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Functions of leptin in tuberculosis and diabetes: multi-omics studies across species

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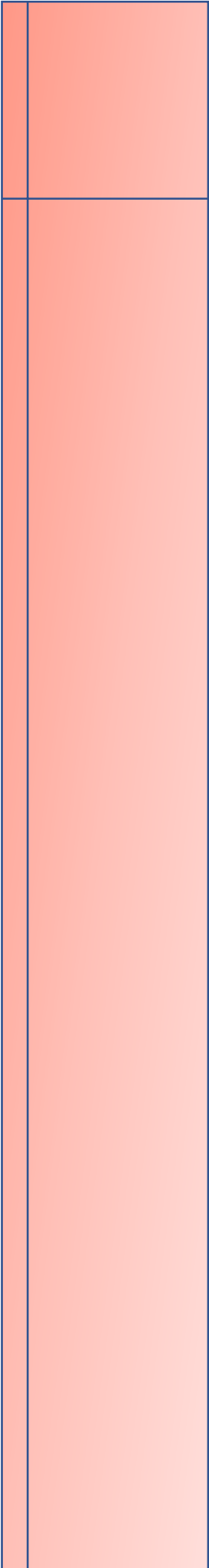


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General introduction

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Functions of leptin in metabolic wasting syndrome

Metabolic wasting syndrome, also called cachexia, is a metabolic disorder characterized by unintentional excessive weight loss (greater than 5% of body weight in preceding 6 months) with the depletion of muscle and adipose tissues [1, 2]. It is different from the weight loss resulting from starvation, which can be recovered by ingestion of food. However, in the condition of wasting syndrome, the weight loss and body composition are hardly reversed by food supplementation [3]. This is because profound irreversible metabolic reprogramming takes place, which results in the degradation of muscle tissues [3, 4]. It usually occurs at the late stage of chronic diseases such as cancer [5], autoimmune disease (rheumatoid arthritis) [6], infectious diseases including AIDS and tuberculosis [7, 8], diabetes [9] and chronic kidney disease [10]. Cachexia increases the mortality and it accounts for 30% of death in patients with chronic diseases [11]. However, currently no pharmacological treatment exists [12, 13].

We have studied the function of the leptin gene in wasting syndrome. Leptin was firstly cloned and identified in obese mice by Friedman in 1994 [14]. It is a 167 amino acid long protein hormone with four-helix bundles (**Figure 1A**) and mainly secreted by adipose tissues. It has been shown that leptin is a key regulator of appetite, body weight, energy regulation, bone metabolism, immunity, reproduction and metabolic homeostasis [15, 16] (**Figure 1B**). Mutation of the *lep* gene (originally called *ob* gene) in mice (*ob/ob*) leads to extreme obesity, insulin resistance and type 2 diabetes mellitus [17]. The mutants in a later stage of diabetes, also display wasting syndrome characterized by the loss of muscle mass [18]. Therefore, *ob/ob* mice are commonly used as a diabetic animal model [17, 19].

This thesis is focused on metabolic effects of tuberculosis and leptin deficiency in mice (*Mus musculus*) and zebrafish (*Danio rerio*) animal models. We used different metabolomic tools including mass spectrometry (MS), nuclear magnetic resonance (NMR) and high-resolution magic-angle-spinning NMR (HR-MAS NMR) spectrometry to acquire the metabolic profiles of samples. Additionally, transcriptomics was used to investigate the mechanism underlying the metabolic disorders of TB and leptin mutation. Furthermore, we tested whether the adult leptin mutant zebrafish have a diabetic phenotype as well as kidney damage resulting from this. In this chapter we will give a short overview of various aspects that determine cachexia in tuberculosis and diabetes and explain the animal models and metabolomics methods that we used to study this.

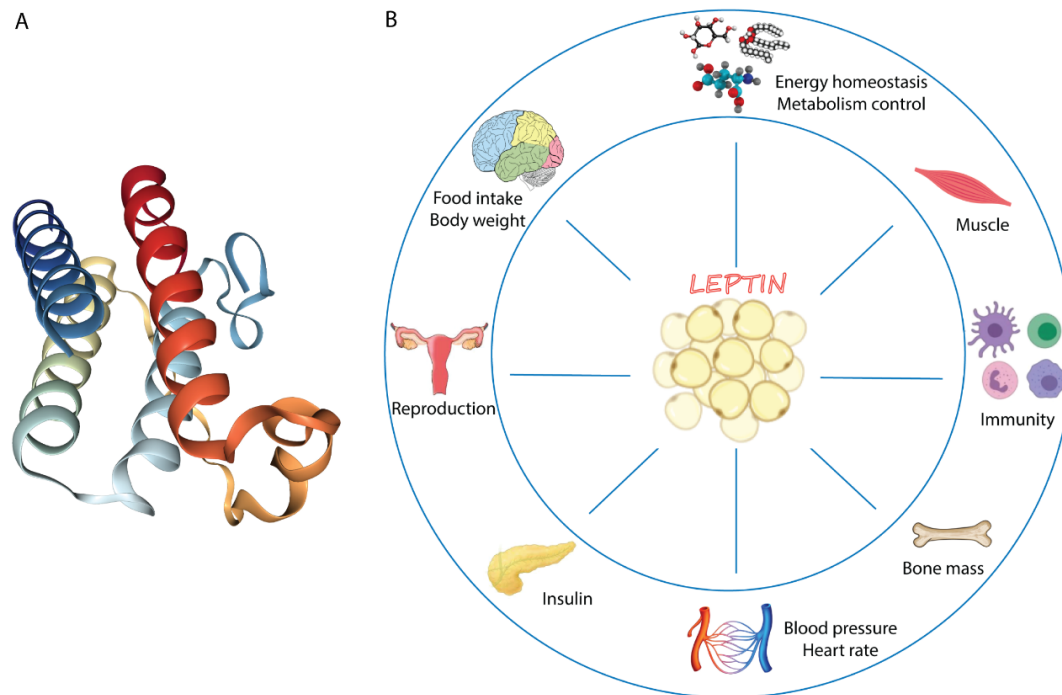


Figure 1. The structure and physiological functions of leptin. **A.** The structure of human leptin protein shows a four-helix bundles similar to that of the long-chain helical cytokine family [20]. **B.** Leptin is produced by a variety of tissues, for instance by white adipose tissues (in the middle) and it has diverse functions in several organs and molecules. Figure 1B is similar to the figure of Zabeau et al., 2016 [21].

Molecular basis of cachexia

Cachexia is an energy wasting syndrome in which energy expenditure exceeds energy intake resulting in muscle wasting and atrophy [22, 23]. Chronic diseases, such as cancer and tuberculosis, consume large amounts of energy, therefore the body triggers compensatory mechanisms to maintain energy homeostasis. The compensatory mechanisms are associated with important metabolic changes in carbohydrate, lipid and amino acid metabolism and the balance of protein synthesis and degradation [23]. In the condition of cachexia, protein synthesis is decreased and myofibrillar protein degradation is increased [24]. There are two main mechanisms for protein degradation: the ubiquitin-dependent proteasome and autophagy pathways [23, 25]. An increase in ubiquitin proteasome system (UPS) activity is involved in the degradation of muscle myofibrillar proteins [26]. In the case of skeletal muscle atrophy, this increase of UPS activity is accompanied by a higher expression level of atrogin-1 and MuRF-1, which are two key E3 ubiquitin ligases [27]. Autophagy is a well-known recycling process that ingests dysfunctional organelles such as mitochondria, eliminating intracellular pathogens as well as clearing up misfolded or aggregated proteins [28]. A block of autophagy signaling and mutation in autophagy genes lead to myofiber disorders and muscle weakness [29, 30]. Excessive activation of the activity of autophagy results in

detrimental consequences on muscle homeostasis and severe muscle loss [31, 32]. Therefore, a balanced autophagy flux is essential to maintain muscle mass and myofiber integrity.

A common effect of cachexia in mammals is chronic inflammation [33]. It is reported that inflammatory responses in many chronic diseases influence various metabolic changes related to wasting syndrome [34]. Chronic systemic production of pro-inflammatory cytokines decreases the appetite and increases the catabolism in the body [13]. Serum levels of the pro-inflammatory cytokines were found to be increased in human patients and in animal models of chronic diseases associated with wasting syndrome [24, 35]. The elevated levels of these cytokines inhibit the secretion and activity of growth hormones such as IGF-1. In addition, the high concentration of the cytokines directly induces signaling pathways that regulate enzymes leading to skeletal muscle protein turnover [24]. These cytokines include TNF- α , IL-6, IL-1 β , interferon- γ , myostatin and activin A [13, 24, 36, 37]. TNF- α , originally named cachectin, is shown to induce cachexia in rats [37] and mice [38] and cause myotube atrophy *in vitro* via the activation of the UPS pathway [39]. Inhibition of IL-6 signaling attenuates cachexia development in mouse cancer models [40]. The blockade of the common receptor ActRIIB of myostatin and activin A, suppresses cachexia progression and reverses prior skeletal muscle loss in several mouse cancer cachexia models [41]. However, the molecular mechanisms underlying the regulation of wasting syndrome by cytokines at the systemic level are not yet known.

Tuberculosis

Tuberculosis (TB) remains one of the top 10 leading causes of human death worldwide because it is an infectious, contagious and often drug-resistant disease [42]. It spreads merely by aerosolized droplets carrying bacteria from the *Mycobacterium tuberculosis* (*Mtb*) complex [43]. It has been a threat to global public health for decades with about 10 million new cases and 1.4 million deaths reported in 2019 [42]. A quarter of the world's population is estimated to have latent TB infection, according to the World Health Organization (WHO) [42]. Approximately one tenth of latent TB is likely to develop to active TB disease at which stage the causative mycobacteria become highly transmittable [44]. The pathogen mainly targets the lung, although extra-pulmonary infection is also very frequently observed [43]. TB is characterized pathologically by the formation of granulomas that consist of clusters of infected macrophages and surrounding uninfected immune cells of various types (**Figure 2**). TB results in many symptoms including fever, cough, chest pains, hemolysis and weight loss [45, 46]. The causes of death by tuberculosis are mainly due to severe tissue damage, oxygen shortage and wasting syndrome [43]. It is claimed that TB has been existing throughout human history, leading to large epidemic proportions in the 18th, 19th and early 20th centuries [47, 48]. Although the disease was thought to be mostly eradicated by the end of the 20th

century, currently TB is spreading rapidly due to the emergence of many antibiotic resistant strains and a lack of proper diagnosis in many countries. The latter problem is aggravated by the current health problems due to the Covid-19 pandemic. Therefore, approaches to prevent *Mtb* transmission as well as to reduce TB morbidity and mortality, should not only rely on good preventions and effective therapies but also on efficient diagnoses [49].

Currently, several tests are applied for the diagnosis of TB [46, 50]. Those methods include tuberculin skin tests (TST), interferon- γ release assays (IGRA), sputum smear microscopy, mycobacterial culture-based methods, chest radiography such as chest X-rays and PET-CT, and commercial molecular tests [46]. TST is simple and cheap, however, it results in false positive responses for people who are Bacille Calmette-Guerin (BCG) vaccinated [51]. IGRA is more specific than the TST, but it gives false negative responses for people who suffer from immunosuppression [45]. Sputum smear microscopy is the most widely used test for TB, but it lacks sensitivity and misses a diagnosis of extra-pulmonary infections [46]. Mycobacterial culture-based methods are the golden standard diagnostic test with a high sensitivity for TB. However, it takes 3-4 weeks to obtain results, which delays diagnosis [52]. Chest radiography examines whether an abnormality is present, but it doesn't give information about the lesion's activity. Commercial molecular tests, such as GeneXpert MTB/RIF, although rapid and detecting antibiotic resistance of the pathogen, are expensive and have high requirements for infrastructure [53].

Given the limitations of the current diagnostic tests, there is an urgent need for the development of new efficient, reproducible and cost effective diagnostic methods to control the disease. Biomarker-based tests for the detection of TB, ideally performed on easily accessible samples such as blood or urine, provide great potential for the detection of TB [53, 54]. Since various biomarkers are used to define an endpoint in clinical trials distinguishing a normal and pathogenic biological state, they can be also directly used for advanced diagnostic tools [55]. Additionally, biomarkers can indicate the effectiveness of a therapeutic treatment and promote development of alternative therapy regimens. Reported biomarkers are either pathogen-derived such as lipoarabinomannan (LAM), or host-derived such as antibodies, cellular immune responses to *Mtb* antigens (i.e., cytokines and chemokines) or derived from both pathogen and host factors using omics-based approaches [53, 56, 57].

Omics-based approaches are capable of accurately predicting disease outcomes and providing insights for disease mechanisms [58]. Omics includes genomics, transcriptomics, proteomics and metabolomics approaches [58]. Trans-omics is defined as a discipline that seeks to reconstruct biomolecular networks not as a group of indirect statistical correlations, but as chains of direct mechanistic molecular interactions by using comprehensive multi-omics data measured under identical conditions, as opposed to heterogeneous sources of data [59, 60]. The metabolome

encompasses a complete set of metabolites in biological processes, and it is the ultimate downstream outcome of the processes measured by the other three omics levels. Therefore, any disruption at the other omics levels could affect the quantities of specific metabolites [61]. Identification and quantification of full metabolomes provide a great possibility for discovering biomarkers of diseases including TB [62]. To date, metabolomics has been applied to identify potential biomarkers of TB from blood [63], urine [64, 65] and sputum [66] of patients. However, they still need to be validated by comparisons of the outcomes of independent studies and tested in clinical trials before they can be used in the clinic. Additionally, TB animal models that for example use mice and zebrafish, provide basic tools to discover new biomarkers for diagnosis. The models can provide mechanistic insights into the biological relevance of the biomarkers, resulting in a more robust theoretical basis for the prediction of their effectiveness.

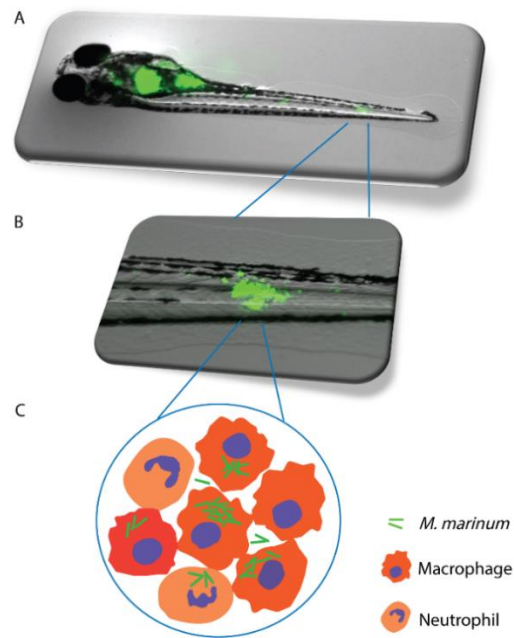
The zebrafish as a versatile model organism for tuberculosis studies

Animal models offer practical advantages to study the progression and the pathogenic mechanisms of TB, therefore they serve as important means for TB research [67]. Although *Mtb* infection in mouse recapitulates some aspects of human TB disease, *Mtb* is a dangerous pathogen to work with and it requires specialized and expensive facilities [43, 67]. Zebrafish larvae offer an excellent animal model for studying the pathogenesis of *Mtb* [68]. The optical transparency of zebrafish larvae allows the visualization of the infection of a fluorescently-labelled pathogen *in vivo*. *Mycobacterium marinum* (*M. marinum*), the closest known genetic relative of the *Mtb* complex, is a natural pathogen for zebrafish [69]. It is much less dangerous to work with than the *Mtb* pathogen because of its restricted temperature range of growth. Infection of zebrafish embryos with *M. marinum* results in the formation of characteristic necrotic and granulomatous lesions which is a hallmark of human TB (**Figure 2**). The granuloma is a highly organized structure characterized by aggregation of immune cells that engulf and confine the pathogens (**Figure 2**). Many publications show that the mechanisms of *M. marinum* infection in zebrafish larvae can be translated at the genetic, cellular and molecular signaling levels to tuberculosis progression in humans [70, 71]. Therefore, zebrafish larvae are a very powerful model organism to study the fundamental mechanism underlying TB progression.

Figure 2 (following page). Zebrafish as an excellent model organism for tuberculosis studies. Zebrafish embryos were injected with 30 colony forming units of *M. marinum* bacteria into the yolk at 4 hours post fertilization. Images were acquired at 5 days post infection. *M. marinum* strain M was transformed with a plasmid encoding the green fluorescent Wasabi protein, therefore it can be visualized with a fluorescent microscope. **A.** A whole zebrafish larva showing *M. marinum* infection in the yolk and tail (Y. Ding unpublished data). **B.** An enlargement of a mycobacterial cluster in the tail of the zebrafish larva. **C.** A

General introduction

schematic diagram illustrates the morphology of the granuloma. The diagram is adapted from Masud et al., 2017 [72].



Diabetes

Diabetes mellitus (DM) is a chronic disease that is caused by a defect in insulin signaling in the body leading to limited glucose uptake from the blood stream. There were around 422 million cases and 1.6 million deaths all over the world per year [73]. Globally, currently one in eleven people are diagnosed with DM [73]. The morbidity and mortality of DM is steadily increasing, therefore, it exerts a big threat to global public health [73, 74]. DM is classified into type 1 diabetes mellitus (T1DM), type 2 diabetes mellitus (T2DM) and gestational diabetes mellitus (GDM) [75]. T1DM is most often caused by autoimmune disorders which results in the dysfunction or death of the beta cells leading to a failure of the pancreas to secrete insulin. The frequency of T1DM is up to 10% of diabetic patients. T2DM is the most common type of diabetes and accounts for approximately 90% of all diabetic cases. It is a chronic metabolic disorder characterized by dysregulation of carbohydrate, fat and protein metabolism [76]. It is caused by peripheral insulin resistance that can lead to impaired insulin production [76]. GDM only occurs in pregnant women, and normally their blood glucose level will be back to normal after pregnancy. However, the risk of developing T2DM is higher both for the mother and child later on during their lifetime [77]. T2DM affects the whole body and is usually accompanied by many complications, including cardiovascular disease and chronic failure of organs such as kidney and eyes [78]. In addition, people with T2DM are more susceptible to infectious diseases including tuberculosis [79]. Obesity is reported to be the most

important factor in the development of metabolic diseases including T2DM [80]. Therefore, both obesity and T2DM are accompanied with insulin resistance [80]. This could be explained by the function of fat tissues in the secretion of hormones and cytokines, such as leptin, that can control whole body metabolism and cause insulin resistance [80]. Serum leptin levels are positively correlated with body mass index and percentage of body fat in T2DM patients [81]. Higher leptin levels have been observed in insulin-resistant patients with T2DM independently of body fat mass [82, 83]. However, several other studies show that leptin levels were decreased in non-obese patients with T2DM [84, 85]. Rodents with leptin deficiency are hyper obese and diabetic [86]. Therefore, leptin has been studied extensively for its role in diabetes [87, 88].

Leptin

Leptin in humans is a 167 amino peptide that is mainly produced and secreted by white adipose tissues, but also found in a variety of tissues including placenta, mammary gland, ovary, skeletal muscle, stomach, pituitary gland, and lymphoid tissue [89]. However, those tissues contribute relatively little to the circulating levels of leptin [90]. The concentration of circulating leptin is considered to be proportional to the amount of fat tissue [91]. Leptin, as both an adipokine and cytokine, plays a key role in various aspects including control of body weight, energy homeostasis and metabolism. In mammals, leptin exerts its role via binding to specific isoforms of the leptin receptor that have the structure of a class I cytokine receptor and are located on the cell membrane of multiple tissues [92]. Leptin binds to leptin receptors resulting in the activation of three main cellular downstream signaling pathways including JAK-STAT, SHP2-ERK and IRS-PI3K pathways (Figure 3).

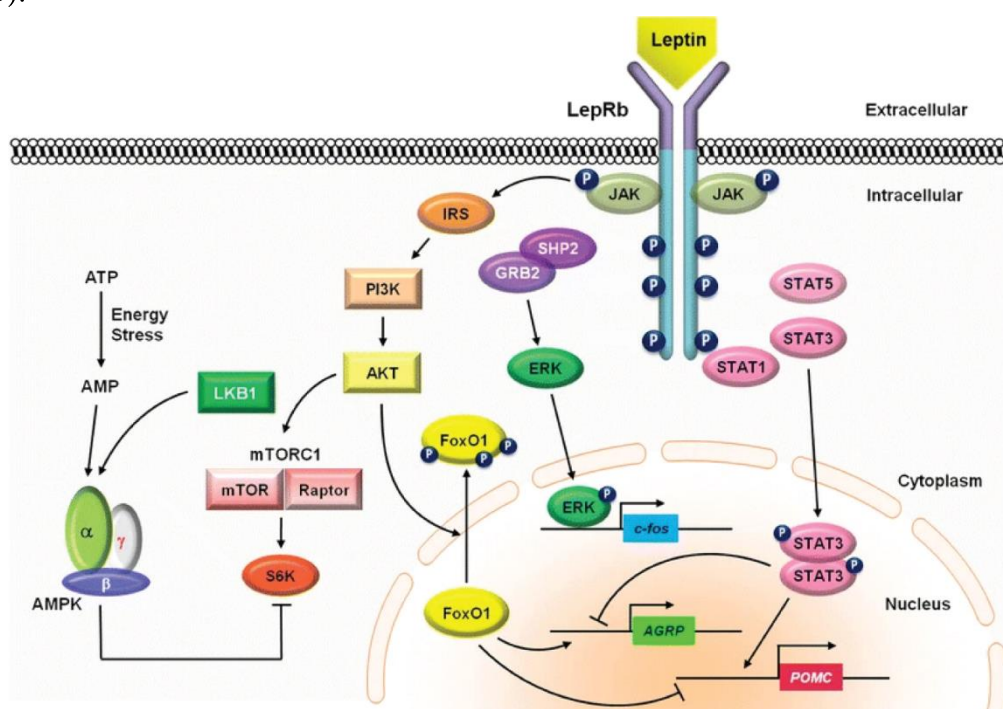


Figure 3 (previous page). Leptin signaling pathways. The figure is adapted from Kwon et al., 2016 [93]. Leptin binds to leptin receptor resulting in the activation of JAK-STAT, SHP2-ERK and IRS-PI3K pathways. The activated JAK prompts the phosphorylation of tyrosine residues which is involved in the dimerization of STAT3 and STAT5. Dimerized STAT3 and STAT5 then translocate into the nucleus and mediate the transcriptional expression of the targeted genes including POMC, which is involved in the regulation of appetite and body weight. In addition, the activation of tyrosine residues recruits SHP2 and GRB2, leading to the phosphorylation of ERK. The activated ERK translocates into the nucleus and regulates the expression of genes associated with energy homeostasis. Furthermore, upon leptin binding, activated JAK phosphorylates IRS, resulting in the activation of PI3K and AKT. This, on one hand, leads to the inactivation of FoxO1 by sequestering from nucleus to cytoplasm. On the other hand, AKT activates mTORC1 that subsequently activates S6K causing the phosphorylation of the AMPK α subunit and inhibition of AMPK activity. This pathway plays important roles in control of appetite and protein metabolism.

Leptin plays many functions in energy homeostasis and metabolism control. It maintains euglycemia by switching carbohydrate metabolism to fat metabolism during starvation [94]. In an early fasting state, glucose is mainly generated from hepatic breakdown of glycogen (glycogenolysis) [95]. In contrast, in a prolonged fasting state, *de novo* glucose production (gluconeogenesis), using lactate, pyruvate, amino acids and glycerol, is a main source for maintaining euglycemia level [95]. Gluconeogenesis is facilitated by changes of hormone levels such as glucagon, insulin, growth hormone and cortisol [95-97]. These signals stimulate lipolysis in fat tissues to generate ketone bodies through mitochondrial β oxidation and ketogenesis. The ketone bodies provide substrate to obligate glucose-utilizing organs such as the brain. The switch from glucose metabolism to fat metabolism is thought to be mainly driven by an increase of glucagon and a decrease of insulin levels. Perry et al [94] show that hypoleptinemia is a key regulator for this shift. A low level of plasma leptin stimulates the hypothalamic-pituitary-adrenal (HPA) axis, thereby mediating the degradation of white adipose tissue (WAT) (**Figure 4**). This results in an increase of hepatic acetyl-CoA content and pyruvate carboxylase flux, which is essential to maintain gluconeogenesis during prolonged starvation (**Figure 4**). The amount of hepatic acetyl-CoA and the activity of pyruvate carboxylase are key indicators for liver and whole body glucose and fat metabolism. Therefore, leptin is an essential regulator for glucose homeostasis during starvation.

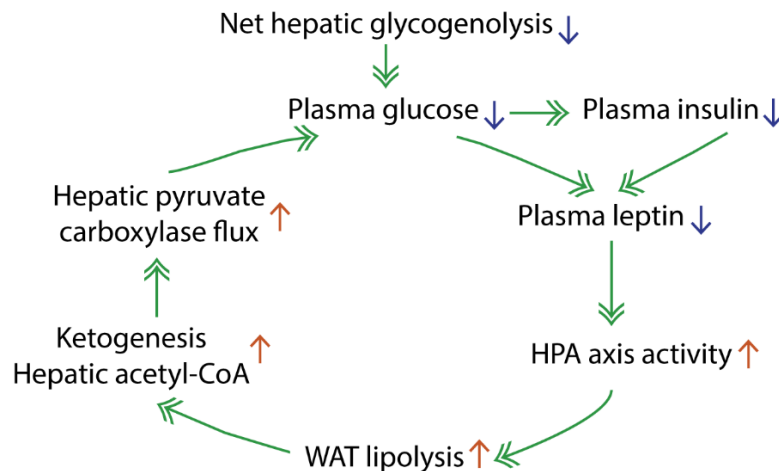


Figure 4. Leptin mediates a glucose-fatty acid cycle to maintain glucose homeostasis in starvation. The figure is adapted from Perry et al., 2018 [94]. In prolonged starvation, glucose generated from hepatic glycogenolysis is reduced, resulting in a decrease of plasma insulin and leptin concentrations. Hypoleptinemia is a critical signal to increase WAT lipolysis via the HPA axis activity. This accordingly results in an increase of hepatic acetyl-CoA content, ketogenesis and hepatic pyruvate carboxylase activity, therefore maintaining the glucose homeostasis during starvation. Orange arrows mean an increase and blue arrows mean a decrease. HPA: hypothalamic-pituitary-adrenal axis, WAT: white adipose tissues.

Congenital leptin mutations in human are extremely rare [98]. Patients with the disease are severely obese, hyperinsulinemic and dyslipidemic [99, 100]. The mean BMI of patients is distinctly decreased after 18 months of leptin replacement [99]. Furthermore, leptin administration normalizes serum glucose, insulin and lipid level [101]. Animal models in rodents with spontaneous mutations in the leptin (*ob/ob*) and leptin receptor (*db/db*) have existed for decades. Mice with the mutation in leptin signaling display hyperphagic, obese and diabetic phenotypes and the circulating leptin level is reduced [102]. Similar with the effects of leptin mutation in humans, recombinant leptin treatment in *ob/ob* mice successfully lowers the body weight, glucose and insulin level [102]. Leptin and leptin receptor are evolutionarily conserved from mammalian to nonmammalian species including zebrafish [103]. Zebrafish have one leptin receptor and two orthologs of the leptin gene, namely *leptin a* (*lepa*) and *leptin b* (*lepb*). Studies show that *lepr* mRNA is also highly expressed in the brain of zebrafish as it is in mammals [104]. The mutation in *lepa* results in an obesity phenotype in adult zebrafish [105]. However, the metabolic effects of leptin mutation in the early development are still largely unknown.

Metabolomics

Metabolomics is defined as large-scale studies of metabolite compositions that are substrates, intermediates and products of biological processes [106]. It focuses on global sets of small molecules with a molecular weight smaller than 1500 Da within cells, tissues, organs and organisms at any given time [107]. The small molecules include sugars, amino acids, organic acids, and lipids. Metabolomics is a powerful approach because the studied metabolites are the end products of cellular processes and they therefore represent an important element of the molecular phenotype [108]. Therefore, the level of metabolites can directly reflect the underlying biochemical condition in a biological sample.

The most common used analytical tools for metabolomics are mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectrometry [109]. In comparison, they both have their specific advantages and disadvantages shown in **Table 1**. MS is a powerful analytical technique as it offers the merits of high sensitivity, selectivity, and high throughput ability [110]. MS provides a mass-to-charge ratio (m/z) of charged molecules to identify components in a sample by determining the molecular weight and its isotopic abundance [107]. MS instruments consist of three main parts: an ionization source, a mass analyzer and a detector. The ionization source converts targeted analytes from liquid and solid phase into gas phase ions [111]. The ions travel through a fixed distance in a mass analyzer before reaching to the detector. The higher m/z ions achieve lower velocity than the lower m/z ions as the ion velocities are inversely proportional to their m/z . Therefore, the ion m/z can be determined by measuring the time of traveling in the mass analyzer [112]. However, samples for MS measurement are destructive. NMR spectroscopy compensates for this limitation of MS.

NMR spectroscopy enables the identification and quantification of metabolites in complex biological samples with a high reproducibility [113]. By applying an external magnetic field, nuclei of molecules can behave like a magnet due to their charge and spin. Thus, the molecular structure of components in a sample can be analyzed by measuring the interaction of nuclear spins. There are solid-state and solution-state NMR methods. In biology, NMR spectrometry is usually applied for the analysis of liquid samples obtained by extraction of tissues with organic solvents. To apply NMR for the analysis of (semi-) solid samples, such as intact tissues, another advanced technique, called high-resolution magic-angle-spinning NMR (HR-MAS NMR) was developed [114]. The most important advantage of HR-MAS NMR is that it directly measures intact samples. It thereby prevents the loss and degradation of metabolites by extraction which are required for the measurements by MS and solution-state NMR. HR-MAS NMR spectrometry spins samples at a magic angle of approximately 54.7° at a frequency of a few thousands of Hertz to reduce the hetero- and homonuclear dipolar coupling of the samples [115, 116]. In this way, it makes it possible to acquire high-resolution spectra of heterogeneous samples based on a small volume (50 microliter).

Therefore, HR-MAS NMR spectrometry has contributed greatly to the metabolomic analysis of small intact organisms such as zebrafish larvae for instance in the work described in this thesis.

Table 1. Advantages and disadvantages of mass spectrometry in comparison with nuclear magnetic resonance spectroscopy as standard analytical tools for metabolomics research [116-118].

	MS	NMR
Sensitivity	High and detection limit reach nanomolar, but can suffer from ion suppression in complex and salty mixtures	Low but can be improved with the use of higher field strength, cryoprobes and dynamic nuclear polarization
Selectivity	Can be used for both selective and nonselective (targeted and nontargeted) analyses	Even though few selective experiments are available such as selective TOCSY, it is in general used for nonselective analysis
Number of detectable metabolites	300-1000+	30-100
Target analysis	Superior for targeted analysis	Inferior for targeted analysis
Sample preparation	More demanding; Needs different LC columns and optimization of ionization conditions	Needs minimal sample preparation
Sample measurement	Usually needs different chromatography techniques for different classes of metabolites	All metabolites that are within the limit of NMR sensitivity can be detected in one measurement
Sample recovery	Destructive technique, but needs a small amount of samples	Nondestructive for HR-MAS NMR; Samples can be recovered and stored for a long time, several analyses can be carried out on the same sample
Tissue samples	Requires tissue extraction. MS can be used to identify metabolites in tissues using MALDI-MS	Using HR-MAS NMR tissue samples can be analyzed directly
In vivo studies	Not possible	Widely used for ^1H magnetic resonance spectroscopy (and to a lesser degree ^{31}P and ^{13}C)

Transcriptomics

Transcriptomics investigates all RNA transcripts of cells, tissues and organisms. It serves as an intermediary omics tool used to study the level of transcription of the entire genome and can also be used to analyze RNA modification, stability and localization [119]. Measuring the expression levels of genes under a particular condition provides information on how the genes are regulated, thereby contributing to better understanding of the genetic functions underlying developmental or disease processes in terms of molecular mechanism. RNA sequencing (RNA-seq) has evolved over a decade and has become an indispensable method to study differential expression of genes [120, 121]. It is a medium throughput technology that has to be combined with computational methodologies to map the sequencing data to the genome. The most commonly used RNA-seq technology is marketed by Illumina, which is mainly used for short read sequencing to a length of 50-500bp [120]. We also used this RNA-seq method extensively in this thesis. The general workflow of short read sequencing starts with RNA isolation of specimens, followed by mRNA enrichment or rRNA depletion, RNA fragmentation, cDNA synthesis, adaptor ligation, PCR amplification, sequencing and analysis (**Figure 5A**). For analysis of eukaryotic gene transcription, the prepared library is commonly sequenced to a read depth of 10-30 million reads per run [120]. The principle of this method is based on detection of four colored fluorophores that are incorporated in synthetic cDNA derived from the RNA (**Figure 5B**). Transcripts can be sequenced in one direction (single-end) as well as both directions (paired-end). The bioinformatic analysis of RNAseq data requires alignment of sequencing reads to an annotated reference genome and normalization between samples [122]. Differential gene expression (DGE) analysis and further analysis such as gene ontology (GO) enrichment are common research tools for the use of RNAseq data sets. The short-read sequencing techniques have the advantages of high throughput, better knowledge of biases and error profiles and applications for degraded RNA. However, imperfections and bias can be introduced due to reproducibility problems in sample preparation and computational analysis. In many eukaryotes, a majority of genes are alternatively spliced to form two or more distinct transcripts. For short-read cDNA sequencing, a significant percentage of reads map ambiguously when an exon is shared between isoforms (**Figure 5C**). This complicates analysis and interpretations of results. Long-read sequencing methods can generate full-length transcript reads, thereby improving differential isoform expression analysis. There are two long read technologies: long-read cDNA sequencing and long-read direct RNA-seq (**Figure 5A**). The long-read cDNA method shares many of the steps of library construction with the short-read method (**Figure 5A**). In the absence of RNA fragmentation, read lengths up to 50kb can be generated. The library preparation of the long-read direct RNA-seq method only requires adaptor ligation with minimal steps. The maximum read length of this method is currently 10kb, but is rapidly improving [123]. The long-read sequencing approaches overcome the limitation of short-read method. It reduces

the ambiguity in the mapping of sequence reads and high rates of false positive splice junction detection (**Figure 5C**). Longer transcripts can be identified, resulting in a more complete capture of transcript diversity. Despite that they are still relatively expensive, it might soon present a challenge to the dominance of Illumina short-read sequencing.

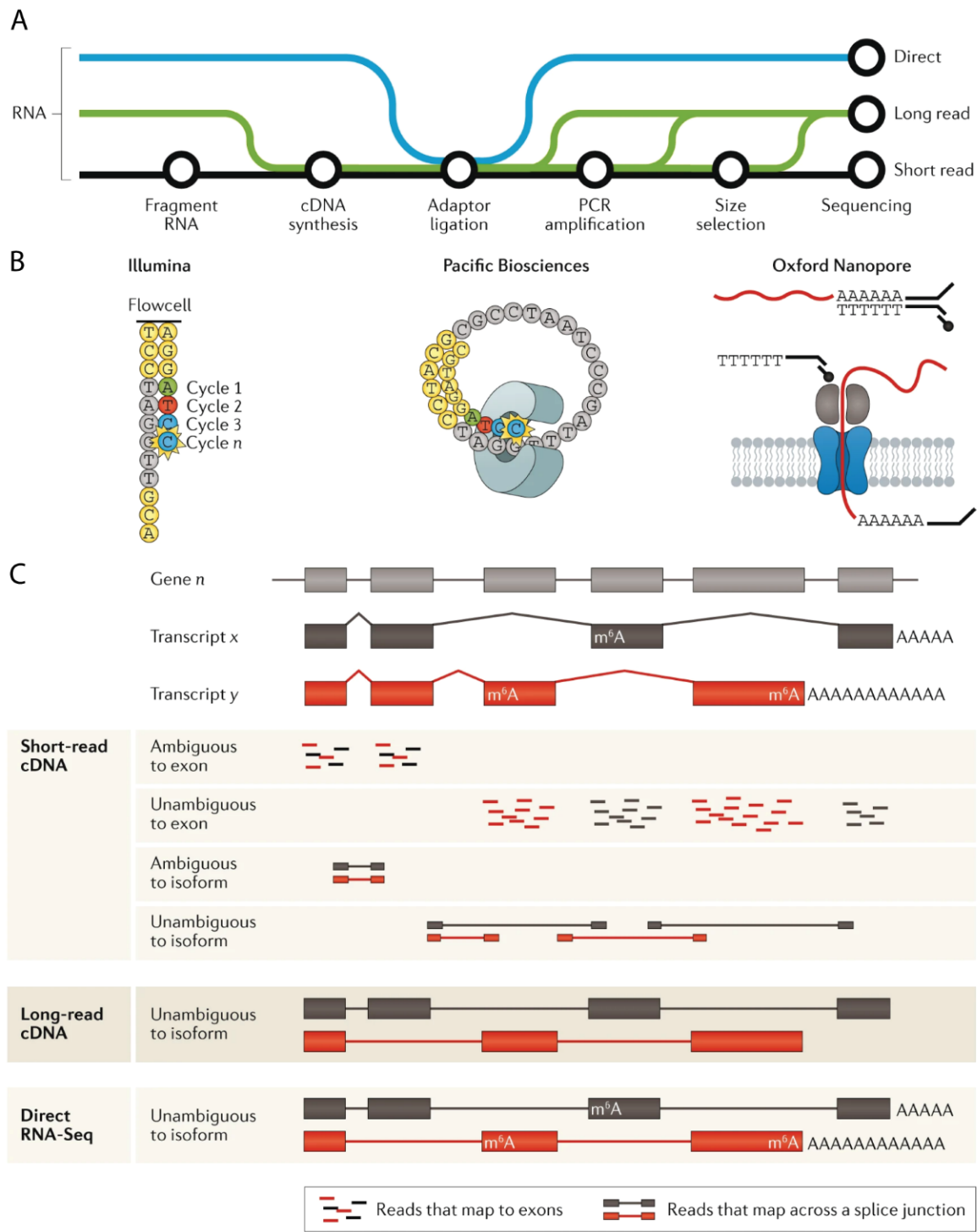


Figure 5 (previous page). Short-read, long-read and direct RNA-seq technologies and workflows. The figure is adapted from Stark 2019 et al [120]. **A.** An overview of library preparation methods for different RNA-seq methods. Black: short-read sequencing. Green: long-read cDNA sequencing. Blue: long-read direct RNA sequencing. **B.** An overview is shown of three main sequencing technologies for RNA-seq. **C.** Comparison of short-read, long-read and direct RNA-seq analysis.

Outline of this thesis

In this thesis, my aims are to study 1) metabolic alterations in tuberculosis related to wasting syndrome in human patients as well as in rodent and fish animal models. 2) effects of the mutation of the leptin gene on cachexia and diabetes in rodent and zebrafish animal models. 3) how tuberculosis infection and resulting metabolic reprogramming are dependent on leptin signaling in mice and zebrafish larvae.

In **Chapter 2**, we obtained metabolic profiles of the blood of TB patients and *Mtb*-infected C57BL/6 mice, and *M. marinum*-infected entire zebrafish larvae and their respective controls measured by MS. Despite that there were huge differences among the tested samples, we found that they shared 10 common biomarkers which could distinguish the disease and healthy state. Furthermore, we confirmed the 10 common biomarkers in zebrafish larvae by using another metabolomic tool NMR spectroscopy.

In **Chapter 3**, we generated and acquired a *lepb* mutant zebrafish line. The leptin mutation in mice leads to hyperphagic, severe obese, insulin resistance and T2DM. In this chapter we described the investigation of the effect of leptin mutation on fat accumulation in tissues and glucose metabolism in adult zebrafish. We found that *lepb* deficiency in zebrafish leads to higher body weight, more visceral fat tissues and higher glucose level, indicating there was a diabetic phenotype similar to that observed in *ob/ob* mice. Moreover, we showed that there was a mild diabetic complication showing in kidneys as observed by thicker glomerular basement membranes.

In **Chapter 4**, we tested and compared the metabolomic profiles of leptin mutants in mice and zebrafish larvae. We found that there are great similarities in terms of metabolite changes in the two species measured by different metabolomic approaches. These metabolites also shared a common set of biomarkers that are found in tuberculosis. To further investigate the mechanisms of the metabolic alterations, we performed deep RNA sequencing. We showed that the transcriptomic signature sets of the mutants of the two species were both enriched in genes with the common GO terms proteolysis and arachidonic acid signaling. In summary, leptin mutation leads to the same evolutionarily conserved metabolic syndrome in adult mice and zebrafish larvae.

In chapter 4, we found there was a connection of metabolite alteration in mycobacterial infection and leptin mutation as well as the reported connection of TB and diabetes in clinical condition. Therefore, in **Chapter 5**, we investigated how mycobacterial infection and leptin mutation affect each other. We tested the metabolic profiles in the leptin mutant mice and zebrafish larvae in the presence and absence of infection. We found that leptin mutation and mycobacterial infection led non-synergistically to a similar metabolic syndrome. In contrast, the RNAseq data of zebrafish larvae samples showed that mycobacteria induce a very different transcriptome signature in the *lepb* mutant compared to the wild type sibling control. Therefore, we concluded that leptin and mycobacterial infection led to a similar dysregulation of metabolism via different genetic mechanisms.

In **Chapter 6**, I summarized and discussed our results and findings described in this thesis and suggested future directions of research.

References

- [1] Berardi E, Madaro L, Lozanoska-Ochser B, Adamo S, Thorrez L, Bouche M, et al. A Pound of Flesh: What Cachexia Is and What It Is Not. *Diagnostics* 2021;11(1).
- [2] Laviano A, Paldino A. Diagnosing cachexia. *Clinical practice* 2014;11(1):71.
- [3] Fearon K, Strasser F, Anker SD, Bosaeus I, Bruera E, Fainsinger RL, et al. Definition and classification of cancer cachexia: an international consensus. *The Lancet Oncology* 2011;12(5):489-95.
- [4] Rosen ED, Spiegelman BM. Adipocytes as regulators of energy balance and glucose homeostasis. *Nature* 2006;444(7121):847-53.
- [5] Fonseca GW, Farkas J, Dora E, von Haehling S, Lainscak M. Cancer Cachexia and Related Metabolic Dysfunction. *International Journal of Molecular Sciences* 2020;21(7).
- [6] Kerekes G, Nurmohamed MT, González-Gay MA, Seres I, Paragh G, Kardos Z, et al. Rheumatoid arthritis and metabolic syndrome. *Nature Reviews Rheumatology* 2014;10(11):691-6.
- [7] Grinspoon S, Mulligan K, Department of H, Human Services Working Group on the P, Treatment of W, Weight L. Weight Loss and Wasting in Patients Infected with Human Immunodeficiency Virus. *Clinical Infectious Diseases* 2003;36(Supplement_2):S69-S78.
- [8] Schwenk A, Macallan DC. Tuberculosis, malnutrition and wasting. *Current Opinion in Clinical Nutrition & Metabolic Care* 2000;3(4).
- [9] Perry BD, Caldow MK, Brennan-Speranza TC, Sbaraglia M, Jerums G, Garnham A, et al. Muscle atrophy in patients with Type 2 Diabetes Mellitus: roles of inflammatory pathways, physical activity and exercise. *Exerc Immunol Rev* 2016;22:94-109.
- [10] Koppe L, Fouque D, Kalantar-Zadeh K. Kidney cachexia or protein-energy wasting in chronic kidney disease: facts and numbers. *J Cachexia Sarcopenia Muscle* 2019;10(3):479-84.
- [11] von Haehling S, Anker MS, Anker SD. Prevalence and clinical impact of cachexia in chronic illness in Europe, USA, and Japan: facts and numbers update 2016. *J Cachexia Sarcopenia Muscle* 2016;7(5):507-9.
- [12] Advani SM, Advani PG, VonVille HM, Jafri SH. Pharmacological management of cachexia in adult cancer patients: a systematic review of clinical trials. *BMC Cancer* 2018;18(1):1174-.
- [13] Webster JM, Kempen LJAP, Hardy RS, Langen RCJ. Inflammation and Skeletal Muscle Wasting During Cachexia. *Frontiers in Physiology* 2020;11:1449.
- [14] Zhang Y, Proenca R, Maffei M, Barone M, Leopold L, Friedman JM. Positional cloning of the mouse obese gene and its human homologue. *Nature* 1994;372(6505):425-32.
- [15] Park H-K, Ahima RS. Physiology of leptin: energy homeostasis, neuroendocrine function and metabolism. *Metabolism: clinical and experimental* 2015;64(1):24-34.
- [16] Farooqi IS, O'Rahilly S. Leptin: a pivotal regulator of human energy homeostasis. *The American Journal of Clinical Nutrition* 2009;89(3):980S-4S.
- [17] Wang B, Chandrasekera PC, Pippin JJ. Leptin- and leptin receptor-deficient rodent models: relevance for human type 2 diabetes. *Curr Diabetes Rev* 2014;10(2):131-45.
- [18] Sáinz N, Rodríguez A, Catalán V, Becerril S, Ramírez B, Gómez-Ambrosi J, et al. Leptin administration favors muscle mass accretion by decreasing FoxO3a and increasing PGC-1 α in ob/ob mice. *PloS one* 2009;4(9):e6808.
- [19] King AJF. The use of animal models in diabetes research. *Br J Pharmacol* 2012;166(3):877-94.

- [20] Zhang F, Basinski MB, Beals JM, Briggs SL, Churgay LM, Clawson DK, et al. Crystal structure of the obese protein leptin-E100. *Nature* 1997;387(6629):206-9.
- [21] Zabeau L, Peelman F, Tavernier J. Leptin and Leptin Receptor. In: Choi S, editor *Encyclopedia of Signaling Molecules*. New York, NY: Springer New York; 2016, p. 1-6.
- [22] Rohm M, Zeigerer A, Machado J, Herzig S. Energy metabolism in cachexia. *EMBO Rep* 2019;20(4):e47258.
- [23] Argilés JM, Busquets S, Stemmler B, López-Soriano FJ. Cancer cachexia: understanding the molecular basis. *Nature reviews Cancer* 2014;14(11):754-62.
- [24] Yoshida T, Delafontaine P. Mechanisms of Cachexia in Chronic Disease States. *Am J Med Sci* 2015;350(4):250-6.
- [25] Sakuma K, Aoi W, Yamaguchi A. Molecular mechanism of sarcopenia and cachexia: recent research advances. *Pflugers Archiv : European journal of physiology* 2017;469(5-6):573-91.
- [26] Kitajima Y, Yoshioka K, Suzuki N. The ubiquitin–proteasome system in regulation of the skeletal muscle homeostasis and atrophy: from basic science to disorders. *The Journal of Physiological Sciences* 2020;70(1):40.
- [27] Bodine SC, Baehr LM. Skeletal muscle atrophy and the E3 ubiquitin ligases MuRF1 and MAFbx/atrogen-1. *Am J Physiol Endocrinol Metab* 2014;307(6):E469-E84.
- [28] Glick D, Barth S, Macleod KF. Autophagy: cellular and molecular mechanisms. *J Pathol* 2010;221(1):3-12.
- [29] Xia Q, Huang X, Huang J, Zheng Y, March ME, Li J, et al. The Role of Autophagy in Skeletal Muscle Diseases. *Frontiers in physiology* 2021;12:638983-.
- [30] Masiero E, Sandri M. Autophagy inhibition induces atrophy and myopathy in adult skeletal muscles. *Autophagy* 2010;6(2):307-9.
- [31] Masiero E, Agatea L, Mammucari C, Blaauw B, Loro E, Komatsu M, et al. Autophagy Is Required to Maintain Muscle Mass. *Cell Metabolism* 2009;10(6):507-15.
- [32] Sandri M, Coletto L, Grumati P, Bonaldo P. Misregulation of autophagy and protein degradation systems in myopathies and muscular dystrophies. *Journal of Cell Science* 2013;126(23):5325-33.
- [33] Webster JM, Kempen LJAP, Hardy RS, Langen RCJ. Inflammation and Skeletal Muscle Wasting During Cachexia. *Frontiers in physiology* 2020;11:597675-.
- [34] Webster JM, Kempen LJAP, Hardy RS, Langen RCJ. Inflammation and Skeletal Muscle Wasting During Cachexia. *Frontiers in Physiology* 2020;11(1449).
- [35] Di Francia M, Barbier D, Mege JL, Orehek J. Tumor necrosis factor-alpha levels and weight loss in chronic obstructive pulmonary disease. *American journal of respiratory and critical care medicine* 1994;150(5 Pt 1):1453-5.
- [36] Mannelli M, Gamberi T, Magherini F, Fiaschi T. The Adipokines in Cancer Cachexia. *International Journal of Molecular Sciences* 2020;21(14).
- [37] Fong Y, Moldawer LL, Marano M, Wei H, Barber A, Manogue K, et al. Cachectin/TNF or IL-1 alpha induces cachexia with redistribution of body proteins. *The American journal of physiology* 1989;256(3 Pt 2):R659-65.
- [38] Oliff A, Defeo-Jones D, Boyer M, Martinez D, Kiefer D, Vuocolo G, et al. Tumors secreting human TNF/cachectin induce cachexia in mice. *Cell* 1987;50(4):555-63.

- [39] Li YP, Chen Y, John J, Moylan J, Jin B, Mann DL, et al. TNF-alpha acts via p38 MAPK to stimulate expression of the ubiquitin ligase atrogin1/MAFbx in skeletal muscle. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 2005;19(3):362-70.
- [40] Narsale AA, Carson JA. Role of interleukin-6 in cachexia: therapeutic implications. *Curr Opin Support Palliat Care* 2014;8(4):321-7.
- [41] Zhou X, Wang JL, Lu J, Song Y, Kwak KS, Jiao Q, et al. Reversal of Cancer Cachexia and Muscle Wasting by ActRIIB Antagonism Leads to Prolonged Survival. *Cell* 2010;142(4):531-43.
- [42] World Health Organization. *Global Tuberculosis Report*. 2020.
- [43] Smith I. *Mycobacterium tuberculosis* pathogenesis and molecular determinants of virulence. *Clin Microbiol Rev* 2003;16(3):463-96.
- [44] Kiazzyk S, Ball TB. Latent tuberculosis infection: An overview. *Can Commun Dis Rep* 2017;43(3-4):62-6.
- [45] Fogel N. Tuberculosis: a disease without boundaries. *Tuberculosis (Edinburgh, Scotland)* 2015;95(5):527-31.
- [46] Pai M, Behr MA, Dowdy D, Dheda K, Divangahi M, Boehme CC, et al. Tuberculosis. *Nature reviews Disease primers* 2016;2:16076.
- [47] Barberis I, Bragazzi NL, Galluzzo L, Martini M. The history of tuberculosis: from the first historical records to the isolation of Koch's bacillus. *J Prev Med Hyg* 2017;58(1):E9-E12.
- [48] Daniel TM. The history of tuberculosis. *Respiratory medicine* 2006;100(11):1862-70.
- [49] Jensen PA, Lambert LA, Iademarco MF, Ridzon R. Guidelines for preventing the transmission of *Mycobacterium tuberculosis* in health-care settings, 2005. 2005.
- [50] Furin J, Cox H, Pai M. Tuberculosis. *Lancet (London, England)* 2019;393(10181):1642-56.
- [51] Mancuso JD, Mody RM, Olsen CH, Harrison LH, Santosham M, Aronson NE. The Long-term Effect of Bacille Calmette-Guérin Vaccination on Tuberculin Skin Testing: A 55-Year Follow-Up Study. *Chest* 2017;152(2):282-94.
- [52] Chopra KK, Singh S. Tuberculosis: Newer diagnostic tests: Applications and limitations. *Indian Journal of Tuberculosis* 2020;67(4, Supplement):S86-S90.
- [53] MacLean E, Broger T, Yerlikaya S, Fernandez-Carballo BL, Pai M, Denkinger CM. A systematic review of biomarkers to detect active tuberculosis. *Nature microbiology* 2019;4(5):748-58.
- [54] Goletti D, Lee MR, Wang JY, Walter N, Ottenhoff THM. Update on tuberculosis biomarkers: From correlates of risk, to correlates of active disease and of cure from disease. *Respirology (Carlton, Vic)* 2018;23(5):455-66.
- [55] Wallis RS, Peppard T. Early Biomarkers and Regulatory Innovation in Multidrug-Resistant Tuberculosis. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* 2015;61Suppl 3:S160-3.
- [56] Goletti D, Petruccioli E, Joosten SA, Ottenhoff THM. Tuberculosis Biomarkers: From Diagnosis to Protection. *Infect Dis Rep* 2016;8(2):6568-.
- [57] Yong YK, Tan HY, Saeidi A, Wong WF, Vignesh R, Velu V, et al. Immune Biomarkers for Diagnosis and Treatment Monitoring of Tuberculosis: Current Developments and Future Prospects. *Frontiers in Microbiology* 2019;10(2789).
- [58] Hasin Y, Seldin M, Lusis A. Multi-omics approaches to disease. *Genome Biol* 2017;18(1):83-.
- [59] Yugi K, Kubota H, Hatano A, Kuroda S. Trans-Omics: How To Reconstruct Biochemical Networks Across Multiple 'Omic' Layers. *Trends in Biotechnology* 2016;34(4):276-90.

- [60] Yugi K, Kuroda S. Metabolism-Centric Trans-Omics. *Cell Systems* 2017;4(1):19-20.
- [61] Preez Id, Luies L, Loots DT. Metabolomics biomarkers for tuberculosis diagnostics: current status and future objectives. *Biomarkers in Medicine* 2017;11(2):179-94.
- [62] Parida SK, Kaufmann SHE. The quest for biomarkers in tuberculosis. *Drug Discovery Today* 2010;15(3):148-57.
- [63] Weiner J, 3rd, Parida SK, Maertzdorf J, Black GF, Repsilber D, Telaar A, et al. Biomarkers of inflammation, immunosuppression and stress with active disease are revealed by metabolomic profiling of tuberculosis patients. *PloS one* 2012;7(7):e40221.
- [64] Banday KM, Pasikanti KK, Chan ECY, Singla R, Rao KVS, Chauhan VS, et al. Use of Urine Volatile Organic Compounds To Discriminate Tuberculosis Patients from Healthy Subjects. *Analytical Chemistry* 2011;83(14):5526-34.
- [65] Luier L, Loots DT. Tuberculosis metabolomics reveals adaptations of man and microbe in order to outcompete and survive. *Metabolomics* 2016;12(3):40.
- [66] Schoeman JC, du Preez I, Loots DT. A comparison of four sputum pre-extraction preparation methods for identifying and characterising *Mycobacterium tuberculosis* using GCxGC-TOFMS metabolomics. *Journal of Microbiological Methods* 2012;91(2):301-11.
- [67] Bucsan AN, Mehra S, Khader SA, Kaushal D. The current state of animal models and genomic approaches towards identifying and validating molecular determinants of *Mycobacterium tuberculosis* infection and tuberculosis disease. *Pathog Dis* 2019;77(4):ftz037.
- [68] Meijer AH. Protection and pathology in TB: learning from the zebrafish model. *Seminars in immunopathology* 2016;38(2):261-73.
- [69] Tobin DM, Ramakrishnan L. Comparative pathogenesis of *Mycobacterium marinum* and *Mycobacterium tuberculosis*. *Cellular microbiology* 2008;10(5):1027-39.
- [70] Cronan MR, Tobin DM. Fit for consumption: zebrafish as a model for tuberculosis. *Disease models & mechanisms* 2014;7(7):777-84.
- [71] Berg RD, Ramakrishnan L. Insights into tuberculosis from the zebrafish model. *Trends in Molecular Medicine* 2012;18(12):689-90.
- [72] Masud S, Torraca V, Meijer AH. Modeling Infectious Diseases in the Context of a Developing Immune System. *Current topics in developmental biology* 2017;124:277-329.
- [73] Lin X, Xu Y, Pan X, Xu J, Ding Y, Sun X, et al. Global, regional, and national burden and trend of diabetes in 195 countries and territories: an analysis from 1990 to 2025. *Scientific Reports* 2020;10(1):14790.
- [74] Roglic G. WHO Global report on diabetes: A summary. *International Journal of Noncommunicable Diseases* 2016;1(1):3.
- [75] Association AD. Diagnosis and classification of diabetes mellitus. *Diabetes care* 2014;37(Supplement 1):S81-S90.
- [76] DeFronzo RA, Ferrannini E, Groop L, Henry RR, Herman WH, Holst JJ, et al. Type 2 diabetes mellitus. *Nature Reviews Disease Primers* 2015;1(1):15019.
- [77] Kim C, Newton KM, Knopp RH. Gestational Diabetes and the Incidence of Type 2 Diabetes. A systematic review 2002;25(10):1862-8.
- [78] American Diabetes A. Diagnosis and classification of diabetes mellitus. *Diabetes care* 2009;32 Suppl 1(Suppl 1):S62-S7.

- [79] Dooley KE, Chaisson RE. Tuberculosis and diabetes mellitus: convergence of two epidemics. *Lancet Infect Dis* 2009;9(12):737-46.
- [80] Al-Goblan AS, Al-Alfi MA, Khan MZ. Mechanism linking diabetes mellitus and obesity. *Diabetes, metabolic syndrome and obesity: targets and therapy* 2014;7:587.
- [81] Asakawa H, Tokunaga K, Kawakami F. Relationship of leptin level with metabolic disorders and hypertension in Japanese type 2 diabetes mellitus patients. *Journal of Diabetes and its Complications* 2001;15(2):57-62.
- [82] Fischer S, Hanefeld M, Haffner SM, Fusch C, Schwanebeck U, Köhler C, et al. Insulin-resistant patients with type 2 diabetes mellitus have higher serum leptin levels independently of body fat mass. *Acta Diabetologica* 2002;39(3):105-10.
- [83] Facey A, Dilworth L, Irving R. A review of the leptin hormone and the association with obesity and diabetes mellitus. *Journal of Diabetes & Metabolism* 2017;8(3).
- [84] Mohammadzadeh G, Zarghami N. Serum leptin level is reduced in non-obese subjects with type 2 diabetes. *Int J Endocrinol Metab* 2013;11(1):3-10.
- [85] Sayeed MA, Azad Khan AK, Mahtab H, Ahsan KA, Banu A, Khanam PA, et al. Leptin Is Reduced in Lean Subjects With Type 2 Diabetes in Bangladesh. *Diabetes Care* 2003;26(2):547-.
- [86] Wang B, Chandrasekera PC, Pippin JJ. Leptin- and leptin receptor-deficient rodent models: relevance for human type 2 diabetes. *Curr Diabetes Rev* 2014;10(2):131-45.
- [87] Meek TH, Morton GJ. The role of leptin in diabetes: metabolic effects. *Diabetologia* 2016;59(5):928-32.
- [88] Amitani M, Asakawa A, Amitani H, Inui A. The role of leptin in the control of insulin-glucose axis. *Frontiers in Neuroscience* 2013;7(51).
- [89] Park H-K, Ahima RS. Physiology of leptin: energy homeostasis, neuroendocrine function and metabolism. *Metabolism* 2015;64(1):24-34.
- [90] Kelesidis T, Kelesidis I, Chou S, Mantzoros CS. Narrative review: the role of leptin in human physiology: emerging clinical applications. *Ann Intern Med* 2010;152(2):93-100.
- [91] Zhang Y, Chua S, Jr. Leptin Function and Regulation. *Compr Physiol* 2017;8(1):351-69.
- [92] Gorska E, Popko K, Stelmazczyk-Emmel A, Ciepiela O, Kucharska A, Wasik M. Leptin receptors. *Eur J Med Res* 2010;15 Suppl 2(Suppl 2):50-4.
- [93] Kwon O, Kim KW, Kim MS. Leptin signalling pathways in hypothalamic neurons. *Cellular and molecular life sciences : CMLS* 2016;73(7):1457-77.
- [94] Perry RJ, Wang Y, Cline GW, Rabin-Court A, Song JD, Dufour S, et al. Leptin Mediates a Glucose-Fatty Acid Cycle to Maintain Glucose Homeostasis in Starvation. *Cell* 2018;172(1):234-48.e17.
- [95] Rui L. Energy metabolism in the liver. *Comprehensive Physiology* 2014;4(1):177-97.
- [96] Aronoff SL, Berkowitz K, Shreiner B, Want L. Glucose Metabolism and Regulation: Beyond Insulin and Glucagon. *Diabetes Spectrum* 2004;17(3):183.
- [97] Kuo T, McQueen A, Chen T-C, Wang J-C. Regulation of Glucose Homeostasis by Glucocorticoids. *Adv Exp Med Biol* 2015;872:99-126.
- [98] Farooqi IS, O'Rahilly S. 20 YEARS OF LEPTIN: Human disorders of leptin action. *Journal of Endocrinology* 2014;223(1):T63-T70.
- [99] Paz-Filho G, Wong ML, Licinio J. Ten years of leptin replacement therapy. *Obesity Reviews* 2011;12(5):e315-e23.

- [100] Montague CT, Farooqi IS, Whitehead JP, Soos MA, Rau H, Wareham NJ, et al. Congenital leptin deficiency is associated with severe early-onset obesity in humans. *Nature* 1997;387(6636):903-8.
- [101] Licinio J, Caglayan S, Ozata M, Yildiz BO, de Miranda PB, O'Kirwan F, et al. Phenotypic effects of leptin replacement on morbid obesity, diabetes mellitus, hypogonadism, and behavior in leptin-deficient adults. *Proceedings of the National Academy of Sciences of the United States of America* 2004;101(13):4531-6.
- [102] Pelleymounter M, Cullen M, Baker M, Hecht R, Winters D, Boone T, et al. Effects of the obese gene product on body weight regulation in ob/ob mice. *Science* 1995;269(5223):540-3.
- [103] Denver RJ, Bonett RM, Boorse GC. Evolution of Leptin Structure and Function. *Neuroendocrinology* 2011;94(1):21-38.
- [104] Liu Q, Chen Y, Copeland D, Ball H, Duff RJ, Rockich B, et al. Expression of leptin receptor gene in developing and adult zebrafish. *General and Comparative Endocrinology* 2010;166(2):346-55.
- [105] Audira G, Sarasamma S, Chen J-R, Juniardi S, Sampurna BP, Liang S-T, et al. Zebrafish Mutants Carrying Leptin a (lepa) Gene Deficiency Display Obesity, Anxiety, Less Aggression and Fear, and Circadian Rhythm and Color Preference Dysregulation. *International Journal of Molecular Sciences* 2018;19(12).
- [106] Clish CB. Metabolomics: an emerging but powerful tool for precision medicine. *Cold Spring Harb Mol Case Stud* 2015;1(1):a000588-a.
- [107] Turi KN, Romick-Rosendale L, Ryckman KK, Hartert TV. A review of metabolomics approaches and their application in identifying causal pathways of childhood asthma. *Journal of Allergy and Clinical Immunology* 2018;141(4):1191-201.
- [108] Johnson CH, Ivanisevic J, Siuzdak G. Metabolomics: beyond biomarkers and towards mechanisms. *Nat Rev Mol Cell Biol* 2016;17(7):451-9.
- [109] Pan Z, Raftery D. Comparing and combining NMR spectroscopy and mass spectrometry in metabolomics. *Analytical and Bioanalytical Chemistry* 2007;387(2):525-7.
- [110] Ren J-L, Zhang A-H, Kong L, Wang X-J. Advances in mass spectrometry-based metabolomics for investigation of metabolites. *RSC Advances* 2018;8(40):22335-50.
- [111] Awad H, Khamis MM, El-Aneed A. Mass Spectrometry, Review of the Basics: Ionization. *Applied Spectroscopy Reviews* 2015;50:158 - 75.
- [112] Glish GL, Vachet RW. The basics of mass spectrometry in the twenty-first century. *Nature Reviews Drug Discovery* 2003;2(2):140-50.
- [113] Emwas A-H, Roy R, McKay RT, Tenori L, Saccenti E, Gowda G, et al. NMR spectroscopy for metabolomics research. *Metabolites* 2019;9(7):123.
- [114] Power WP. High Resolution Magic Angle Spinning-Applications to Solid Phase Synthetic Systems and Other Semi-Solids. *ANNUAL REPORTS OF NMR SPECTROSCOPY* 2003;51:261-96.
- [115] Corsaro C, Cicero N, Mallamace D, Vasi S, Naccari C, Salvo A, et al. HR-MAS and NMR towards Foodomics. *Food Research International* 2016;89:1085-94.
- [116] Augustijn D, de Groot HJM, Alia A. HR-MAS NMR Applications in Plant Metabolomics. *Molecules* 2021;26(4).
- [117] Emwas A-HM, Salek RM, Griffin JL, Merzaban J. NMR-based metabolomics in human disease diagnosis: applications, limitations, and recommendations. *Metabolomics* 2013;9(5):1048-72.
- [118] Emwas A-HM. The strengths and weaknesses of NMR spectroscopy and mass spectrometry with particular focus on metabolomics research. *Metabonomics*. Springer; 2015, p. 161-93.

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- [119] Lowe R, Shirley N, Bleackley M, Dolan S, Shafee T. Transcriptomics technologies. PLoS computational biology 2017;13(5):e1005457.
- [120] Stark R, Grzelak M, Hadfield J. RNA sequencing: the teenage years. Nature reviews Genetics 2019;20(11):631-56.
- [121] Wang Z, Gerstein M, Snyder M. RNA-Seq: a revolutionary tool for transcriptomics. Nature reviews Genetics 2009;10(1):57-63.
- [122] Conesa A, Madrigal P, Tarazona S, Gomez-Cabrero D, Cervera A, McPherson A, et al. A survey of best practices for RNA-seq data analysis. Genome Biol 2016;17(1):13.
- [123] Amarasinghe SL, Su S, Dong X, Zappia L, Ritchie ME, Gouil Q. Opportunities and challenges in long-read sequencing data analysis. Genome Biol 2020;21(1):30.

