supraphysiological dose of ITLN1 (1–2  $\mu$ g/ml) led to the arrest of cell cycling and subsequent apoptosis in hepatocellular carcinoma cell lines (47). Supraphysiological ITLN1 also inhibited the proliferation and promoted the apoptosis of colon cancer stem cells in a time-dependent manner (48). In neuroblastoma cell lines and human tumors, increased ITLN1 seemed to have a PI3K/Akt-mediated protective effect by increasing the expression of N-myc downstream-regulated gene 2 (NDRG2) and by reducing tumor volume and metastatic potential (32). Notably, higher ITLN1 expression in human neuroblastoma tumors improved the probability of survival (32). Similarly, higher tumor expression of ITLN1 was associated with improved prognosis in patients with colorectal cancer (17, 18). These results suggest that ITLN1 may have potential as an anticancer agent. However, the levels of ITLN1 used in many of these studies were in the  $\mu$ g/ml range, whereas the normal physiological concentration of ITLN1, even in cancer or cell culture supernatant, was in the ng/ml range. Therefore, there may be unanticipated physiological effects if elevated levels of ITLN1 are used as an anticancer agent.

### Intelectin, Obesity, and Cancer Risk

Visceral adipose tissue is the primary source of ITLN suggesting that the relationship between this adipokine and various cancer mechanisms could be mediated by BMI or adiposity.

The presence of excess adipose tissue alters adipokine production which may contribute to the development of cancer, indicating the potential role of ITLN1 (44, 49). Also, it has been previously demonstrated that obese individuals present a higher risk of developing cancer compared with people with normal weight (50). Obesity has been linked to lower levels of circulating ITLN1 in several studies (5, 51). However, low circulating ITLN1 levels in people with impaired glucose regulation and untreated type 2 diabetes (52), women with metabolic syndrome secondary to polycystic ovary syndrome (53), and Japanese men with a higher number of metabolic risk factors (54) as well as in women with gestational diabetes and BMI <30 (52) suggest that low ITLN1 may be a marker for the metabolic effects of obesity rather than obesity itself. Although a recent meta-analysis of studies examining the relationship between body weight and circulating ITLN1 levels found an overall effect, there was very high study heterogeneity (93%) and evidence of publication bias (55). Furthermore, subgroup analysis revealed that higher-quality studies suggested no relationship and that serum ITLN1 level was significantly lower in overweight but not obese individuals (55). In summary, the available evidence suggests that circulating ITLN1 may be a marker of metabolic dysregulation rather than overweight/obesity per se.

In their review, Arjmand and colleagues (12) found no overall relationship between circulating ITLN1 and cancer. As we note above, evidence suggests that the direction of change for circulating ITLN1 may be different in the presence of different tumors. Notably, levels of circulating ITLN1 were consistently higher than control groups in gastrointestinal and prostate cancer and lower than control groups in renal, lung, bladder, endometrial, and breast cancer (12). Arjmand et al. (12) also suggested that ITLN1 levels were higher in studies examining



cancer patients with a mean BMI >25 as opposed to studies that included individuals with an average BMI <25. This could indicate an interaction between cancer presence, adiposity, and intelectin. However, as noted above, the evidence base for an association between circulating ITLN1 and overweight/obesity is weak (55). Arjmand et al. (12) also grouped studies based on average BMI values but did not consider the variation around the mean for several studies (7, 9, 22, 28, 33, 56). Not all of the patients included in these studies had a BMI >25 according to their published variance statistics. Including these studies in the “BMI >25” subgroup therefore does not represent a valid statistical approach. Thus, tumor-specific directional changes in circulating ITLN1 levels, local versus global changes in ITLN1, and aggregate views of overweight/obesity or metabolic status in patient groups could lead to misinterpretations of the relationship between circulating ITLN1 and cancer risk or status.

### Future Directions

Changes in circulating ITLN1 levels have been associated with several cancers. Tumor site and/or type seems to be important in the direction of the change. Further work is required to identify whether ITLN1 has utility as a biomarker for cancer occurrence or re-occurrence. This work would have to further confirm and take account of the possible tumor-specific direction of change in ITLN1. As we noted in our work on adipose tissue in upper gastrointestinal cancer, increased production of visceral adipose tissue ITLN1 is a feature of cancer-induced weight loss (11). Future studies with larger cohort sizes should also investigate whether cancer-induced weight loss leads to increased circulating ITLN1. The mechanisms by which ITLN1 may lead to either positive or negative change in cancer also deserve research attention. Further work on the signaling pathways and the roles of ITLN1 in both normal and dysregulated metabolism should help identify the mechanisms through which this adipokine influences tumor biology and the metabolic consequences of cancer.

### CONCLUSION

The available literature indicates that ITLN1 might have a role in cancer formation and development since ITLN1 level was highly variable but different from healthy controls when patients with various cancer types were examined. High concentrations of ITLN1 were found in patients with gastrointestinal (8, 13) and prostate cancer (23) compared with control groups. Conversely, women with breast (27) and gynecological cancer (29) expressed less ITLN1 as opposed to healthy individuals. Although these are

relevant observations, a meta-analysis with a reliable methodological approach is required to quantify ITLN1 levels in cancer patients and healthy participants. Most available studies, including a recent systematic review (12), only measured differences between groups. To date, no study discussed the variability in ITLN1 levels observed in both healthy and diseased individuals. For instance, in the review by Arjmand et al. (12), mean ITLN1 varied from 1.8 to 618.0 ng/ml in the cancer groups, while the range in the control groups was 1.6 to 756.4 ng/ml. Therefore, the source of ITLN1 variability and its physiological concentration should be determined by future research.

Several studies (40, 41) assessed the mode of action of ITLN1 and observed that it activates the PI3k/Akt pathway. The improper regulation of this pathway could lead to the proliferation of mesenchymal cells that favor the progression and development of cancer. Low ITLN1 levels are a marker of the metabolic effects of obesity and might contribute to a deregulation of the PI3k/Akt pathway. Otherwise, local changes in ITLN1 could favor a protumor environment. Further research is required to clarify the role of ITLN1 in carcinogenesis and to describe the pathways through which this adipokine interacts with other cells. Moreover, future studies should aim to assess ITLN1 expression in cancer patients by measuring its concentration in the blood, cancer tissue, and subcutaneous and visceral adipose tissue. Animal models and *in-vitro* investigations might focus on determining the direction of the causal relationship between ITLN1 and cancer. This area of research has been emerging during recent years and there are numerous promising pathways that can be explored.

### AUTHOR CONTRIBUTIONS

DP: conceptualization, methodology, writing of the drafts and the final version of the manuscript, and editing. TV: conceptualization and writing of the drafts and the final version of the manuscript. RS: conceptualization and writing of the drafts and the final version of the manuscript. IG: conceptualization, methodology, writing of the drafts and the final version of the manuscript, editing, and supervision. All authors contributed to the article and approved the submitted version.

### SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fonc.2022.767859/full#supplementary-material>

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## Influence of resistance training load on measures of skeletal muscle hypertrophy and improvements in maximal strength and neuromuscular task performance: A systematic review and meta-analysis


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To cite this article: Martin C. Refalo, D. Lee Hamilton, D. Robert Paval, Iain J. Gallagher, Simon A. Feros & Jackson J. Fyfe (2021): Influence of resistance training load on measures of skeletal muscle hypertrophy and improvements in maximal strength and neuromuscular task performance: A systematic review and meta-analysis, *Journal of Sports Sciences*, DOI: [10.1080/02640414.2021.1898094](https://doi.org/10.1080/02640414.2021.1898094)

To link to this article: <https://doi.org/10.1080/02640414.2021.1898094>

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 Published online: 19 Apr 2021.

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## Influence of resistance training load on measures of skeletal muscle hypertrophy and improvements in maximal strength and neuromuscular task performance: A systematic review and meta-analysis

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### ABSTRACT

This systematic review and meta-analysis determined resistance training (RT) load effects on various muscle hypertrophy, strength, and neuromuscular performance task [e.g., countermovement jump (CMJ)] outcomes. Relevant studies comparing higher-load [ $>60\%$  1-repetition maximum (RM) or  $<15\%$  RM] and lower-load ( $\leq 60\%$  1-RM or  $\geq 15\%$  RM) RT were identified, with 45 studies (from 4713 total) included in the meta-analysis. Higher- and lower-load RT induced similar muscle hypertrophy at the whole-body (lean/fat-free mass; [ES (95% CI) = 0.05 (-0.20 to 0.29),  $P = 0.70$ ]), whole-muscle [ES = 0.06 (-0.11 to 0.24),  $P = 0.47$ ], and muscle fibre [ES = 0.29 (-0.09 to 0.66),  $P = 0.13$ ] levels. Higher-load RT further improved 1-RM [ES = 0.34 (0.15 to 0.52),  $P = 0.0003$ ] and isometric [ES = 0.41 (0.07 to 0.76),  $P = 0.02$ ] strength. The superiority of higher-load RT on 1-RM strength was greater in younger [ES = 0.34 (0.12 to 0.55),  $P = 0.002$ ] versus older [ES = 0.20 (-0.00 to 0.41),  $P = 0.05$ ] participants. Higher- and lower-load RT therefore induce similar muscle hypertrophy (at multiple physiological levels), while higher-load RT elicits superior 1-RM and isometric strength. The influence of RT loads on neuromuscular task performance is however unclear.

### ARTICLE HISTORY

Accepted 26 February 2021

### KEYWORDS



Strength; muscle hypertrophy; resistance training; load; systematic review


### 1. Introduction

Resistance training (RT) is the only non-pharmacological intervention known to improve strength and induce skeletal muscle hypertrophy (Folland & Williams, 2007). While the manipulation of various RT parameters (e.g., volume (Schoenfeld, Ogborn et al., 2017), intensity (Schoenfeld, Grgic et al., 2017), frequency (Heaselgrave et al., 2019), and rest periods (Grgic et al., 2018)) can influence RT outcomes, both the volume [defined as either volume load (sets \* repetitions \* load) or set volume (number of sets completed irrespective of repetitions and load)] and intensity of RT seem to have the greatest influence on muscle hypertrophy and strength development (Morton et al., 2019). Defining RT intensity is contentious (Fisher & Smith, 2012; Steele, 2014) and may describe either the loads lifted (which define absolute and relative intensity), or the degree of effort applied, during a set (Morton et al., 2019). Previous studies exploring the influence of RT loads on physiological adaptations have shown comparable muscle hypertrophy across a wide spectrum of loads (Lopez et al., 2020; Schoenfeld, Grgic et al., 2017), and greater dynamic, but not isometric, strength gains with higher- versus lower loads (Schoenfeld, Grgic et al., 2017). For example, a meta-analysis by Schoenfeld and colleagues (Schoenfeld, Grgic et al., 2017) found muscle hypertrophy and isometric strength development was similar with higher-load ( $>60\%$  1-RM or  $\leq 15\%$  RM) versus lower-load

( $\leq 60\%$  1-RM or  $>15\%$  RM) RT, while higher-load RT promoted greater dynamic 1-RM strength gain. Lopez and colleagues (Lopez et al., 2020) also noted superior dynamic 1-RM strength gain for both high-load ( $\leq 8\%$  RM) and moderate-load (9–15-RM) RT versus low-load RT ( $>15\%$  RM), and no influence of RT load on muscle hypertrophy.

Current meta-analytic evidence (Lopez et al., 2020; Schoenfeld, Grgic et al., 2017) therefore highlights the versatility of RT loads for promoting muscle hypertrophy and the superiority of higher RT loads for improving dynamic 1-RM strength. There are, however, a number of methodological considerations when interpreting the role of RT load in promoting muscle hypertrophy and maximal strength development. Assessing muscle hypertrophy with RT is particularly complex, due not only to ambiguity in its definition as a biological construct but also given the many tools available to assess indices of muscle hypertrophy at multiple physiological levels (e.g., whole-body versus whole-muscle or muscle fibre-specific measures), with variability in aspects of validity, reliability, and specificity between measures (Haun et al., 2019). Such complexities are highlighted by the divergent magnitudes of muscle hypertrophy observed at different physiological levels after the same RT intervention [e.g., greater changes in muscle fibre versus whole-muscle vastus lateralis CSA (cross-sectional area)] (Aagaard et al., 2001; Esmarck et al., 2001; Narici et al., 1996), and that certain measures [e.g.,

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 Supplemental data for this article can be accessed online <https://doi.org/10.1080/02640414.2021.1898094>.

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whole-body measures such as DXA (dual x-ray absorptiometry)] are less sensitive for detecting RT-induced muscle hypertrophy versus other gold-standard measures of whole-muscle size [e.g., MRI (magnetic resonance imaging) or CT (computed tomography)] (Delmonico et al., 2008). For these reasons, the measures used to assess muscle hypertrophy can strongly influence conclusions on the influence of RT parameters, including load, on these outcomes. While some meta-analyses examining the influence of RT load on physiological adaptations analysed different muscle hypertrophy outcomes (i.e., lean body mass, whole-muscle CSA, and muscle fibre CSA) separately (Schoenfeld, Grgic et al., 2017), others combined various indices of muscle hypertrophy into a single outcome (Lopez et al., 2020). The latter approach is likely problematic (Fisher et al., 2017), as it precludes insight into the influence of RT load on muscle hypertrophy outcomes known to respond divergently to RT interventions. An updated analysis of the influence of RT loads on various indices of muscle hypertrophy separately is therefore warranted to ensure conclusions are specific to the measures of muscle hypertrophy used in individual studies.

In addition to muscle hypertrophy outcomes, various methodological considerations apply when determining the influence of RT load on maximal strength. Strength may be assessed using multiple methods, including dynamic strength [typically the one-repetition maximum (1-RM)], isometric strength, or isokinetic strength. Because strength is a highly task-specific phenomenon (Morrissey et al., 1995), improvements in strength with RT depend on various elements of specificity (e.g., movement pattern, range of motion, lifting velocity, and intensity/load specificity). Because of these factors, the magnitude of strength gain with RT is largest when the measures used to assess strength mimic the RT intervention itself. This concept is highlighted by observations that higher-load RT elicits superior strength gains versus lower-load RT when strength is assessed during measures that mimic higher-load RT (i.e., dynamic 1-RM strength) (Lopez et al., 2020; Schoenfeld, Grgic et al., 2017), but not when assessed using measures non-specific to either loading condition (i.e., isometric strength) (Mitchell et al., 2012; Schoenfeld, Grgic et al., 2017). It is therefore recommended that studies comparing strength outcomes between multiple RT conditions (e.g., higher- versus lower-load RT) assess multiple strength measures to avoid potentially biased outcomes due to task specificity (Buckner et al., 2017). Only one (Schoenfeld, Grgic et al., 2017) of the three (Lopez et al., 2020; Schoenfeld, et al., 2016; 2017) meta-analyses performed to date analysed the effects of RT load on multiple strength outcomes (i.e., both dynamic 1-RM and isometric strength, while the meta-analysis of isokinetic strength outcomes was not possible due to insufficient data (Schoenfeld, Grgic et al., 2017)), with one combining multiple strength assessments into a single outcome (Schoenfeld et al., 2016), and the other only assessing dynamic 1-RM strength (Lopez et al., 2020). Determining the influence of RT load on multiple strength outcomes is therefore necessary to determine whether advantages of higher-load RT for dynamic 1-RM strength gain are likely mediated by task specificity, or whether these benefits transfer to strength gain during non-specific measures (i.e., isometric or isokinetic strength).

To control for factors independent of RT load *per se* that might influence physiological adaptation to RT, such as intensity-of-effort (commonly defined as the proximity to which a set is taken to momentary muscular failure (Drinkwater et al., 2005)), previous meta-analyses (Lopez et al., 2020; Schoenfeld, et al., 2016; 2017) have only included studies whereby higher- and lower-load RT sets were performed to muscular failure. While this approach theoretically ensures the degree of muscle activation – and therefore presumably the stimuli for muscle hypertrophy – is similar for higher- and lower-load RT, there are also limitations with this approach. In particular, while intensity-of-effort is considered an important determinant of muscle hypertrophy with RT, it appears less important for strength development (Morton et al., 2019). Excluding studies that compared higher- versus lower-load RT performed at sub-maximal intensities-of-effort therefore precludes insight into the influence of RT load on physiological adaptations (particularly strength outcomes) independent of the proximity to which RT is performed to muscular failure. Such insights are of high practical importance, as consistently performing RT to muscular failure is not feasible for many individuals due to differences in motivation and tolerances to exertion and discomfort (Fisher & Steele, 2017). In addition, perceptions of discomfort may limit an individuals' ability to reach true muscular failure, particularly with lighter RT loads (Fisher et al., 2017) invalidating the assumption that higher- and lower-load RT performed to muscular failure involves near-equivalent muscle activation. Further work is therefore required to elucidate the influence of load on physiological adaptations to RT involving various intensities-of-effort, and to determine whether intensity-of-effort may have independently influenced these adaptations.

Previous systematic reviews and meta-analyses investigating the influence of RT load have focused on strength and muscle hypertrophy outcomes (Lopez et al., 2020; Schoenfeld et al., 2016, 2017; Lopez et al., 2020), but the influence of RT load on changes in sport-specific (e.g., jumping, sprinting and change of direction) or neuromuscular [e.g., countermovement jump (CMJ) and isometric mid-thigh pull (IMTP)] performance tasks has not been investigated. Maximal strength contributes to improvements in sport-specific performance tasks such as jumping, sprinting and change of direction (Seitz et al., 2014; Suchomel et al., 2016), and improved strength enhances mechanical power and rates of force development, both of which are key components of athletic performance (Suchomel et al., 2016). It is therefore possible that because strength relates to performance in sport-specific and neuromuscular performance tasks, performance in these tasks will be further improved with RT that optimizes strength development (Seitz et al., 2014; Suchomel et al., 2016). It is unclear, however, whether RT load influences changes in performance during sport-specific tasks or in tests related to neuromuscular performance (e.g., CMJ or IMTP).

Various other methodological factors may also influence the role of RT load in physiological adaptations to RT and therefore contribute to heterogeneity between studies. For example, the dose–response relationship between RT volume, which can be modified independently of RT load *per se*, and muscle hypertrophy (up to an undetermined threshold) (Schoenfeld, Ogborn et al., 2017) may influence comparisons



of muscle hypertrophy following high- versus low-load RT interventions not equated for total volume. Other methodological factors, including the age (Phillips et al., 2017) and training experience (Wetmore et al., 2020) of participants, may also moderate the influence of RT load on physiological adaptations and should be considered when interpreting the available evidence. This systematic review and meta-analysis therefore aimed to further elucidate the role of RT load in developing various indices of maximal strength (i.e., dynamic 1-RM, isometric, and isokinetic strength), muscle hypertrophy (i.e., lean body/fat-free mass, and both whole-muscle and muscle fibre CSA), and sport-specific or neuromuscular task performance. We also aimed to explore the influence of additional methodological factors (i.e., participant age, training status, and RT intensity-of-effort) that may influence the role of RT load in physiological adaptation to RT.

## 2. Methods

### 2.1. Criteria for study selection

#### 2.1.1. Population

Studies of participants of any age, sex, and training history were included. Studies of participants with chronic diseases (e.g., heart disease, type 2 diabetes, cancer, and hypertension) were excluded.

#### 2.1.2. Resistance training intervention

Studies that incorporated resistance training of at least six weeks in duration (which was considered an acceptable duration for substantial changes in both strength and muscle hypertrophy to occur, and was consistent with previous meta-analyses (Lopez et al., 2020; Schoenfeld, Grgic et al., 2017; Schoenfeld et al., 2016), and included at least one group that performed higher-load RT (>60% 1-RM or <15-RM) and at least one other group that performed lower-load RT ( $\leq 60\%$  1-RM or  $\geq 15\%$ -RM), were included. Studies incorporating additional modalities that may influence the role of RT load in physiological adaptation (e.g., blood flow restriction or hypoxia) were excluded. In the case of a study that included more than one group undertaking either higher- or lower-load RT, and applied additional factors (e.g., blood flow restriction or a deliberately slow tempo) to some of the groups that may differentially influence adaptation to RT, these additional group(s) were excluded from the analysis (see Table 1).

#### 2.1.3. Assessment of strength, muscle hypertrophy, and sport-specific or neuromuscular task performance

Studies that included a measure of either maximal strength (dynamic 1-RM or  $\leq 5$ -RM strength, isometric [maximal voluntary isometric contraction (MVIC)] strength, or isokinetic strength) and/or muscle hypertrophy (muscle thickness, whole-limb or muscle CSA or volume, muscle fibre CSA (fCSA), or lean body/fat-free mass via dual x-ray absorptiometry (DXA) or bioelectrical impedance analysis (BIA)) and/or sport-specific (e.g., jumping, sprinting, or changing-of-direction) or neuromuscular (e.g., CMJ or IMTP) task performance were included.

### 2.2. Search strategy and study identification

The literature search followed the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) guidelines

(Moher et al., 2009). Literature searches of the PubMed, SCOPUS and SPORTDiscus databases were conducted in August 2020 using the following search terms for each individual database:

- (1) "resistance training" OR "resistance exercise" OR "strength training"
- (2) "high load" OR "high-load" OR "low load" OR "low-load" OR "high intensity" OR "high-intensity" OR "low intensity" OR "low-intensity"
- (3) "Strength" OR "maximal strength" OR "isometric strength" OR "isokinetic strength" OR "maximal force" OR "MVC OR MVIC OR 1RM OR 1-RM" OR "1 RM" OR "one repetition maximum" OR "one-repetition maximum" OR "sprint" OR "vertical jump" OR "countermovement jump" OR "CMJ" OR "isometric mid-thigh pull" OR "isometric mid thigh pull" OR "isometric midhigh pull" OR "IMTP,"
- (4) "muscle hypertrophy" OR "muscle size" OR "muscle growth" OR "muscle mass" OR "muscle thickness" OR "cross-sectional area"

An overview of the article identification process is shown in Figure 1. Conference abstracts, review articles, commentaries, or duplicated data in publications were excluded from the analysis. The article identification process was completed independently by two authors (MR and JF) with any disagreement resolved by mutual discussion.

### 2.3. Data extraction

Relevant characteristics of each study were extracted into an Excel spreadsheet (Supplementary File A). Where study outcomes were presented in figures instead of numerical data, data was extracted using an online tool (WebPlotDigitizer, San Francisco, California, USA). Study characteristics included the age (<60 years for younger,  $\geq 60$  years for older), sex, and training status (trained or untrained as pertains to RT) of participants, details of the RT intervention including the number of sets, repetitions and loads used, duration of the intervention, training frequency per week, muscle groups trained, and raw data from pre- and post-intervention for all relevant outcome measures. A summary of the characteristics of each included study and sub-group included in the meta-analysis is presented in Table 1.

### 2.4. Methodological quality assessment

Evaluation of methodological study quality was conducted using the tool for the assessment of study quality and reporting in exercise (TESTEX) scale (Smart et al., 2015) and is shown in Table 2. The TESTEX scale is an exercise science-specific scale, designed for use by exercise specialists, to assess the quality and reporting of exercise training trials. The scale contains 12 criteria that can either be scored a "one" or not scored at all; 1, eligibility; 2, randomization; 3, allocation concealment; 4, groups similar at baseline; 5, assessor blinding; 6, outcome measures assessed in 85% of patients (3 possible points); 7, intention-to-treat; 8, between-group statistical comparisons (2 possible points); 9, point-estimates of all measures included; 10,

Table 1. Summary of the characteristics of all included studies.

Study	Participants	Age (mean $\pm$ SD)	RT intervention	Intervention duration (sessions/week)	Interventions equated for total volume	RT performed to volitional failure	Outcome measures	Key findings
Aagaard et al. (1994)	Younger male elite soccer players (n=24)	23 $\pm$ 3.4 y	High-load: 4 * 8-RM Low-load: 4 * 24-RM Loaded kicking movements#	12 weeks (3/week)	No	Yes	Isokinetic strength (knee extension/flexion)	$\uparrow$ Isokinetic strength for the high-load group only.
Anderson & Kearney (1982)	Younger untrained males (n=43)	20.7 $\pm$ 1.8 y	Control group (no exercise)# High-load: 3 * 6-8-RM Low load: 3 * 30-40-RM	9 weeks (3/week)	No	Yes	1-RM strength (bench press)	$\uparrow$ 1-RM strength for both groups, with greater $\uparrow$ in 1-RM strength for the high-load vs. low-load groups.
Au et al. (2017)	Younger trained males (n=46)	23 $\pm$ 2.3 y	Low load: 1 * 100-150-RM High-load: 3 * 8-12 reps (75-90% 1-RM) Low-load: 3 * 20-25 reps (30-50% 1-RM) Control group (maintained physical activity)#	12 weeks (4/week)	No	Yes	1-RM strength (bench press and leg press) Lean body mass (BodyPod)	$\uparrow$ 1-RM strength for both groups, with greater $\uparrow$ in 1-RM bench press strength for the high-load group. $\uparrow$ LBM for both groups, with no between-group differences.
Beneka et al. (2005)	Older males and females (n=64)	68.8 $\pm$ 4.2 y	High-load 1: 3 * 4-6 reps (90% 1-RM) High-load 2: 3 * 8-10 reps (70% 1-RM) Low-load: 3 * 12-14 reps (50% 1-RM) Control group (no exercise)#	16 weeks (3/week)	No	No	Isokinetic strength (knee extension)	$\uparrow$ Isokinetic strength at all velocities other than 180° s <sup>-1</sup> for all groups, with greater $\uparrow$ in the high-load group for 60, 90 and 120° s <sup>-1</sup> .
Bezerra et al. (2019)	Older untrained males (n=18)	63.4 $\pm$ 6.1 y	High-load: 3 * 5-RM Low-load: 1 * 15-RM Control group (no exercise)#	12 weeks (2/week)	No	Yes (only for seated row)	5-RM strength (seated row)	$\uparrow$ 5-RM strength for both groups, with no between group differences.
Campos et al. (2002)	Younger untrained males (n=32)	22.5 $\pm$ 5.8 y	High-load 1: 4 * 3-5-RM High-load 2: 3 * 9-11-RM Low-load: 2 * 20-28-RM Control group (no exercise)#	8 weeks (2-3/week)	Yes	Yes	1-RM strength (squat, leg press, knee extension) Muscle fCSA (biopsy; VL)	$\uparrow$ 1-RM strength for all groups, with greater $\uparrow$ in the high-load groups. $\uparrow$ VL fCSA for the high-load
De Vos et al. (2005)	Older untrained males (n=100)	68.5 $\pm$ 5.7 y	High-load: 3 * 8 reps (80% 1-RM) Low-load 1: 3 * 8 reps (20% 1-RM) Low-load 2: 3 * 8 reps (50% 1-RM) Control group (no exercise)#	8-12 weeks (2/week)	Yes	No	1-RM strength (leg press, chest press, knee extension, seated row, leg curl)	Total 1-RM strength for all groups, with greater $\uparrow$ in the high-
Fatouros et al. (2006)	Older untrained males (n=50)	70.4 $\pm$ 3.8 y	High-load: 3 * 10 reps (80% 1-RM) Low-load 1: 3 * 10 reps (40% 1-RM) Low-load 2: 3 * 10 reps (60% 1-RM) Control group (no exercise)#	24 weeks (3/week)	Yes	No	1-RM strength (leg press, chest press)	$\uparrow$ 1-RM strength for all groups, with greater $\uparrow$ in the high-load group.
Fink et al. (2016)	Younger male gymnastics athletes (n=21)	23.3 $\pm$ 2.7 y	Control group (no exercise)# High-load: 3 sets at 80% 1-RM Low-load: 3 sets at 30% 1-RM Mixed load: - alternated protocols every 2 weeks#	8 weeks (3/week)	No	Yes	MVIC strength (elbow flexors) Muscle CSA (MRI; elbow flexors)	$\uparrow$ MVIC strength for the high-load group only. $\uparrow$ Elbow flexor CSA for all groups, with no between-group differences.
Franco et al. (2019)	Younger untrained females (n=32)	23.7 $\pm$ 3.9 y	High-load: 3 * 8-10-RM Low-load: 3 * 30-35-RM	9 weeks (2/week)	No	Yes	1-RM strength (leg extension) Fat and bone free lean mass (DXA)	$\uparrow$ 1-RM strength for both groups, with no between-group differences. $\uparrow$ Fat-free lean mass for both groups, with greater $\uparrow$ in the low-load group.

(Continued)



Table 1. (Continued).

Study	Participants	Age (mean ± SD)	RT intervention	Intervention duration (sessions/week)	Interventions equated for total volume	RT performed to volitional failure	Outcome measures	Key findings
Harris et al. (2004)	Older untrained males and females (n = 61)	71.2 ± 5.1 y	High-load 1: 4 * 6-RM High-load 2: 3 * 9-RM Low-load: 2 * 15-RM Control group (no exercise)†	18 weeks (2/ week)	No	Yes	1-RM strength (knee extension, leg press, leg curl, biceps curl, triceps extension, lat pull-down, shoulder press, bench press)	↑ Total 1-RM strength in all groups, with no between-group differences.
Hisaeda et al. (1996)	Younger untrained females (n = 11)	20.1 ± 1.6 y	High-load: 8–9 sets of 4–5-RM Low-load: 5–6 sets of 15–20-RM	8 weeks (3/ week)	Yes	N/A	Isokinetic strength (knee extension) Muscle CSA (MRI; QF, RF, VL, VM, VI)	↑ Isokinetic strength for both groups, with no between-group differences. ↑ Thigh (VL, VM, RF) CSA for both groups, with no between-group differences.
Holm et al. (2008)	Younger untrained males (n = 11)	24.7 ± 1.1 y	High-load: 10 * 8 reps (70% 1-RM) Low-load: 10 * 36 reps (15.5% 1-RM)	12 weeks (3/ week)	Yes	No	1-RM strength (knee extension) MVIC strength (knee extension) Muscle CSA (MRI; QF)	↑ 1-RM strength for both groups, greater increases in the high-load group. ↑ MVIC and isokinetic strength for the high-load group only. ↑ VL CSA for both groups, greater ↑ in the high-load group.
Hortobágyi et al. (2001)	Older untrained males (n = 37)	72 ± 4.7 y	High-load: 5 * 4–6 reps (80% 1-RM) Low-load: 5 * 8–12 reps (40% 1-RM) Control group (no exercise)‡	10 weeks (3/ week)	No	No	1-RM strength (leg press)MVIC strength (knee extension) Isokinetic strength (knee extension)	↑ 1-RM and MVIC strength for both groups, with no between-group differences. ↑ Concentric and eccentric isokinetic strength only with both conditions combined, with no between-group differences.
Ikezoe et al. (2020)	Younger untrained males (n = 15)	23.1 ± 2.6 y	High-load: 3 * 8 reps (80% 1-RM) Low-load: 12 * 8 reps (30% 1-RM)	8 weeks (3/ week)	No	No	1-RM strength (knee extension) MVIC strength (knee extension) Muscle thickness (ultrasound; RF)	↑ 1-RM and MVIC strength for both groups, with no between-group differences. ↑ RF thickness for both groups, with no between-group differences.
Jenkins et al. (2017)	Younger untrained males (n = 26)	23.1 ± 4.7 y	High-load: 3 sets at 80% 1-RM Low-load: 3 sets at 30% 1-RM	6 weeks (3/ week)	No	Yes	1-RM strength (knee extension) MVIC strength (knee extension)Muscle thickness (ultrasound; VL, VM, RF)	↑ 1-RM and MVIC strength for both groups, with greater ↑ in the high-load group. ↑ Muscle thickness (VL, VM, RF) for both groups, with no between-group differences.
Jessee et al. (2018)	Younger untrained males and females (n = 40)	21 ± 2 y	High-load: 4 sets at 70% 1-RM Low-load 1: 4 sets at 15% 1-RM Low-load 2: 4 sets at 15% 1-RM (40% arterial occlusion pressure)‡ Low-load 3: 4 sets at 15% 1-RM (80% arterial occlusion pressure)‡	8 weeks (2/ week)	No	Yes	1-RM strength (knee extension) MVIC strength (knee extension) Isokinetic strength (knee extension) Muscle thickness (ultrasound; anterior and lateral thigh at 30%, 40%, 50%, and 60% femur length)	↑ 1-RM strength for the high-load group only. ↑ MVIC and isokinetic (at 180°·s <sup>-1</sup> but not 60°·s <sup>-1</sup> ) strength for both groups, with no between-group differences. ↑ Thigh muscle thickness for both groups, with no between-group differences.
Jones et al. (2001)	Younger trained males (n = 26)	20.6 ± 1.4 y	High-load: 4 * 3–10 reps (70–90% 1-RM) Low-load: 4 * 5–15 reps (40–60% 1-RM)	10 weeks (2/ week)	No	No	1-RM strength (squat) Jump performance (set angle jump, squat jump, depth jump)	↑ 1-RM strength for both groups, with greater increases in the high-load group. ↑ Peak force for both groups in 50% 1-RM squat jump and set angle jump only. ↑ Peak power for both groups in 30% 1-RM and 50% 1-RM squat jump only. ↑ Peak velocity for both groups in depth jump only. No between-group differences.

(Continued)

Table 1. (Continued).

Study	Participants	Age (mean $\pm$ SD)	RT intervention	Intervention duration (sessions/week)	Interventions equated for total volume	RT performed to volitional failure	Outcome measures	Key findings
Kalaotharakos et al. (2004)	Older untrained males ( $n=33$ )	64.9 $\pm$ 4.2 y	High-load: 3 * 8-RM Low-load: 3 * 15 reps (60% 1-RM) Control group (no exercise)#	12 weeks (3/ week)	No	No	1-RM strength (knee extension, knee flexion, elbow extension, elbow flexion, lat-pulldown, chest press)Isokinetic strength (knee extension/flexion)Muscle CSA (CT; mid-thigh)	$\uparrow$ 1-RM strength for both groups, with greater $\uparrow$ in the high-load group. $\uparrow$ Isokinetic strength for both groups at 60 and 180° s <sup>-1</sup> , with greater $\uparrow$ in the high-load group. $\uparrow$ Midthigh CSA for both groups, with greater $\uparrow$ in the high-load group. $\uparrow$ 1-RM strength for both groups, with no between-group differences.
Kerr et al. (1996)	Postmenopausal females ( $n=56$ )	57.1 $\pm$ 4.2 y	High load: 3 * 8-RM Low load: 3 * 20-RM	52 weeks (2/ week)	No	No	1-RM strength (wrist curl, reverse wrist curl, wrist pronation/supination, biceps curl, triceps pushdown, hip extension, hip flexion, hip abduction, hip adduction, leg press)	$\uparrow$ 1-RM strength for all groups, with greater $\uparrow$ in the high-load 1 (80% 1-RM) group. $\uparrow$ Elbow flexor and VL thickness for all groups, with greater $\uparrow$ in the high-load 1 (80% 1-RM) group. $\uparrow$ 1-RM strength for all groups, with greater $\uparrow$ in the high-load groups. $\uparrow$ QF muscle CSA for both high loads groups and low-load to failure, with no-between group differences. In outcome measures for low load not to failure.
Lasevicius et al. (2018)	Younger untrained males ( $n=30$ )	24.5 $\pm$ 2.4 y	High-load 1: 3 sets at 80% 1-RM High-load 2: 3 sets at 60% 1-RM Low-load 1: 3 sets at 20% 1-RM Low-load 2: 3 sets at 40% 1-RM	12 weeks (2/ week)	Yes	Yes	1-RM strength (leg press, elbow flexion) Muscle thickness (ultrasound; elbow flexors, VL)	$\uparrow$ 1-RM strength for all groups, with greater $\uparrow$ in the high-load groups. $\uparrow$ QF muscle CSA for both high loads groups and low-load to failure, with no-between group differences.
Lasevicius et al. (2019)	Younger untrained males ( $n=25$ )	19–34 y (overall mean $\pm$ SD not provided)	High-load 1: 3 sets at 80% 1-RM (2 min) Low-load 1: 3 sets at 30% 1-RM (2 min)High-load 2: 3+ sets at 80% 1-RM (2 min)Low-load 2: 3+ sets at 30% 1-RM (2 min)#	8 weeks (2/ week)	Yes	Yes YesNoNo	1-RM strength (knee extension) Muscle CSA (MRI; QF)	$\uparrow$ 1-RM strength for either group. $\uparrow$ Isokinetic strength for low-load 2 at 240° s <sup>-1</sup> , observed in the other groups. $\uparrow$ Type I muscle fibre CSA for high-load and low-load 2, with observed in low-load 1. $\uparrow$ Type II muscle fibre CSA were found in all groups. No between-group differences. $\uparrow$ MVIC and 1-RM strength for all groups, with greater $\uparrow$ in 1-RM strength in the high load groups. $\uparrow$ QF CSA and VL fCSA for all groups, with no between-group differences.
Lim et al. (2019)	Younger untrained males ( $n=21$ )	Mean ages 23–24 y per group (overall mean $\pm$ SD not provided)	High-load: 3 sets at 80% 1-RM Low-load 1: 3 sets at 30% 1-RM (volume-matched to high-load) Low-load 2: 3 sets at 30% 1-RM	10 weeks (3/ week)	No	Yes	1-RM strength (leg extension)Isokinetic strength (knee extension)Muscle fCSA (biopsy; VL)	$\uparrow$ 1-RM strength for both groups, with greater $\uparrow$ in the high-load groups. $\uparrow$ Type I muscle fibre CSA for high-load and low-load 2, with observed in low-load 1. $\uparrow$ Type II muscle fibre CSA were found in all groups. No between-group differences. $\uparrow$ MVIC and 1-RM strength for all groups, with greater $\uparrow$ in 1-RM strength in the high load groups. $\uparrow$ QF CSA and VL fCSA for all groups, with no between-group differences.
Mitchell et al. (2012)	Younger untrained males ( $n=18$ )	21 $\pm$ 0.8 y	High-load 1: 3 sets at 80% 1-RM High-load 2: 1 set at 80% 1-RM#Low-load: 3 sets at 30% 1-RM to the point of fatigue	10 weeks (3/ week)	No	NoYesNo	1-RM strength (knee extension)MVIC strength (knee extensors)Muscle CSA (MRI; QF) Muscle fCSA (biopsy; VL)	$\uparrow$ 1-RM strength for both groups, with no between-group differences. $\uparrow$ LBM and VL fCSA for both groups, with no between-group differences. $\uparrow$ 1-RM strength for all groups, with a greater $\uparrow$ in the high-load group. $\uparrow$ Elbow flexor CSA for the low-load (2) group only.
Morton et al. (2016)	Younger trained males ( $n=49$ )	23 $\pm$ 1 y	High-load: 3 * 8–12 reps (75–90% 1-RM) (1 min) Low-load: 3 * 20–25 reps (30–50% 1-RM) (1 min)	12 weeks (4/ week)	No	Yes	1-RM strength (bench press, leg press, shoulder press, knee extension) Lean body mass (DXA) Muscle fCSA (biopsy; VL)	$\uparrow$ 1-RM strength for all groups, with a greater $\uparrow$ in the high-load group. $\uparrow$ Elbow flexor CSA for the low-load (2) group only.
Moss et al. (1997)	Younger trained males ( $n=30$ )	23.2 $\pm$ 3.2 y	High-load: 3–5 * 2 at 90% 1-RM Low-load 1: 3–5 * 10 at 15% 1-RM Low-load 2: 3–5 * 7 at 35% 1-RM	9 weeks (3/ week)	No	No	1-RM strength (elbow flexion) Muscle CSA (CT; elbow flexors)	$\uparrow$ 1-RM strength for both groups, with no between-group differences. $\uparrow$ LBM and VL fCSA for both groups, with no between-group differences. $\uparrow$ 1-RM strength for all groups, with a greater $\uparrow$ in the high-load group. $\uparrow$ Elbow flexor CSA for the low-load (2) group only.

(Continued)

Table 1. (Continued).

Study	Participants	Age (mean ± SD)	RT intervention	Intervention duration (sessions/week)	Interventions equated for total volume	RT performed to volitional failure	Outcome measures	Key findings
Nobrega et al. (2018)	Younger untrained males (n=27)	23 ± 3.6 y	High-load 1: 3 sets at 80% 1-RM (2 min) High-load 2: 3 sets at 80% 1-RM (2 min) Low-load 1: 3 sets at 30% 1-RM (2 min) Low-load 2: 3 sets at 30% 1-RM (2 min)	12 weeks (2/week)	No	Yes/No	1-RM strength (knee extension) Muscle CSA (ultrasound; VL)	↑ 1-RM strength for all groups, with no between-group differences. ↑ VL muscle CSA for all groups, with no between-group differences.
Ogasawara et al. (2013)	Younger untrained males (n=9)	25 ± 3 y	High-load: 3 * 75% 1-RM (3 min) Low-load: 4 * 30% 1-RM (3 min)	6 weeks (3/week)	No	No	1-RM strength (bench press) MVIC strength (elbow extensors) Muscle CSA (MRI; PM and TB)	↑ 1-RM and MVIC strength for both groups, with greater ↑ in the high-load group. ↑ TB and PM CSA for both groups, with no between-group differences.
Popov et al. (2006)	Younger untrained males (n=18)	21 ± 2 y	High-load: 3 and 7 * 80% MVC (10 min) Low-load: 3 and 4 * 50% MVC (10 min)	8 weeks (3/week)	No	No	MVIC strength (knee extensors) Muscle CSA (MRI; QF and GM)	↑ MVIC strength for both groups, with no between-group differences. ↑ QF and GM CSA for both groups, with no between-group differences.
Rana et al. (2008)	Younger untrained females (n=26)	21.1 ± 2.7 y	High load 1 (TS): 3 * 6-10 RM (80-85% 1-RM) High load 2 (SS): 3 * 6-10 RM (80-85% 1-RM) at intentionally slow velocity# Low load (TE): 3 * 20-30 RM (40-60% 1-RM)	6 weeks (2-3/week)	No	Yes	1-RM strength (squat, leg press, knee extension) Lean body mass (BodyPod) Vertical jump height	↑ 1-RM strength for all groups. Greater ↑ in 1-RM leg press and knee extension strength in the high-load, normal velocity group vs. other groups. ↑ LBM for all groups, with no between-group differences. ↑ in vertical jump height.
Ribeiro et al. (2020)	Older untrained females (n=27)	71.5 ± 5.3 y	Control group (no exercise)# High-load: 3 * 10-RM Low-load: 3 * 15-RM	8 weeks (3/week)	No	Yes	1-RM strength (chest press, knee extension, preacher curl) Fat-free mass (DXA)	↑ 1-RM strength and fat-free mass for both groups, with no between-group differences.
Richardson et al. (2019)	Older untrained males and females (n=40)	66.5 ± 5.3 y	High-load (once-weekly): 3 * 7 (80% 1-RM) High-load 2 (twice-weekly): 3 * 7 (80% 1-RM) Low-load 1 (once-weekly): 3 * 14 (40% 1-RM) Low-load 2 (twice-weekly): 3 * 14 (40% 1-RM)	10 weeks (1-2/week)	No	Yes	1-RM strength (leg press, calf raise, leg extension, leg curl, seated row, chest press, tricep extension, bicep curl) Lean body mass (bioelectrical impedance analysis)	↑ 1-RM strength for all groups, with greater ↑ in the high-load 2 group. ↑ in LBM for any of the groups.
Schoenfeld et al. (2015)	Younger untrained males (n=24)	23.3 (range 18-33) y	Control (usual activities)# High-load: 3 * 8-12-RM Low-load: 3 * 25-35-RM	8 weeks (3/week)	No	Yes	1-RM strength (squat and bench press) Muscle thickness (ultrasound; elbow flexors and extensors, QF)	↑ 1-RM bench press strength for the high-load group only. ↑ 1-RM squat strength for both groups, with a greater ↑ in the high-load group. ↑ Upper arm and QF thickness for both groups, with no between-group differences.
Schoenfeld et al. (2020)	Younger untrained males (n=26)	22.5 y (SD not provided)	High-load: 4 * 6-10-RM Low-load: 4 * 20-30-RM	8 weeks (2/week)	No	Yes	MVIC strength (plantar flexors) Muscle thickness (ultrasound; medial and lateral gastrocnemius, soleus)	↑ Isometric strength and muscle thickness for both groups, with no between-group differences.

(Continued)



Table 1. (Continued).

Study	Participants	Age (mean $\pm$ SD)	RT intervention	Intervention duration (sessions/week)	Interventions equated for total volume	RT performed to volitional failure	Outcome measures	Key findings
Schuenke et al. (2012)	Younger untrained females (n = 34)	21.1 $\pm$ 2.7 y	High load 1 (TS): 3 * 6–10 RM (80–85% 1-RM) High load 2 (SS): 3 * 6–10 RM (80–85% 1-RM) at intentionally slow velocity# Low load (TE): 3 * 20–30 RM (40–60% 1-RM) Control group (no exercise)# High-load: 3 * 8 reps (80% 1-RM) Low-load: 3 * 8 reps (40% 1-RM) Control (placebo) group: 3 * 8 reps (unloaded)#	6 weeks (2–3/week)	No	Yes	Muscle fCSA (biopsy; VL) Fat-free mass (skinfolds)	$\uparrow$ Mean fCSA only for the TS group, with no between-group differences; in fat-free mass for neither group.
Seynnes et al. (2004)	Older untrained males (n = 14)	82 $\pm$ 2.6 y	Control group (no exercise)# High-load: 3 * 8 reps (80% 1-RM) Low-load: 3 * 8 reps (40% 1-RM) Control (placebo) group: 3 * 8 reps (unloaded)#	10 weeks (3/week)	Yes	No	1-RM strength (knee extension)	$\uparrow$ 1-RM strength for both groups, with a greater $\uparrow$ in the high-load group.
Stefanaki et al. (2019)	Younger females not engaging in more than 2 hours of RT/wk (n = 13)	29.7 $\pm$ 4.7 y	High-load: 1 * 80% of 1-RM Low-load: 1 * 30% 1-RM	6 weeks (2/week)	No	Yes	1-RM strength (knee extension and elbow flexion) Muscle thickness (ultrasound; VL and BB)	$\uparrow$ 1-RM strength for all groups, with no between-group differences. $\uparrow$ BB and VL muscle thickness for all groups, with no between-group differences.
Stone & Coulter (1994)	Younger untrained females (n = 50)	23.1 $\pm$ 3.5 y	High-load (3 * 6–8-RM)	9 weeks (3/week)	No	Unclear	1-RM strength (squat, bench press)	$\uparrow$ 1-RM strength for all groups, with no between-group differences.
Taaffe et al. (1996)	Older untrained females (n = 25)	67.3 $\pm$ 0.4 y	Low-load 1 (3 * 15–20-RM) Low-load 2 (3 * 30–40-RM) High-load: 3 * 7 reps (80% 1-RM) Low-load: 3 * 14 reps (40% 1-RM) Control (no exercise)#	52 weeks (3/week)	No	No	1-RM strength (leg press, knee extension, knee flexion) Muscle fCSA (biopsy; VL) Lean body mass (DXA)	$\uparrow$ 1-RM strength for both groups, with no between-group differences. $\uparrow$ Type I fCSA for both groups but in type II fCSA, with no between-group differences. in thigh LBM in both groups. $\uparrow$ 1-RM strength for all groups, with no between-group differences.
Tanimoto & Ishii (2006)	Younger untrained males (n = 24)	19.4 $\pm$ 0.6 y	High-load: 3 * 80% 1-RM (normal tempo) Low-load 1: 3 * 50% 1-RM (slow tempo)# Low-load 2: 3 * 50% 1-RM (normal tempo)	12 sessions (3/week)	No	Yes	1-RM (knee extension) MVIC strength (knee extensors) isokinetic strength (knee extensors) Muscle CSA (MRI; QF)	$\uparrow$ MVIC strength for the high-load group only. in isokinetic strength at 90, 200 and 300° s <sup>-1</sup> in low-load group at 90 and 200° s <sup>-1</sup> but not at 300° s <sup>-1</sup> . No between-group differences. $\uparrow$ QF CSA for the high-load group only.
Tanimoto et al. (2008)	Younger untrained males (n = 24)	19.3 $\pm$ 0.6 y	High-load: 3 * 80–90% 1-RM (normal tempo) Low-load: 3 * 55–60% 1-RM (slow tempo) Control group (no exercise)#	13 weeks (2/week)	No	Yes	1-RM strength (squat, chest press, lat pull-down, ab bend, back extension, knee extension) Muscle thickness (ultrasound; chest, anterior and posterior upper arm, abdomen, subscapula, anterior and posterior thigh) Lean body mass (DXA)	$\uparrow$ 1-RM strength for both groups, with no between-group differences. $\uparrow$ Chest, upper arm, abdomen, subscapula and thigh thickness for both groups, with no between-group differences. $\uparrow$ LBM for both groups, with no between-group differences.

(Continued)

Table 1. (Continued).

Study	Participants	Age (mean ± SD)	RT intervention	Intervention duration (sessions/week)	Interventions equated for total volume	RT performed to volitional failure	Outcome measures	Key findings
Van Roie, Baumann et al. (2013)	Younger untrained males and females (n= 36)	21.8 ± 2.1 y	High-load: 1 * 8–12 reps (80% 1RM) Low-load 1: 60 repetitions at 20–25% 1-RM, followed by 1 * 10–12 reps (40% 1-RM) Low-load 2: 1 * 10–12 reps (40% 1-RM) ‡	9 weeks (3/ week)	No	Yes	1-RM strength (knee extension) MVIC strength (knee extension) isokinetic strength (knee extension)	↑ 1-RM strength for both groups, with a greater ↑ in the high-load group. ↑ MVIC strength for the high-load group only. ↑ Isokinetic strength for the low-load group only.
Van Roie, Delecluse et al. (2013)	Older untrained males and females (n= 56)	67.9 ± 5.1 y	High-load: 2 * 10–15 reps (80% 1-RM) Low-load 1: 1 * 80–100 reps (20% 1-RM) Low-load 2: 1 * 60 reps (20% 1-RM) + 1 x 10–20 reps (40% 1-RM)	12 weeks (3/ week)	No	Yes	1-RM strength (leg press, knee extension) MVIC strength (knee extension) isokinetic strength (knee extension) Muscle CSA (CT; thigh)	Greater ↑ in 1-RM strength in the high-load and low-load (2) group vs. low-load (1) group. ↑ MVIC strength for all groups, with no between-group differences. ↑ Isokinetic strength for the high-load group only. ↑ High muscle CSA for all groups, with no between-group differences. ↑ LBM for the high-load group only.
Vargas et al. (2019)	Younger trained males (n= 20)	27.6 ± 6.7 y	High-load: 3 * 6–8 RM Low-load: 3 * 20–25 RM Control group (continue with usual exercise habits) ‡	8 weeks (4/ week)	No	Yes	Lean body mass (DXA)	
Wallerstein et al. (2012)	Older untrained males (n= 30)	64.3 ± 4 y	High-load: 2–4 * 4–10 reps (70–90% 1-RM) Low-load: 2–4 * 4–7 reps (30–50% 1-RM) Control group (no exercise) ‡	16 weeks (2/ week)	Yes	No	1-RM strength (chest press, leg press) Muscle CSA (MRI, QF)	↑ 1-RM strength for both groups, with no between-group differences. ↑ QF CSA for both groups, with no between-group differences.

**Abbreviations:** 1-RM, one-repetition maximum; BB, barbell; BB, biceps brachii; CSA, cross-sectional area; CT, computerized tomography; DXA, dual x-ray absorptiometry; fCSA, fibre cross-sectional area; GM, gluteus maximus; LBM, lean body mass; MRI, magnetic resonance imaging; MVIC, maximum voluntary isometric contraction; PM, pectoralis major; QF, quadriceps femoris; RF, rectus femoris; Reps, repetitions; RM, repetition maximum; TB, triceps brachii; VL, vastus lateralis; VM, vastus medialis; ‡ = group excluded from the meta-analysis; † = increased; ‡ = decreased; = ↔ no change or difference.

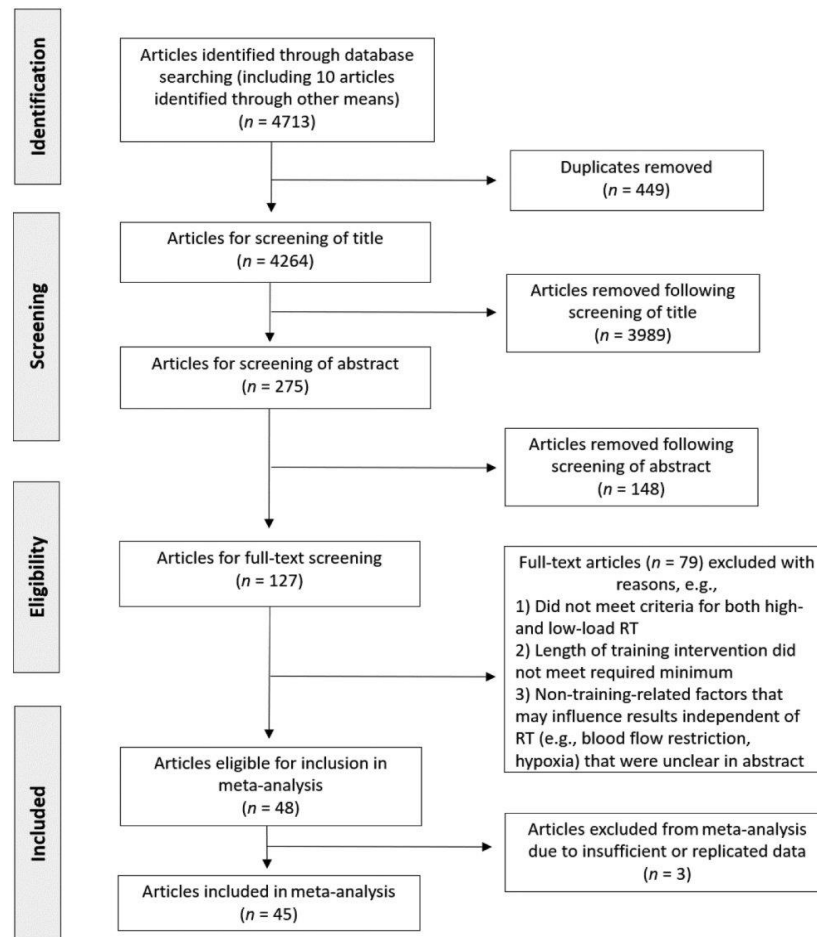


Figure 1. PRISMA flow chart of systematic literature search and article selection process.

activity monitoring in control groups; 11, relative exercise intensity remained constant; 12, exercise parameters recorded. As items 5 and 6 each have three sub-criteria (with two of the sub-criteria for item 5 scored as yes/no and therefore not scored numerically), and item 8 has two sub-criteria, the best possible total score is 15 points.

### 2.5. Calculation of effect size and statistical analysis

Within the studies, the average value of the means and average standard deviation for each outcome measure at both pre- and post-intervention was calculated for both high-load and low-load groups. For the analysis of muscle hypertrophy outcomes, studies that assessed changes in whole-muscle size (i.e., muscle thickness, muscle cross-sectional area, or muscle volume using ultrasound, MRI, or CT) were combined, while studies assessing muscle fCSA (via muscle biopsy) or lean body/fat-free mass (via DXA, BIA, or BodPod) were analysed separately. The average standard deviation was calculated using the formula proposed in the Cochrane Handbook for Systematic Reviews (Higgins &

Deeks, 2008). After calculating the average mean and the average standard deviation pre- and post-intervention for each study, we determined the mean change (post minus pre) and the standard deviation change (Higgins et al., 2008) for the high-load and low-load groups. These values were used in RevMan5 (Review Manager (RevMan), V.5.4; Cochrane Collaboration) with a Random-Effects model to calculate the standardized mean difference (SMD) between treatments (high-load versus low-load). Effect size (ES) values were interpreted according to Cohen (1988), whereby values of 0.2 to 0.49 indicate small, 0.50 to 0.79 indicate medium, and  $\geq 0.80$  indicate large, effects. Heterogeneity was assessed using the  $I^2$  statistic and/or the standard deviation (SD) derived from the study-estimate random effect (represented as  $\tau^2$ ).

## 3. Results

### 3.1. Search results

Three of the 48 studies eligible for inclusion after full-text screening were excluded as either the raw pre/post-



Table 2. Methodological quality for each included study assessed using the (TESTEX) scale.

Study	TESTEX scale item															Total score		
	1	2	3	4	5a	5b	5 c	6a	6b	6 c	7	8a	8b	9	10		11	12
Aagaard et al. (1994)	1	0	0	1	No	No	0	0	0	0	0	0	0	1	0	1	1	5
Anderson & Kearney (1982)	1	1	0	1	No	No	0	0	0	0	0	1	1	1	0	1	1	8
Au et al. (2017)	1	0	0	1	No	No	0	1	0	1	0	1	1	1	0	1	1	9
Beneka et al. (2005)	1	0	0	1	No	No	0	0	0	0	0	1	1	1	0	0	1	6
Bezarra et al. (2019)	1	1	0	1	No	No	0	1	1	1	0	1	1	1	0	1	1	11
Campos et al. (2002)	1	0	0	1	No	No	0	0	0	0	0	1	1	1	0	1	1	7
De Vos et al. (2005)	1	0	0	1	No	No	0	1	1	1	0	1	1	1	0	1	1	10
Fatouros et al. (2006)	1	0	0	1	No	No	0	1	0	1	0	1	1	1	0	1	1	9
Fink et al. (2016)	1	1	0	1	No	No	0	0	0	0	0	1	0	1	0	1	1	7
Franco et al. (2019)	1	1	0	1	No	No	0	0	0	0	0	1	1	1	0	1	1	8
Harris et al. (2004)	1	1	1	1	No	No	0	1	1	1	0	1	0	1	0	1	1	11
Hisaeda et al. (1996)	1	1	0	1	No	No	0	0	0	0	0	1	1	1	0	1	1	8
Holm et al. (2008)	1	1	0	1	No	No	0	1	0	1	0	1	1	1	0	1	1	10
Hortobagyi et al. (2001)	1	1	0	1	No	No	0	1	1	1	0	1	1	1	0	1	1	11
Ikezoe et al. (2020)	1	0	0	1	No	No	0	0	1	0	0	1	1	1	0	1	1	8
Jenkins et al. (2017)	1	1	0	0	No	No	0	1	1	1	0	1	1	1	0	1	1	10
Jessee et al. (2018)	1	1	0	0	No	No	0	1	1	1	0	1	1	0	0	0	1	8
Jones et al. (2001)	0	1	0	1	No	No	0	0	0	0	0	1	1	1	0	0	1	6
Kalapotharakos et al. (2004)	1	1	1	1	No	No	0	1	0	1	0	1	1	1	0	0	1	10
Kerr et al. (1996)	1	1	0	1	No	No	0	0	1	1	0	1	1	1	0	1	1	10
Lasevicius et al. (2018)	0	1	0	1	No	No	0	0	0	0	0	1	1	1	0	1	1	7
Lasevicius et al. (2019)	1	1	0	1	No	No	0	0	0	0	0	1	1	1	0	1	1	8
Lim et al. (2019)	1	1	0	1	No	No	0	0	0	0	0	1	1	1	0	1	1	8
Mitchell et al. (2012)	1	1	0	1	No	No	0	0	0	0	0	0	1	1	0	0	0	5
Morton et al. (2016)	1	1	0	1	No	No	0	1	1	1	0	1	1	1	0	1	1	11
Moss et al. (1997)	0	1	1	1	No	No	0	0	0	0	0	1	1	1	0	1	1	8
Nobrega et al. (2018)	1	1	1	1	No	No	0	1	0	1	0	1	1	1	0	0	1	10
Ogasawara et al. (2013)	1	0	0	1	No	No	0	1	0	1	0	1	1	1	0	1	1	9
Popov et al. (2006)	0	0	0	1	No	No	0	0	0	0	0	1	1	1	0	0	0	4
Rana et al. (2008)	1	0	0	1	No	No	0	1	0	0	0	1	1	1	0	0	0	6
Riberio et al. (2020)	1	1	0	1	No	No	1	1	0	1	0	0	0	1	0	1	1	9
Richardson et al. (2019)	1	1	0	1	No	No	0	0	1	0	0	1	1	1	0	1	1	9
Schoenfeld et al. (2015)	1	1	0	1	No	No	0	1	1	1	0	1	1	1	0	1	1	11
Schoenfeld et al. (2020)	1	1	0	1	No	No	1	1	1	1	0	1	1	1	0	1	1	12
Schuenke et al. (2012)	1	1	0	1	No	No	0	0	0	0	0	1	1	1	0	1	1	8
Seynnes et al. (2004)	1	1	1	1	No	No	0	1	1	1	0	1	1	1	0	1	1	12
Stefanaki et al. (2019)	1	1	0	1	No	No	0	1	0	1	0	1	1	1	0	0	1	9
Stone & Coulter (1994)	0	0	0	1	No	No	0	1	0	1	0	1	1	1	0	1	1	8
Taaffe et al. (1996)	1	1	0	1	No	No	0	0	0	1	0	1	1	1	0	1	1	9
Tanimoto & Ishii (2006)	1	1	0	1	No	No	0	0	0	0	0	1	1	1	0	1	1	8
Tanimoto et al. (2008)	1	1	0	1	No	No	0	0	0	0	0	1	1	1	0	1	1	8
Van Roie et al. (2013a)	1	1	0	1	No	No	0	0	0	0	0	1	1	1	0	1	1	8
Van Roie et al. (2013b)	1	1	0	1	No	No	0	1	0	1	0	1	1	1	0	1	1	10
Vargas et al. (2019)	1	1	0	1	No	No	0	0	0	0	0	1	1	1	0	1	1	8
Wallerstein et al. (2012)	1	1	0	1	No	No	0	0	0	0	0	1	1	1	0	1	1	8

intervention data could not be extracted/could not be provided by the authors (Fink et al., 2018; Fisher & Steele, 2017) or included data previously published in another included study (Kalapotharakos et al., 2005). Additionally, one study (Jessee et al., 2018) was excluded from the analyses of whole-muscle size, isometric (MVIC) strength, and isokinetic strength, and one study (Taaffe et al., 1996) was excluded from the analyses of lean body mass and muscle fCSA analysis (Taaffe et al., 1996), as the raw pre/post-intervention data could not be extracted and could not be provided by the authors for these measures. Thus, 45 studies were included in the meta-analysis. Only two studies (Jones et al., 2001; Rana et al., 2008) included measures of sport-specific or neuromuscular task performance, and the findings of these studies are therefore summarized qualitatively.

### 3.2. Methodological quality assessment

The methodological quality of included studies was assessed using the TESTEX scale (Smart et al., 2015). Study

quality scores ranged from 4 to 12 (out of a possible 15), with mean and median scores of 9 and 8, respectively (Table 2). Based on the range of study quality scores, we defined low, medium and high-quality scores as between 4 and 7 ( $n = 9$ ), between 8 and 10 ( $n = 29$ ), and  $\geq 11$  ( $n = 7$ ), respectively, which ensured an approximately even distribution of studies across subgroups.

We then performed subgroup analyses to examine whether study quality may have contributed to the heterogeneity observed for the 1-RM, isometric, and isokinetic strength analyses. For the 1-RM strength analysis, no heterogeneity ( $I^2 = 0\%$ ) was observed in the high-quality group ( $n = 6$ ), while both the moderate- ( $I^2 = 64\%$ ) and low-quality ( $I^2 = 43\%$ ) subgroups showed high degrees of heterogeneity (Supplementary Figure 1). There was, however, no difference in outcomes between methodological quality subgroups ( $P = 0.36$ ).

Study quality appeared to influence heterogeneity in the isometric strength analysis (Supplementary Figure 2), with an inverse relationship between study quality and heterogeneity ( $I^2$  values of 0%, 31%, and 68% for high-, moderate-, and low-

quality studies, respectively). There was, however, no difference in outcomes between methodological quality subgroups ( $P = 0.14$ ). Study quality did not explain heterogeneity in the isokinetic strength meta-analysis (Supplementary Figure 3), with  $I^2$  values of 0% and 47% for moderate- and low-quality studies, respectively, while only a single high-quality study was included. There was no difference in outcomes between methodological quality subgroups ( $P = 0.96$ ). While low heterogeneity ( $I^2 = 0\text{--}10\%$ ) was observed in the analyses for whole-muscle size, muscle fibre CSA, and lean body/fat-free mass, subgroup analyses (Supplementary Figures 4–6) nevertheless confirmed there was no influence of study quality on heterogeneity or outcomes.

### 3.3. Meta-analysis results

#### 3.3.1. Dynamic 1-RM strength

A total of 36 studies measured dynamic 1-RM strength in one or more of the following exercises: bench press, chest press, overhead press, seated row, lat pulldown, forearm flexion, elbow extension, elbow flexion, leg press, squat, knee extension, knee flexion, back extension, and abdominal bend. Twenty (Anderson & Kearney, 1982; Au et al., 2017; Campos et al., 2002; Fatouros et al., 2006; Holm et al., 2008; Jenkins et al., 2017; Jessee et al., 2018; Jones et al., 2001; Kalapotharakos et al., 2004; Lasevicius et al., 2019, 2018; Mitchell et al., 2012; Moss et al., 1997; Ogasawara et al., 2013; Rana et al., 2008; Richardson et al., 2019; Schoenfeld et al., 2015; Seynnes et al., 2004; Van Roie, Bautmans et al., 2013; De Vos et al., 2005) out of the 36 studies found greater improvements in 1-RM strength with high-load compared to low-load RT, while equivalent improvements between both loading conditions were noted in 15 studies (Franco et al., 2019; Harris et al., 2004; Hortobágyi et al., 2001; Ikezoe et al., 2020; Kerr et al., 1996; Morton et al., 2016; Nobrega et al., 2018; Ribeiro et al., 2020; Stefanaki et al., 2019; Stone & Coulter, 1994; Taaffe et al., 1996; Tanimoto & Ishii, 2006; Tanimoto et al., 2008; Van Roie, Delecluse et al., 2013; Wallerstein et al., 2012). One study (Bezerra et al., 2019) measured dynamic 5-RM strength (for the seated row exercise) and found equivalent improvements between high-load and low-load RT.

Meta-analytic outcomes for dynamic 1-RM strength are shown in Figure 2 and included 537 and 650 ES values from 36 studies for high-load and low-load RT, respectively. There was an advantage for high-load RT versus low-load RT on dynamic 1-RM strength (ES = 0.34, 95% CI: 0.15 to 0.52;  $P = 0.0003$ ). Moderate heterogeneity amongst studies was observed ( $\text{Tau}^2 = 0.17$ ,  $I^2 = 55\%$ ,  $P = 0.0001$ ).

Sub-group analyses for dynamic 1-RM strength outcomes revealed an advantage for high-load versus low-load RT in untrained (ES = 0.37, 95% CI: 0.15 to 0.59;  $P = 0.0009$ ) but not trained (ES = 0.21, 95% CI:  $-0.14$  to 0.55;  $P = 0.24$ ) participants (Figure 2). There was also a larger advantage for high-load versus low-load RT in younger (ES = 0.41, 95% CI: 0.14 to 0.68;  $P = 0.003$ ) versus older (ES = 0.20, 95% CI: 0.00 to 0.40;  $P = 0.05$ ) participants (Supplementary Figure 7). However, there were no statistically significant differences between dynamic 1-RM strength

outcomes for studies in untrained versus trained participants ( $P = 0.59$ ) or in younger versus older participants ( $P = 0.23$ ).

#### 3.3.2. Isometric [maximum voluntary isometric contraction (MVIC)] strength

A total of 15 studies measured isometric (MVIC) strength, with eight of these studies (Aagaard et al., 1994; Fink et al., 2016; Holm et al., 2008; Jenkins et al., 2017; Mitchell et al., 2012; Ogasawara et al., 2013; Tanimoto & Ishii, 2006; Van Roie, Bautmans et al., 2013) showing an advantage of high-load RT, and no studies suggesting an advantage of low-load RT. The remaining seven studies (Hisaeda et al., 1996; Hortobágyi et al., 2001; Ikezoe et al., 2020; Jessee et al., 2018; Popov et al., 2006; Schoenfeld et al., 2020; Van Roie, Delecluse et al., 2013) found equivalent improvements between loading conditions.

Meta-analytic outcomes for isometric (MVIC) strength are shown in Figure 3 and included 136 and 166 ES values from 14 studies for high-load and low-load RT, respectively. Overall there was an advantage for high-load RT versus low-load RT on isometric (MVIC) strength (ES = 0.41, 95% CI: 0.07 to 0.76;  $P = 0.02$ ). Moderate heterogeneity amongst studies was observed ( $\text{Tau}^2 = 0.20$ ,  $I^2 = 49\%$ ,  $P = 0.02$ ).

Sub-group analyses (Figure 3) showed an advantage for high-load RT versus low-load RT on isometric (MVIC) strength in younger participants (ES = 0.53, 95% CI: 0.13 to 0.92;  $P = 0.009$ ), while only two studies used older participants.

There was also an advantage for high-load RT versus low-load RT on isometric (MVIC) strength in untrained participants (ES = 0.42, 95% CI: 0.04 to 0.80;  $P = 0.03$ ), but not for participants whose training status was unclear (ES = 0.40, 95% CI:  $-0.78$  to 1.58;  $P = 0.51$ ; Supplementary Figure 8). No included studies measured isometric (MVIC) strength in trained participants.

However, there were no statistically significant differences in isometric (MVIC) strength outcomes for untrained versus trained participants ( $P = 0.97$ ) or younger versus older participants ( $P = 0.06$ ).

#### 3.3.3. Isokinetic strength

A total of 11 studies investigated the effects of high-load and low-load RT on isokinetic strength and showed inconsistent results. Five (Aagaard et al., 1994; Beneka et al., 2005; Holm et al., 2008; Kalapotharakos et al., 2004; Van Roie, Delecluse et al., 2013) of the 11 studies demonstrated greater increases in isokinetic strength with high-load compared to low-load RT, four studies (Hisaeda et al., 1996; Hortobágyi et al., 2001; Jessee et al., 2018; Tanimoto & Ishii, 2006) found equivalent increases for both loading conditions, and two studies (Lim et al., 2019; Van Roie, Bautmans et al., 2013) showed an advantage to low-load RT.

Meta-analytic outcomes for isokinetic strength are shown in Figure 4 and included 121 and 143 ES values from 10 studies for high-load and low-load RT, respectively. Overall there was no difference between high-load and low-load RT for isokinetic strength (ES = 0.19, 95% CI:  $-0.10$  to 0.49;  $P = 0.20$ ). Low heterogeneity between studies was observed ( $\text{Tau}^2 = 0.05$ ,  $I^2 = 23\%$ ,  $P = 0.24$ ).

Sub-group analyses (Figure 4) revealed no difference between high-load RT versus low-load RT in younger



participants (ES = 0.25, 95% CI: -0.34 to 0.83;  $P = 0.41$ ) or older participants (ES = 0.16, 95% CI: -0.18 to 0.50;  $P = 0.35$ ).

There was also no difference between high-load RT versus low-load RT on isokinetic strength on isokinetic strength in untrained participants (ES = 0.19, 95% CI: -0.17 to 0.56;  $P = 0.29$ ), while the training status of participants was unclear in one study (Aagaard et al., 1994) (Supplementary Figure 9). No included studies that measured isokinetic strength used trained participants.

There were no statistically significant differences between isokinetic strength outcomes for untrained versus trained ( $P = 0.93$ ) or younger versus older participants ( $P = 0.81$ ).

### 3.3.4. Whole-muscle size

A total of eight studies measured muscle thickness via ultrasound at multiple measurement sites including the upper thigh, lower arm, upper arm and chest. Seven (Ikezoe et al., 2020; Jenkins et al., 2017; Jessee et al., 2018; Schoenfeld et al., 2015, 2020; Stefanaki et al., 2019; Tanimoto et al., 2008) of the eight studies identified equivalent increases in muscle thickness between high-load and low-load RT, and one study (Lasevicius et al., 2018) found greater improvements for high-load RT.

Similar findings to studies measuring muscle thickness were noted in the 13 studies that measured whole-muscle CSA via magnetic resonance imaging (MRI) (Fink et al., 2016; Hisaeda et al., 1996; Holm et al., 2008; Lasevicius et al., 2019; Mitchell et al., 2012; Ogasawara et al., 2013; Popov et al., 2006; Tanimoto & Ishii, 2006; Wallerstein et al., 2012), computerized tomography (CT) scan (Kalapotharakos et al., 2004; Moss et al., 1997; Van Roie, Delecluse et al., 2013), or ultrasound (Nobrega et al., 2018). This is perhaps not surprising, as muscle thickness (measured by ultrasound) correlates well with muscle CSA as measured by CT or MRI (Franchi et al., 2018). Of the 13 studies, nine (Fink et al., 2016; Hisaeda et al., 1996; Lasevicius et al., 2019; Mitchell et al., 2012; Nobrega et al., 2018; Ogasawara et al., 2013; Popov et al., 2006; Van Roie, Delecluse et al., 2013; Wallerstein et al., 2012) identified a similar increase in whole-muscle CSA between high-load and low-load RT groups, three (Holm et al., 2008; Kalapotharakos et al., 2004; Tanimoto & Ishii, 2006) demonstrated an advantage to high-load RT, and only one (Moss et al., 1997) found greater improvements in the low-load condition.

Meta-analytic outcomes for whole-muscle size are shown in Figure 5 and included 229 and 304 ES values from 20 studies for high-load and low-load RT, respectively. Overall there was no difference between high-load and low-load RT for changes in whole-muscle size (ES = 0.06, 95% CI: -0.11 to 0.24,  $P = 0.47$ ). Low heterogeneity amongst studies was observed ( $\text{Tau}^2 = 0$ ,  $I^2 = 0\%$ ,  $P = 1.00$ ).

### 3.3.5. Muscle fibre cross-sectional area (fCSA)

A total of six studies measured muscle fCSA via muscle biopsy. Four of the six studies (Lim et al., 2019; Mitchell et al., 2012; Morton et al., 2016; Taaffe et al., 1996) demonstrated equivalent improvements in muscle fCSA amongst both loading

conditions and two studies (Campos et al., 2002; Schuenke et al., 2012) revealed greater improvements for high-load RT.

Meta-analytic outcomes for muscle fibre cross-sectional area (fCSA) are shown in Figure 6 and included 73 and 65 ES values from five studies for high-load and low-load RT, respectively. There was no difference between high-load and low-load RT on changes in muscle fCSA (ES = 0.29, 95% CI: -0.09 to 0.66,  $P = 0.13$ ). Low heterogeneity amongst studies was observed ( $\text{Tau}^2 = 0.02$ ,  $I^2 = 10\%$ ,  $P = 0.35$ ).

### 3.3.6. Lean body mass (LBM) or fat-free mass

A total of 10 studies used either DXA (Franco et al., 2019; Morton et al., 2016; Ribeiro et al., 2020; Taaffe et al., 1996; Tanimoto et al., 2008; Vargas et al., 2019), BodPod (Au et al., 2017; Rana et al., 2008), bioelectrical impedance analysis (Richardson et al., 2019) or skinfolds (Schuenke et al., 2012) to measure changes in lean body mass (LBM) or fat-free mass. Six (Au et al., 2017; Morton et al., 2016; Rana et al., 2008; Ribeiro et al., 2020; Schuenke et al., 2012; Tanimoto et al., 2008) of the 10 studies found no differences between loading conditions, one study (Vargas et al., 2019) demonstrated an advantage for high-load RT, while another (Franco et al., 2019) showed the opposite effect. Two studies (Richardson et al., 2019; Taaffe et al., 1996) found no change in LBM from pre- to post-training in both loading conditions.

Meta-analytic outcomes for LBM/fat-free mass are shown in Figure 7 and included 140 and 127 ES values from nine studies for high-load and low-load RT, respectively. There was no difference between high-load and low-load RT on changes in LBM or fat-free mass (ES = 0.05, 95% CI: -0.20 to 0.29,  $P = 0.70$ ). Low heterogeneity amongst studies was observed ( $\text{Tau}^2 = 0$ ,  $I^2 = 0\%$ ,  $P = 0.76$ ).

### 3.3.7. Sport-specific or neuromuscular task performance

Given the limited availability of data, it was not possible to conduct a meta-analysis evaluating the influence of training load on sport-specific or neuromuscular task performance. Of the two studies included that measured sport-specific or neuromuscular task performance (Jones et al., 2001; Rana et al., 2008), one study (Jones et al., 2001) used several jump tests (i.e., set angle jump, depth jump, weighted squat jump) and the other study (Rana et al., 2008) used a maximal jump height test. No change in jump height in response to high-load and low-load RT from pre- to post-training was observed in one study (Rana et al., 2008), whilst the other (Jones et al., 2001) found equivalent improvements in jump task performance between both loading conditions.

## 4. Discussion

The findings of this systematic review and meta-analysis provide further comprehensive evidence that higher- and lower-load RT are similarly effective for improving multiple indices of muscle hypertrophy (i.e., changes in whole-body lean/fat-free mass and in both whole-muscle and muscle fibre-specific CSA), and extend previous findings by demonstrating higher-load RT is advantageous for improving both dynamic 1-RM and isometric (MVIC) strength, but not for isokinetic strength. Due to



limited available evidence, it remains unclear whether the superiority of higher-load RT for dynamic 1-RM and isometric strength development translates to greater improvements in sport-specific and neuromuscular task performance.

#### 4.1. Influence of RT load on strength development

The superior improvements in dynamic (1-RM) and isometric strength, but not isokinetic strength, with higher-load RT are in partial agreement with previous findings (Lopez et al., 2020; Schoenfeld, Grgic et al., 2017). Both Schoenfeld and colleagues (2017) and Lopez et al. (2020) found superior improvements in dynamic 1-RM strength with higher- versus lower-load RT (with the latter also showing an advantage of moderate-load RT), while Schoenfeld, Grgic et al. (2017) found equivalent isometric strength gains between loading conditions. In the present study, sub-group analyses showed an advantage of higher-load RT for improving dynamic (1-RM) strength in untrained, but not in trained, participants (consistent with others (Lopez et al., 2020)), and a greater advantage for higher-load RT in younger versus older participants. While the present data therefore consolidate the superior influence of higher-load RT on dynamic 1-RM strength, it is possible a wider spectrum of RT loads may positively influence aspects of strength in older and/or resistance-trained individuals. The present findings of greater improvements in isometric strength with higher-load RT, and no clear influence of RT load for isokinetic strength development, provide novel insights from previous studies (Lopez et al., 2020; Schoenfeld et al., 2016, 2017). The present findings therefore provide a more comprehensive overview and advance the current understanding of the effects of RT load and moderating influence of participant characteristics on various strength outcomes.

The analysis of changes in multiple measures of strength with higher- versus lower-load RT allows insight into the potential mechanisms by which RT loading conditions may influence strength development. Similar to previous work (Schoenfeld, Grgic et al., 2017), we found variability in the magnitude of advantage of higher-load RT across different strength outcomes. Specifically, there was a similar advantage of higher-load RT for improving both dynamic 1-RM strength and isometric strength ( $ES = 0.34$  and  $0.41$ , respectively), with no difference between loading conditions for isokinetic strength development ( $ES = 0.19$ ). Previous observations of greater dynamic 1-RM, but similar isometric, strength development with higher-load RT (Mitchell et al., 2012; Schoenfeld, Grgic et al., 2017) may be attributed to the task-specificity of strength development (related to load/intensity specificity in particular) that favours higher-load RT interventions in dynamic 1-RM assessments (Rasch & Morehouse, 1957; Rutherford et al., 1986). Given strength improvements with RT are most specific to the exercises performed during RT (Rasch & Morehouse, 1957; Rutherford et al., 1986), and the exercises during which dynamic 1-RM strength is assessed are typically incorporated into the RT intervention, we anticipated a greater advantage for higher-load RT for improving dynamic 1-RM strength versus isometric strength. In contrast, our finding that higher-load RT is similarly advantageous for improving both dynamic 1-RM and isometric strength suggests the superiority of higher-load

RT may instead be mediated by non-task-specific neuromuscular adaptations. For example, the load-dependent effects of RT on improvements in neural drive (Jenkins et al., 2017), which may stimulate greater neural adaptations (e.g., improved agonist activation, motor unit synchronization, motor unit firing rates, and reduced antagonist co-activation) that underpin strength gain with RT (Zourdos et al., 2015), may at least partially explain the similar advantage of higher-load RT for both dynamic 1-RM and isometric strength gain. Each of the strength outcomes included in the present meta-analysis require maximal neuromuscular activation, which is further improved with higher- versus lower-load RT (Jenkins et al., 2017). It therefore remains possible that despite less task-specificity between the RT exercises in the included studies and isometric strength assessments, the greater neural adaptations likely elicited by higher- versus lower-load RT may have contributed to the superiority of higher load RT for both isometric and dynamic 1-RM strength. In addition, the observation that muscle hypertrophy was similar between RT loading conditions also adds weight to the notion that the superiority of higher-load RT for dynamic 1-RM and isometric strength gain was attributed to non-hypertrophic (i.e., neural) mechanisms.

A number of methodological factors must be considered when interpreting the evidence for the influence of RT load on strength development. In particular, variation in the RT protocols used by individual studies was a likely contributor to the heterogeneity observed. While our analysis broadly classified the RT protocols used by included studies as either higher or lower load, there was considerable variation within the definitions of higher- and lower-load RT, both in terms of load and training volume. Indeed, higher-load RT protocols varied from examples including 8–9 sets at 4–5-RM (Hisaeda et al., 1996) and 1 set at 80% 1-RM (Stefanaki et al., 2019), while lower-load RT protocols varied from examples including 3 sets of 12–14 (50% 1-RM) (Beneka et al., 2005) to 1 x 100–150-RM (Anderson & Kearney, 1982). Such differences in the magnitude of divergence between higher-load and lower-load RT conditions would undoubtedly influence the magnitude of effects favouring either loading condition on outcome measures. In addition, variability in both the duration (ranging from 6 to 52 weeks) and weekly frequency (ranging from 1 to 4 times per week) of the RT interventions, as well as the muscle group specificity (e.g., upper- vs. lower-body) of the exercises used in the RT intervention may all influence strength development with RT and therefore contribute to the observed heterogeneity.

Taken together, the synergistic effects of greater improvements in neural drive and task specificity (albeit less so for isometric strength) may explain the greater improvements in dynamic 1-RM and isometric strength observed with higher-load RT. Similarly, the lack of a load-dependent influence on isokinetic strength is likely explained by similar mechanisms, since the task demands of isokinetic strength tests are not replicated in common RT interventions.

#### 4.2. Influence of RT load on skeletal muscle hypertrophy

Consistent with previous findings (Lopez et al., 2020; Schoenfeld, Grgic et al., 2017), we also observed that muscle

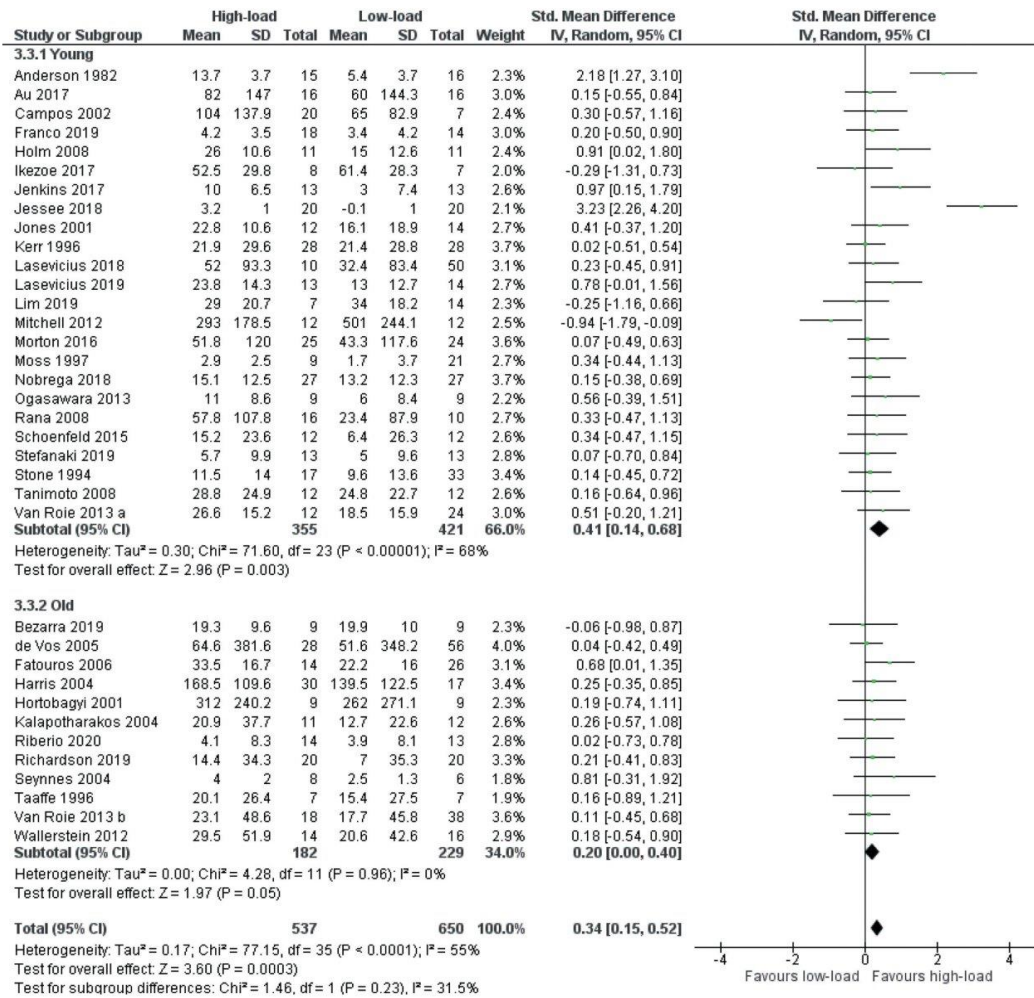


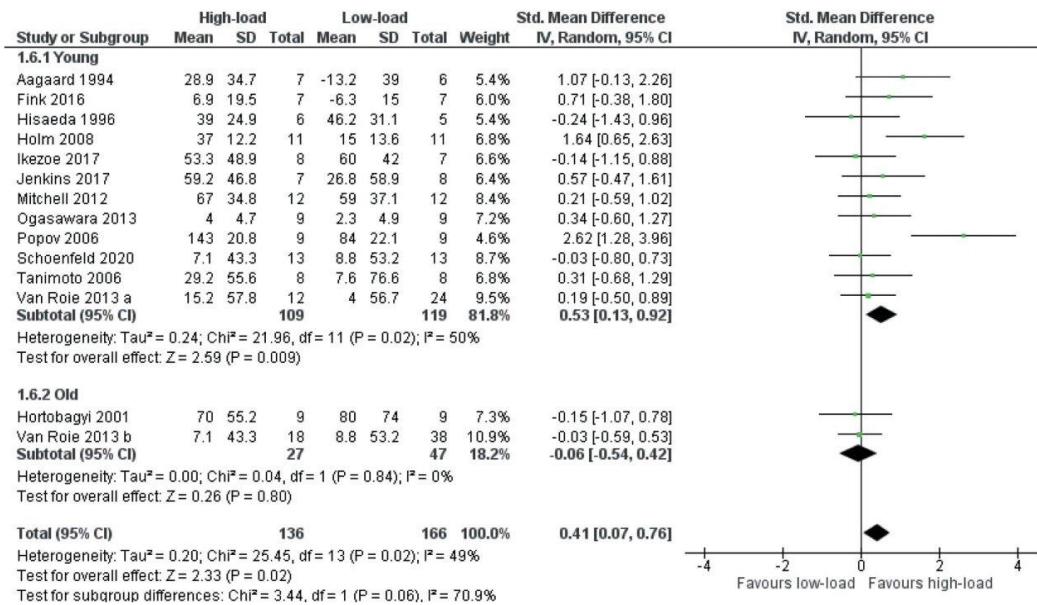
Figure 2. Influence of high-load vs. low-load RT on dynamic 1-RM strength development with subgroup analyses based on studies in younger (<60 years) versus older (≥60 years) participants. Point estimates and error bars signify the standardized mean difference between high-load and low-load groups and 95% confidence interval (CI) values, respectively.

hypertrophy responses were independent of RT load. This finding is in agreement with the notion that high (but not necessarily maximal) intensities-of-effort coupled with adequate RT volume, rather than RT load *per se*, are key stimuli for muscle hypertrophy (Morton et al., 2019; Schoenfeld, Grgic et al., 2017). Together with previous evidence (Lopez et al., 2020; Schoenfeld, Grgic et al., 2017), the findings therefore further highlight the versatility of RT loads that may be used to develop muscle hypertrophy.

Like the interpretation of the strength outcomes, a number of methodological factors must be considered when interpreting the influence of RT load on muscle hypertrophy. In particular, intensity-of-effort (proximity to muscular failure or the degree of internal focus/effort applied during a set) is a key stimulus for muscle hypertrophy due to its implications for motor unit recruitment and the exposure of active muscle

fibres to mechanical tension (Morton et al., 2019). Regardless of the RT load, maximal motor unit/muscle fibre recruitment can occur providing intensity-of-effort is high (Morton et al., 2019). Whether or not RT sets are taken to (or close to) muscular failure may therefore influence study outcomes. However, there is evidence that training to muscular failure is not obligatory, and may even be detrimental, for muscle hypertrophy and strength outcomes (Izquierdo et al., 2006; Martorelli et al., 2017). Notably, previous meta-analyses on this topic (Lopez et al., 2020; Schoenfeld, Grgic et al., 2017; Schoenfeld et al., 2016) excluded studies whereby both higher-load and lower-load RT was not performed to muscular failure, presumably to control for differences in intensity-of-effort across studies that may influence outcomes (e.g., muscle hypertrophy in particular). Since training to muscular failure (i.e., maximal intensities-of-effort) 1) does not necessarily ensure equivalent muscle





**Figure 3.** Influence of high-load vs. low-load RT on isometric (MVIC) strength development with subgroup analyses based on studies in younger (<60 years) versus older (≥60 years) participants. Point estimates and error bars signify the standardized mean difference between high-load and low-load groups and 95% confidence interval (CI) values, respectively.

activation during higher- and lower-load RT conditions due to greater difficulties in reaching true muscular failure with lighter RT loads (Fisher et al., 2017), 2) may be of greater importance to muscle hypertrophy than strength development (Morton et al., 2019), and 3) may not always be feasible or sustainable in practice (Fisher & Steele, 2017; Fisher et al., 2017), we chose to include all relevant studies independent of whether sets were performed to muscular failure or not. This approach resulted in a significantly larger number of included studies (45 studies in the present review vs. nine in Schoenfeld et al. (2016), 21 in Schoenfeld, Grgic et al. (2017), and 28 in Lopez et al. (2020)), and allowed qualitative insight into whether training to muscular failure influenced study outcomes independently of RT load. Of the studies included in the present meta-analysis, approximately 55% (24 of 45 studies) had participants in all groups perform RT to muscular failure. Despite between-study variability in whether RT was performed to muscular failure, the findings of similar muscle hypertrophy with higher- versus lower-load RT was highly consistent between studies, with low heterogeneity in study outcomes ( $I^2 = 0\%$  for both whole-muscle hypertrophy and lean body/fat-free mass, and  $I^2 = 10\%$  for muscle fibre-specific hypertrophy). It therefore appears the intensities-of-effort employed in the included studies were sufficiently high for both higher- and lower-load RT to expose muscle fibres to sufficient mechanical tension and stimulate muscle hypertrophy. These observations also provide further evidence that training to muscular failure is not obligatory for maximizing muscle hypertrophy when RT is performed with either heavier or lighter loads.

From a practical perspective, it therefore may not be necessary for individuals to consistently apply near-maximal intensities-of-

effort (particularly in trained individuals, who may be able to recruit higher-threshold motor units at greater proximities from muscular failure versus untrained individuals (Van Den Tillaar et al., 2019)) to induce additional muscle hypertrophy. Since higher intensities-of-effort during RT are associated with negative affective responses (particularly when lower loads are used) such as discomfort (Fisher & Steele, 2017) in some individuals, consistently applying a high intensity-of-effort may exacerbate fatigue and potentially compromise adherence and long-term training outcomes.

The total volume of RT performed may also influence muscle hypertrophy outcomes and can be manipulated independently of RT load *per se*. There is indeed evidence for a dose-response influence of RT on muscle hypertrophy, with higher weekly RT volumes leading to greater muscle growth (Schoenfeld, Ogborn et al., 2017). It is therefore possible that whether or not high- and low-load RT protocols were matched for total volume performed may influence study outcomes. Twenty-six of the included studies that assessed muscle hypertrophy outcomes did not equate total RT volume between higher- and lower-load groups, which may advantage the higher-volume (i.e., lower-load) group from a advantage hypertrophy perspective. However, any potential advantage for lower-load RT was not evident in our findings, since 19 of the 26 studies in which higher- and lower-load RT was not volume-equated found no difference in muscle hypertrophy between loading conditions. These findings further highlight that sufficiently high intensities-of-effort may somewhat override the potential importance of total RT volume on muscle hypertrophy. While these findings suggest a limited role of RT volume in muscle hypertrophy, providing intensity-of-effort is sufficient, it is possible that RT volume may become more important for

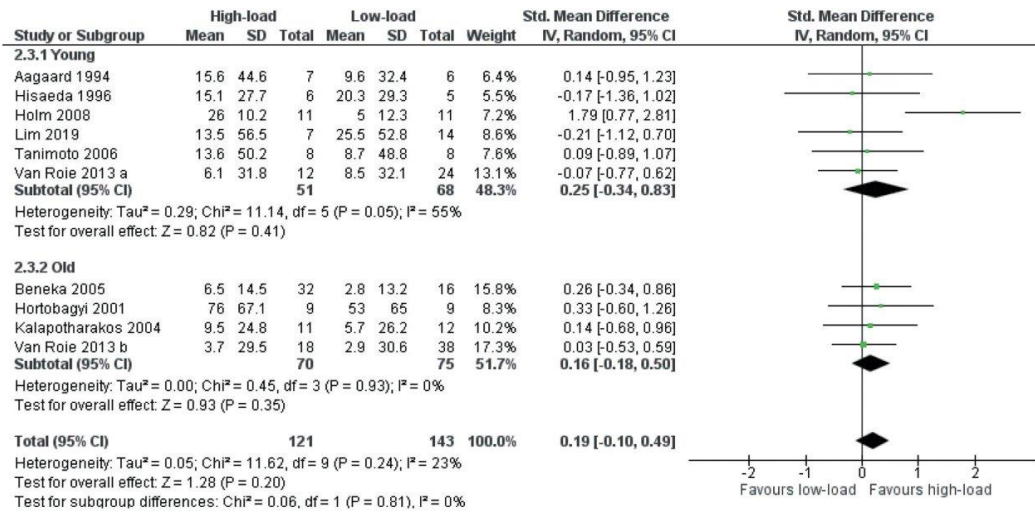


Figure 4. Influence of high-load vs. low-load RT on isokinetic strength development with subgroup analyses based on studies in younger (<60 years) versus older (≥60 years) participants. Point estimates and error bars signify the standardized mean difference between high-load and low-load groups and 95% confidence interval (CI) values, respectively.

muscle hypertrophy as training experience increases – a notion supported by greater muscle hypertrophy observed with higher RT volumes in trained men (Schoenfeld et al., 2019).

A major limitation to the current understanding of the role of RT load (and by extension, any potential moderating influence of equating for RT volume) in muscle hypertrophy is the lack of evidence in participants with RT experience. Indeed, none of the 22 studies included in this review that measured changes in whole-muscle size (and 1 of the 6 studies that measured changes in muscle fibre size) was performed in trained participants. Future studies investigating the influence of RT load in physiological adaptation to RT should, where possible, incorporate participants with some degree of RT experience.

### 4.3. Influence of resistance training load on sport-specific or neuromuscular task performance

While there is evidence that RT is associated with improved sport-specific task performance (e.g., jumping, sprinting, and changing-of-direction) (Cholewa et al., 2018; Suhomel et al., 2016), there is limited evidence for any RT load-dependent influence on improvements in these parameters. The two studies (Jones et al., 2001; Rana et al., 2008) included in this review that measured sport-specific or neuromuscular task performance showed contrasting results, with one study (Jones et al., 2001) finding a similar improvement in various measures of jump performance (set angle jump, squat jump, depth jump)

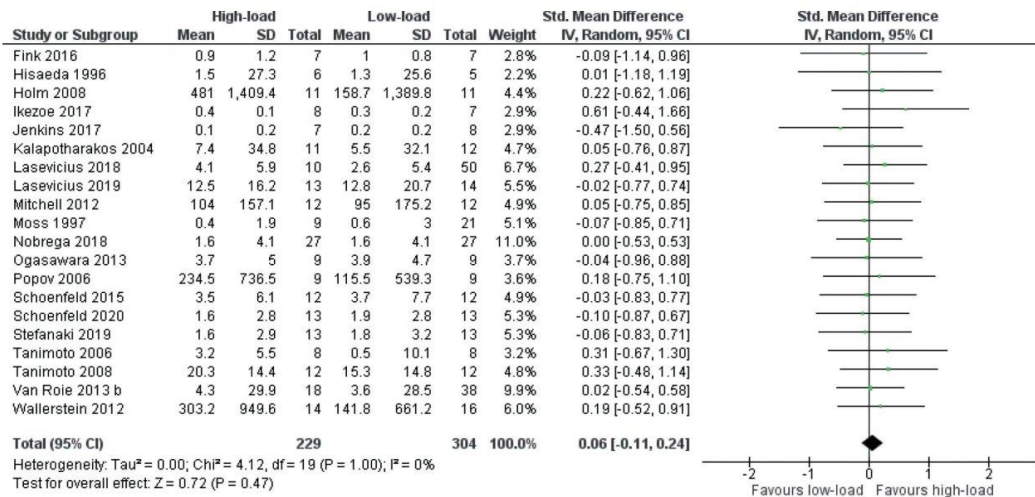


Figure 5. Influence of high-load vs. low-load RT on whole-muscle size. Point estimates and error bars signify the standardized mean difference between high-load and low-load groups and 95% confidence interval (CI) values, respectively.



between loading conditions, and the other (Rana et al., 2008) showing no improvement in vertical jump performance for both conditions. The limited available evidence therefore makes clear interpretations difficult. Nevertheless, since higher-load RT likely promotes greater neural adaptations (Jenkins et al., 2017) that underpin the superiority of higher-load RT for dynamic and isometric strength outcomes, and both neural adaptations and strength likely mediate improvements in sport-specific task performance (Suchomel et al., 2016), future studies may observe greater improvements in these measures with higher-load RT. It is also possible that optimizing improvements in these measures may require other forms of power-specific training, such as complex/contrast or plyometric training, particularly when incorporating exercises that closely mimic the demands of sport-specific or neuromuscular performance tasks.

**4.4. Limitations of current research and future directions**

A number of limitations must be considered when interpreting the findings of the current systematic review and meta-analysis. The majority of included studies involved participants with minimal or no RT experience, making it difficult to elucidate any potential training experience-dependent effects on outcomes. Nonetheless, the limited number of studies conducted on participants with RT experience had similar findings to those in untrained participants, suggesting potential training status-dependent effects on outcomes may be limited. Further evidence in trained participants is nevertheless needed to more firmly draw this conclusion. Although we did not perform any sub-group analyses based on participant sex, only 14 of 45 total

studies included female participants. While study outcomes appeared qualitatively similar between those studies including male or female participants, future research is required to elucidate any potential sex-dependent moderating effects on the influence of RT load on outcomes. Another major limitation was the ages of the participants in the included studies, which was biased towards younger participants. We conducted sub-group analyses based on younger (<60 years) and older (≥60 years) participants, and identified only 13 studies that included older participants, with only a single study (Kerr et al., 1996) in which participants were aged between 30 and 60 years old. Future studies should therefore aim to include participants aged 30 and above to improve understanding of the potential moderating influence of age on responses to higher- versus lower-load RT.

The influence of other methodological differences on study outcomes, such as the rest periods used for the RT protocols, and individual factors such as tolerance to discomfort, must also be considered when interpreting the current findings. For example, the RT protocol used by Campos et al. (2002) had between-set rest periods that varied between the higher-load (3 min) and lower-load (1 min) groups. It is possible this discrepancy in rest periods could influence the total RT volume accumulated by each group, and potentially advantage the group that accumulate a higher RT volume. Nevertheless, between-set rest periods may have limited influence on strength (Grgic et al., 2018) and hypertrophy (Grgic et al., 2017) responses to RT, although longer rest periods may be more important in trained individuals (Grgic et al., 2017). Furthermore, participants may be limited by their perception of effort and the degree of discomfort experienced, particularly

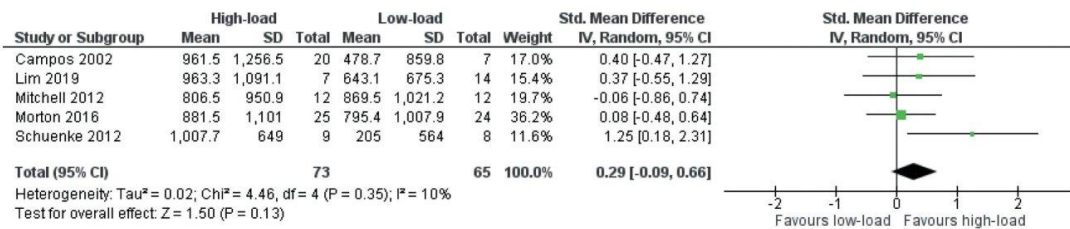


Figure 6. Influence of high-load vs. low-load RT on muscle fibre cross-sectional area (fCSA). Point estimates and error bars signify the standardized mean difference between high-load and low-load groups and 95% confidence interval (CI) values, respectively.

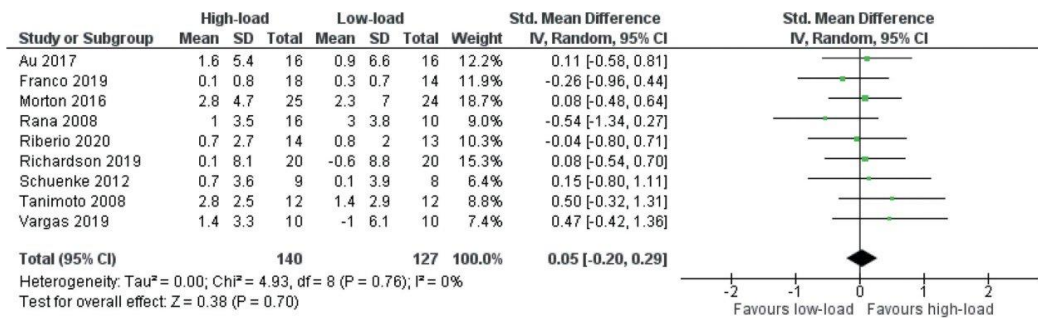


Figure 7. Influence of high-load vs. low-load RT on lean body mass (LBM) or fat-free mass. Point estimates and error bars signify the standardized mean difference between high-load and low-load groups and 95% confidence interval (CI) values, respectively.



during low-load conditions (Fisher et al., 2017), leading to lowered intensities-of-effort and a diminished ability to maximize muscle hypertrophy. It is therefore possible that individuals undertaking a low-load RT intervention may volitionally terminate their sets due to discomfort as opposed to reaching true momentary muscular failure, which may influence comparisons with higher-load conditions that may reach closer to muscular failure. Future studies comparing low-load and high-load conditions performed to muscular failure should therefore ensure participants can effectively gauge their intensity-of-effort and distinguish between momentary muscular failure and volitional termination of a set due to discomfort.

The majority of included studies did not equate total RT volume between low-load and high-load conditions, and a volume-dependent influence on outcomes was not evident. This may be due to most studies being conducted on untrained (or relatively untrained) participants that may require lower training volumes to stimulate physiological adaptations versus trained participants. It is therefore possible that equating for RT volume between higher- versus lower-load RT groups may be more important for future studies conducted in participants with RT experience.

#### 4.5. Practical applications of key findings

The findings of this meta-analysis overall suggest higher RT loads (>60% 1-RM) promote greater dynamic and isometric strength gains compared to lighter RT loads ( $\leq$ 60% 1-RM), whereas a wider spectrum of loads may elicit muscle hypertrophy. Higher RT loads are therefore recommended for dynamic and isometric strength development, whereas for muscle hypertrophy, loads may be selected based on individual preferences and tolerance to discomfort experienced with high intensities-of-effort (which may be greater with lower RT loads). There are, however, additional practical considerations beyond load *per se* for maximizing strength and muscle hypertrophy outcomes with RT. Firstly, task (or exercise) specificity has clear implications for strength development with RT and should be considered when designing RT programs. For this reason, exercises that are specific to the measure of strength used (and vice versa) should be integrated into an RT program focused on strength development to provide an accurate representation of the effectiveness of the intervention. Since motor learning forms a large component of strength development, it is possible that greater repetition practice opportunities may facilitate additional strength gains with RT, particularly in relatively untrained individuals or those learning new exercises or movement patterns. While lower-load RT is likely sub-optimal for long-term maximal strength development, it may facilitate the development of motor learning patterns during certain training phases that may provide the foundations for the subsequent implementation of higher-load RT. For this reason, lower-load RT involving larger repetition numbers, and/or higher training frequencies, may be used during certain training phases to facilitate greater repetition practice opportunities and associated motor learning. Ultimately, while higher-load RT is optimal for strength development, RT prescription should be tailored to the target strength outcome (e.g., 1-RM vs. 6-RM). It should also be considered that higher-load RT may require

longer between-set rest intervals to limit excessive fatigue accumulation and maintain high levels of neural drive during subsequent exercises and sets.

In line with previous work (Schoenfeld, Grgic et al., 2017), the present findings suggest various loads may be used to elicit muscle hypertrophy with RT, providing intensity-of-effort is sufficiently high (but not necessarily maximal). Performing RT with close proximity-to-failure may therefore be a strategy to maximize muscle hypertrophy independently of the load used. As in strength development, exercise selection is an important consideration when determining the suitability of performing RT close to muscular failure with higher or lower loads. In particular, exercises performed close to muscular failure should be selected to allow for safe execution and high levels of effort throughout a set, and to limit the accumulation of excessive fatigue that may compromise intensity-of-effort in subsequent exercises and sets. Exercises where risk of injury is likely higher due to increased movement complexity and/or less stability (e.g., barbell squat versus leg press) may be less suitable for training close to muscular failure. In addition, since multi-joint exercises engage more muscle mass and thus involve higher neurological and aerobic demands (Paoli et al., 2017) than single-joint exercises, training close to muscular failure with numerous multi-joint exercises per session may exacerbate fatigue and impair subsequent training quality. For this reason, high intensities-of-effort should be performed on a limited number of multi-joint exercises per session, with single-joint exercises performed closer to muscular failure to enhance the hypertrophic stimulus.

Compared with higher-load RT, lower-load RT induces greater metabolic stress within the active musculature due to prolonged anaerobic energy provision during longer duration sets. The metabolic stress elicited throughout lower-load, higher-repetition sets promotes higher levels of discomfort (Fisher et al., 2017) that may impair the ability to reach high intensities-of-effort depending on individual tolerance to discomfort. It is therefore recommended that individuals select loads that allow them to reach a close proximity to muscular failure, and that individuals with less tolerance to discomfort prioritize higher versus lower loads.

#### 5. Conclusion

The findings of this systematic review and meta-analysis suggest higher- and lower-load RT induce comparable skeletal muscle hypertrophy (assessed as either changes in lean body/fat-free mass, or in whole-muscle and muscle fibre-specific CSA), improvements in lean/fat-free mass, and isokinetic strength development, while higher-load RT is superior for improving both dynamic (1-RM) and isometric (MVIC) strength. The advantage of higher-load RT for improving dynamic (1-RM) strength was more evident in untrained and younger participants. Due to limited available evidence, the influence of RT load on sport-specific or neuromuscular task performance measures was unable to be determined. Higher-load RT is therefore recommended for improving dynamic and isometric strength, while elements of specificity including exercise/task and repetition range specificity should be considered when prescribing RT for maximizing strength. Since a wide spectrum of RT loads



may promote muscle hypertrophy, load selection may be informed by individual preferences, tolerance to levels of exertion and discomfort (which likely varies based on loading condition), and the suitability of a given exercise to a specific loading condition (e.g., complex exercises may be less suited to low-load RT performed close to failure). When aiming to maximize the muscle hypertrophic response from a given exercise, we advise selecting a load that a) does not limit safe exercise execution, b) allows for high levels of effort to be achieved within a given set, and c) limits the accumulation of excessive fatigue that may impair intensity-of-effort in subsequent exercises and sets, thereby maximizing mechanical tension and the hypertrophic stimulus imparted on the active musculature. The findings of this systematic review and meta-analysis therefore suggest higher-load RT is superior for improving both dynamic (1-RM) and isometric strength (but not isokinetic strength) compared with lower-load RT, and muscle hypertrophy occurs independently of RT load and regardless of whether intensity-of-effort is maximal. A lack of studies in both trained and older participants was a clear limitation of the available literature and should be addressed in future studies. There is also limited evidence on the influence of RT load for improving sport-specific (i.e., jumping, sprinting, and changing-of-direction) or neuromuscular (e.g., CMJ and IMTP) performance tasks.

### Disclosure statement

The authors declare that they have no conflicts of interest or competing interests.

### Funding

No funding was used to directly support the preparation of this review.

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### Availability of data and material

The data sets generated and analysed for this article are available in supplementary information (Supplementary File A).

### Author contributions

Article conceptualisation: MCR, DLH, SAF, and JJF; literature search: MCR and JJF; data analysis: DRP and JLG; drafted manuscript: MCR and JJF; critically revised manuscript: all authors.

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