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## **The molecular basis of metabolic syndrome: studies in zebrafish**

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### **Citation**

Nowik, N. (2022, March 30). *The molecular basis of metabolic syndrome: studies in zebrafish*. Retrieved from <https://hdl.handle.net/1887/3281256>

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# Chapter 1

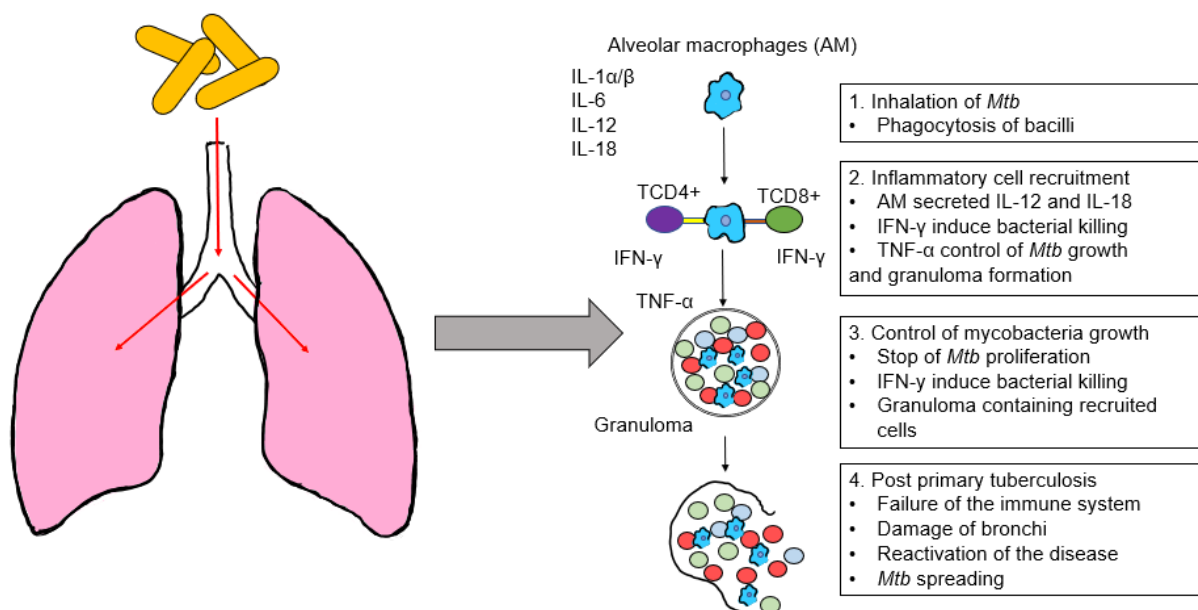
## Introduction

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## I. Tuberculosis

*Mycobacterium tuberculosis* (*Mtb*) infection is currently one of the leading infectious diseases worldwide that has infected approximately one-third of the world population<sup>1</sup> and is a main cause of infection-related death<sup>2</sup>. Although the occurrence of tuberculosis (TB) in Europe is still at relatively stable levels, in some regions of Southeast Asia, Africa and the former Soviet Union, TB in combination with HIV infections contributes greatly to the TB epidemic<sup>3</sup>. What is making TB a global problem is the development of drug resistance by the bacterium. It is mainly caused by mutations that lead to alteration or overproduction of drug target proteins, which results in an altered drug target or titration of the drug respectively<sup>4</sup>. Moreover, it was shown that almost half of all TB-positive patients were infected by multiple-drug-resistant forms of the bacterium, whereas one quarter of them appeared to be resistant to all six drugs commonly used against the infection<sup>5</sup>. What is more, human immunodeficiency virus (HIV) infection has been associated with TB drug resistance, also resulting in inadequate responses to the available drug treatments<sup>6</sup>.

TB results in primary immune responses of an organism<sup>7</sup>. During TB infection, bacteria invade and replicate in macrophages that results in cellular death by apoptosis or necrosis, and formation of granulomas<sup>8</sup>. Further bacterial replication is prevented by antigen specific T cells that promote an effective antimicrobial response via activation of cytokines and targeting the infected macrophages (Fig.1)<sup>9</sup>. In addition, secondary metabolic changes are observed during TB<sup>8</sup>. Prolonged infection leads to an alteration in carbohydrate metabolism and insulin activity that results in impaired glucose tolerance<sup>10</sup>. TB can also have an impact on pancreatic activity that results in pancreatitis and higher susceptibility to inflammation and amyloidosis<sup>11</sup>. Moreover, small numbers of bacteria can be disseminated to the visceral adipose tissue, which could be a cause for the development of a systemic metabolic syndrome in infected patients<sup>12</sup>, because the pathogens can use locally stored fatty acids as a source of carbon. Interestingly, upregulation of some adipokines like MCP-1 (Monocyte Chemoattractant Protein-1), could be advantageous for the bacteria, since it contributes to recruitment of macrophages to the adipose tissue and to the development of insulin resistance<sup>12</sup>. Furthermore, patients with TB suffer from a lowered total cholesterol level and lowered albumin level, mainly during drug-resistant infection and HIV co-infection<sup>13</sup>. Eventually, the metabolic changes result in general undernutrition and lowered body mass index (BMI) of infected patients<sup>14</sup>. The mechanism of this process, which is also known as wasting syndrome, still remains unclear, but a low BMI has been linked to an increased risk of relapse of TB and strongly contributes to the mortality of the disease<sup>15</sup>. In most cases the patients gain weight during the treatment, which is taken as a positive marker of successful response to the medication<sup>16</sup>.



**Figure 1. TB pathogenesis.** TB pathogenesis can be divided in four stages. Macrophages interact through cellular receptors with inhaled mycobacteria which results in phagocytosis of the pathogens (stage 1). Mycobacteria survive and proliferate in the infected macrophages, which induces production of proinflammatory cytokines. The progression of inflammation induces the recruitment of monocytes, neutrophils and dendritic cells to the site of infection (stage 2). Expression of TNF- $\alpha$  leads to control of bacteria growth, followed by induction of T cells, which are organized in characteristic structures called granulomas that stop mycobacteria proliferation and spreading. A characteristic feature of granulomas is the presence of foam cells resulting from the differentiation of chronically activated macrophages (stage 3). At this stage infection can become latent and can become reactivated when immunosuppression occurs (stage 4)<sup>17</sup>.

## II. Tuberculosis and diabetes mellitus type 2

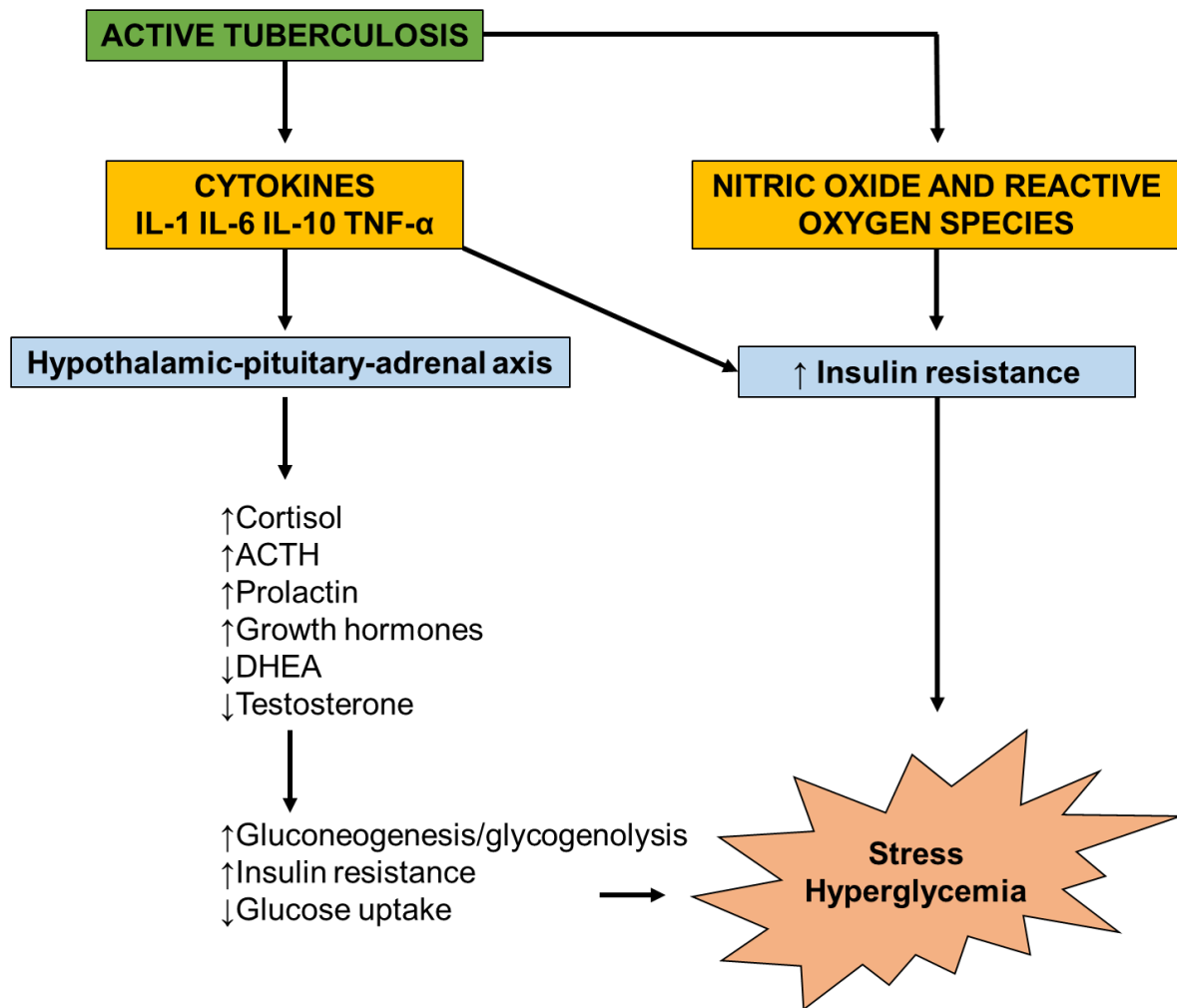
Diabetes mellitus (DM) has become a new worldwide epidemic, as a result of changes in diet, reduction in physical activity and increasing obesity<sup>18</sup>. Like many other factors, such as HIV, that lead to a higher susceptibility to TB, DM increases the risk of TB infection<sup>19</sup>. There are two types of DM which both increase the risk of TB infection about three-fold, as a result of chronic hyperglycemia, although the insulin-resistant DM type 2 (DM2) seems to have a larger contribution to the infection<sup>20,21</sup>. DM2 patients comprise around 90% of the global cases of DM and form a large proportion of the people afflicted with a dual TB/DM burden<sup>22</sup>. Autoimmune type 1 DM (DM1) is also associated with TB susceptibility, but to a smaller extent<sup>23</sup>. The exact mechanisms underlying this increased susceptibility is unclear, but in general it has been shown that patients that suffer from chronic inflammation show hyperglycemia and impaired glucose tolerance<sup>24</sup>. Moreover, an inflammatory state enhances secretion of pro-inflammatory cytokines, some of which have been shown to inhibit insulin signaling<sup>25</sup>. Studies performed in diabetic mice show that hyperglycemic animals have lower interferon- $\gamma$  (IFN- $\gamma$ ) levels, impaired cellular immune responses to TB, higher bacterial burden and broadened infection<sup>26,27</sup>. In turn, TB leads to a higher prevalence of DM2 among infected

patients<sup>28</sup>. Infection affects the glucose metabolism, resulting in stress hyperglycemia and glucose intolerance in TB positive patients<sup>29</sup> and, as a result, a higher predisposition to DM2<sup>30</sup>. Stress hyperglycemia is a result of counter-regulation from multiple signaling pathways including hormones and cytokines. TB-induced hyperglycemia is possibly caused by changes in host immunity, metabolism and the endocrine system that occur during the disease, leading to an increased hepatic glucose production and peripheral insulin resistance<sup>31</sup>. During *Mtb* infection, pro- and anti-inflammatory cytokines, such as interleukin (IL)-1, IL-6, IL-10, interferon (IFN)- $\gamma$  and tumor necrotic factor (TNF)- $\alpha$ , are produced. Furthermore, macrophages generate nitric oxide and reactive oxygen species, as well as activation of T-cells, and natural killer (NK) cells. Increased levels of pro-inflammatory cytokines, reactive oxygen species, and nitric oxide cause hyperglycemia that, through a cascade of inflammatory pathways, results in insulin resistance<sup>32</sup> and decreased glucose uptake<sup>33</sup>. In addition, metabolic and endocrine changes that contribute to the development of hyperglycemia include increased production of cortisol, adrenocorticotropic hormone (ACTH), prolactin and growth hormone that lead to higher glucose production and activated glycogenolysis in the liver and muscles. As a result of the described processes, chronic infection may lead to prolonged stress hyperglycemia (Fig.2) which may eventually develop into pre-diabetes or diabetes, that can persist even after a successful TB treatment<sup>34</sup>.

### III. Animal models in tuberculosis and diabetes research

The current challenges of TB research are connected with the complex pathology of TB infection and the difficulties in treatment. Therefore, there are various experimental animal models to study different aspects of TB, such as clinical signs, pathological changes, bacterial burden, progression of the disease and immunological parameters<sup>35</sup>. An ideal TB model would be able to mimic clinical signs, pathological lesions and metabolic changes that occur in humans. Mice, guinea pigs, rabbits, non-human primates and zebrafish are the most popular research models in TB research<sup>35,36</sup>. Each animal model is used for different research goals. Acute TB in mice models is suitable for evaluation of the efficacy of anti-TB drugs, owing to the possibility to use them to quantify pathological changes and bacterial burden<sup>37</sup>. Guinea pigs are characterized by a strong immune response, which is mostly used to evaluate anti-TB vaccines<sup>38</sup>, whereas monkey TB models develop similar clinical signs and granuloma structures to human TB patients<sup>39</sup>.

The zebrafish is a relatively new alternative animal model to study TB infection, but it is gaining popularity because of several important advantages compared to the other models. TB in zebrafish is caused by a natural pathogen *Mycobacterium marinum*, which causes a systemic disease that shows high similarity with human TB<sup>41</sup>. In humans, infection with *M. marinum* can cause a skin disease called 'tank granuloma'. By using zebrafish larvae, it is possible to track the mycobacterial infection *in vivo*, since larvae are small and transparent and have a fully functional innate immune system. Interestingly, larval zebrafish rely only on



**Figure 2. The role of tuberculosis in the pathophysiology of type 2 diabetes mellitus.** Pro-inflammatory and anti-inflammatory cytokines released during active tuberculosis induce production of cortisol, ACTH, prolactin and growth hormone that leads to gluconeogenesis and glycogenolysis in liver and muscles, increase insulin resistance and decrease glucose uptake, which results in hyperglycemia. IL: Interleukin; TNF: tumor necrosis factor; ACTH: adrenocorticotrophic hormone; T3 & T4: thyroid hormones; DHEA: dehydroepiandrosterone<sup>40</sup>.

the innate immune system and have no developed adaptive immunity, which enables separate studies on innate immunity<sup>42</sup>.

Animal models for DM include models with insulin resistance and models with dysfunction of insulin-producing pancreatic  $\beta$ -cells. Many DM2 animal models display obesity, similarly to humans, and suffer from glucose intolerance and insulin resistance<sup>43</sup>. One of the most popular animal models for diabetes is the Zucker Diabetic Fatty Rat (ZDF), which is characterized by a mutation in the leptin receptor that induces hyperphagia, obesity and glucose intolerance<sup>44</sup>. Another popular DM model is the *ob/ob* mouse, which is characterized by defective leptin signaling due to a mutation in the leptin (*Ob*) gene. The *ob/ob* mice suffer from severe obesity, hyperglycemia and hyperinsulinemia. Other metabolic dysfunctions include hyperlipidemia, lower physical activity and infertility<sup>45</sup>. In a similar model, the *db/db* mouse, alteration in the leptin receptor in also leads to alterations in glucose metabolism and insulin signaling. These

mice are hyperphagic, obese, hyperglycemic and diabetic, and they develop ketosis a few months after birth and have a shorter lifespan than wild types<sup>46</sup>.

The zebrafish is a versatile animal model to study glucose metabolism and development of DM2. Zebrafish larvae become insulin resistant when injected with a high dose of human recombinant insulin, and this way they are used as a model to study insulin signaling and associated disorders<sup>47</sup>. The larvae provide an advantage over other models since they can be used in research on insulin resistance and immunity in a non-obese state. A mutation in the leptin receptor in larval zebrafish results in an increase in  $\beta$ -cell number and dysregulation in the expression of genes that are involved in glucose metabolism in the liver, whereas the adults show normal glucose levels and an increased  $\beta$ -cell mass<sup>48</sup>. Finally, zebrafish can become diabetic after diet-induced obesity (DIO) by overfeeding with artemia. The DIO zebrafish show higher blood glucose levels and insulin resistance after one week of overfeeding, which can be reversed by anti-diabetic treatment<sup>49</sup>. Recent research in adult and larval zebrafish show that it is a useful animal model to study DM2 and associated metabolic disorders<sup>49,50</sup>.

#### **IV. The role of leptin in tuberculosis and diabetes**

As described above, the pathophysiology of TB and DM2 are strongly intertwined. During both conditions there are many organs and cell types that are involved in disease progression. One of the tissues being actively involved in both diseases is adipose tissue. Adipocytes secrete a number of pro-inflammatory cytokines, some of which, such as leptin, affect insulin signaling<sup>51</sup>. Leptin is a hormone, that belongs to the group of cytokines called adipocytokines, as it is mainly produced by adipocytes<sup>52</sup>. Leptin acts both as a hormone and as a cytokine. As a hormone, it regulates endocrine functions, bone metabolism, glucose and energy homeostasis, as well as food intake<sup>53</sup>. As a cytokine, leptin is involved in the inflammatory response and may cause autoimmune diseases<sup>54</sup>.

Recently, it has been shown that leptin may have a significant role in the modulation of the immune responses during TB<sup>55</sup>. TB patients show lowered leptin levels, probably due to the loss of body weight<sup>56</sup>. Generally, during inflammation, infection or sepsis, leptin levels are elevated and correlate with the survival rate of patients<sup>57</sup>. Leptinemia, that occurs during bacterial infection, results from TNF- $\alpha$  and IL-6 activation and has been suggested to enhance pathogen clearance. In contrast, leptin-deficient *ob/ob* mice are more susceptible to infection than wildtype mice, and their increased susceptibility to infection can be reversed by leptin administration<sup>58</sup>. Neutrophils from leptin mutant mice were significantly attenuated in their response to infection, and alveolar macrophages are defective in defense against infection. Importantly, leptin administration restores the function of these cells<sup>58</sup>. Macrophages are especially important for leptin function since they express leptin receptor (LepRb), an isoform of the leptin receptor that can lead to activation of Signal Transducer And Activator Of

Transcription 3 (STAT3)<sup>59</sup>. Studies in *ob/ob* and *db/db* mice have shown that leptin expression is essential for a successful immune response against *Mtb*<sup>60</sup>. Leptin-deficient mice infected with *Mtb* suffer from dysregulated immune responses and impaired bacterial containment. Taken together, these studies indicate an important role of leptin in the response to infection, in particular in TB.

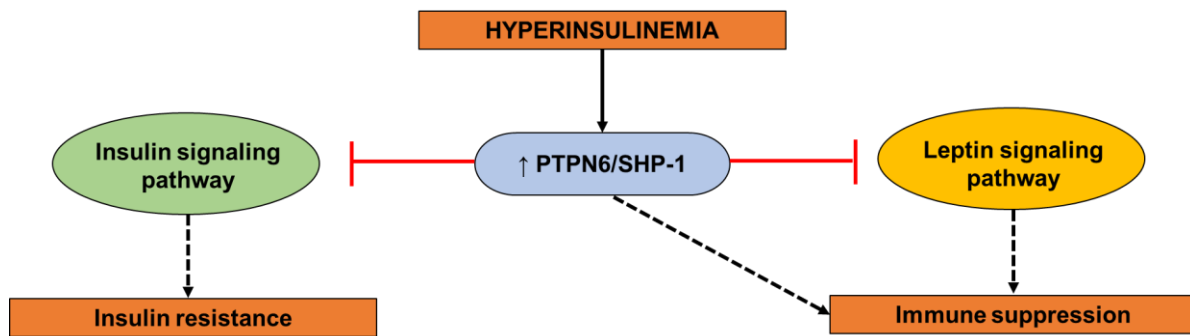
Additionally, leptin contributes to the development of DM2 by modulating glucose homeostasis and the inhibition of insulin synthesis and secretion<sup>61</sup>. Leptin-deficient *ob/ob* mice are characterized by insulin resistance and diabetes<sup>62</sup>, whereas leptin injection lowers blood glucose and insulin levels<sup>51</sup>. Furthermore, leptin injection increases glucose uptake and oxidation in skeletal muscle and decreases the hepatic production of glucose<sup>63</sup>. The leptin signaling pathway works via JAK-STAT, affecting cAMP production and activation of phosphoinositide 3-kinase (PI3K)<sup>64</sup>. The mouse leptin receptor gene gives rise to six isoforms, from which only the long form of leptin receptor (LepRb) is capable to activate JAKs. Janus kinase 2 (JAK2) activation leads to autophosphorylation of multiple tyrosine kinase residues, that create a binding site for STAT molecules<sup>65</sup>. Phosphorylation of STAT3 leads to dimerization and nuclear translocation of this transcription factor, enabling it to regulate the transcription of various genes, such as suppressor of cytokine signaling 3 (SOCS3) that acts in a negative feedback loop to leptin signaling after prolonged stimulation<sup>66</sup>. Taken together, these data suggest that leptin regulates glycemic control in addition to energy homeostasis. Therefore, further studies on leptin signaling could contribute to the development of alternative therapies for restoration of glucose homeostasis.

## **V. The role of protein tyrosine phosphatases in immunity and glucose metabolism**

Protein tyrosine phosphatases (PTPs) are key signaling regulators in many physiological processes. There are eight subtypes of PTPs, which are characterized by a single transmembrane spanning domain, variable N-terminal extracellular regions and a phosphatase domain<sup>67,68</sup>. Regulation of PTP activity occurs through dimerization of the phosphatase domain which results in an inhibition of the phosphatase activity<sup>69</sup>. Mutations in genes encoding PTPs are associated with many diseases, such as cancer<sup>70</sup>, immune disorders<sup>71</sup> and diabetes<sup>72</sup>.

The *motheaten* mouse carries a mutation in the Protein Tyrosine Phosphatase Non-Receptor Type 6 (*Ptpn6*) gene, which encodes the hematopoietic PTP SHP-1. This mouse develops autoimmune disease and inflammation<sup>73</sup> that are marked by alopecia, glomerulonephritis, dermatitis, inflammation of the paws, pneumonitis and high mortality<sup>74</sup>. Moreover, the mutant mice are characterized by overproduction and accumulation of macrophages and neutrophils in the lungs and skin, as well as a higher concentration of pro-inflammatory cytokines, serum immunoglobulins and auto-antibodies<sup>74</sup>. SHP-1 regulates macrophage





**Figure 3. Model for hyperinsulinemia-induced immune suppression and insulin resistance via PTPN6/SHP-1.** Hyperinsulinemia inhibits the insulin signaling pathway, leading to insulin resistance via induction of PTPN6, which encodes the phosphatase SHP-1. SHP-1 plays a role as a negative immune regulator by inhibiting the leptin signaling pathway that results in immune suppression<sup>47</sup>.

function, downregulates IFN- $\gamma$ -mediated macrophage nitric oxide production and IFN- $\gamma$ -inducible gene expression<sup>75</sup>. SHP-1 phosphorylation is increased after TB infection and the protein co-localizes with TB phagosomes, indicating that bacterial phagocytosis by macrophages results in SHP-1 activation and its recruitment to the phagosome. Further phagosome maturation is regulated by the activity of (PI3K), which is inhibited by SHP-1 activation<sup>76</sup>. These findings are important for further TB research and imply the possible use of therapeutics that target SHP-1 for host-directed therapy against TB.

SHP-1-deficient mice show higher insulin sensitivity and glucose tolerance suggesting that this PTP is also involved in glucose metabolism and development of insulin resistance<sup>77</sup>. SHP-1 has also been studied in zebrafish larvae<sup>47</sup>, and it was shown that zebrafish larvae are susceptible to injected human insulin, leading to the inhibition of gluconeogenesis and transient hypoglycemia.

Moreover, larvae treated with a high dose of human insulin develop insulin resistance and a loss of their primary insulin sensitivity. Interestingly, knockdown of *ptpn6* by morpholino oligonucleotides prevents the development of an insulin-resistant state as well as deregulation of other genes involved in the insulin signaling pathway such as genes encoding leptin, (phosphoenolpyruvate carboxykinase 1 (Pck1) and the insulin receptor<sup>47</sup>. Furthermore, *Ptpn6* activation leads to JAK2 dephosphorylation, that is involved in the leptin signaling pathway. These results support the hypothesis that hyperinsulinemia downregulates the insulin signaling pathway resulting in insulin resistance via activation of SHP-1. On the other hand, Shp-1 can play a role as a negative immune regulator by inhibiting the leptin signaling pathway<sup>47</sup>. In summary, SHP-1 regulates the transcription of metabolic and immune signaling pathways, acting as a mediator between insulin signaling regulation and the immune system (Fig.3).

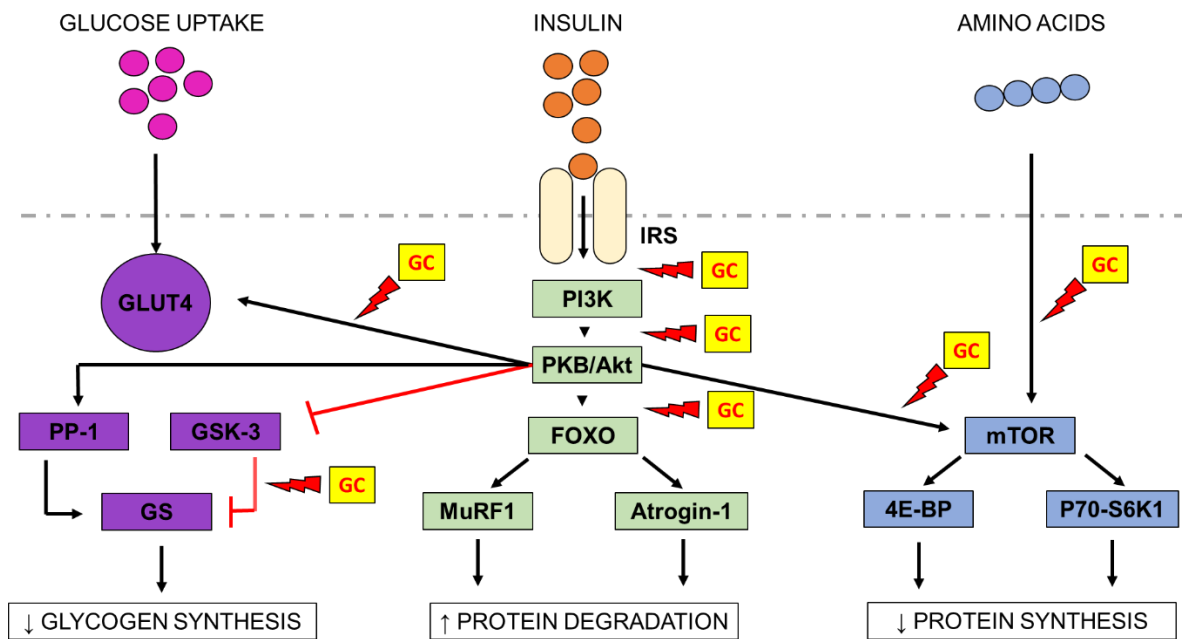
## VI. The effects of glucocorticoids on tuberculosis and diabetes

Glucocorticoids are a class of steroid hormones that regulate various processes in our body and are secreted in response to stress by the adrenal gland<sup>78</sup>. The main endogenous glucocorticoid in our body is cortisol, and its secretion is tightly regulated by the hypothalamic-pituitary-adrenal axis. During their general mode of action, glucocorticoids bind to the glucocorticoids receptor (GR)<sup>79</sup>, which modulates the transcription of genes via two basic mechanisms: regulation of gene expression via interaction with glucocorticoid-responsive elements in the DNA, direct protein-protein interaction with transcription factors such as NF- $\kappa$ B and activator protein 1 (AP-1)<sup>80</sup>.

Because of their immune-suppressive and anti-inflammatory effects, synthetic glucocorticoids such as dexamethasone and prednisolone are frequently prescribed drugs to control inflammation in diverse conditions such as infections and autoimmune diseases. They are widely used in many respiratory diseases like asthma, bronchiolitis, cystic fibrosis, COVID-19 and tuberculosis<sup>81</sup>. Currently, glucocorticoids, such as dexamethasone, are the only approved supporting chemotherapeutics for TB<sup>82</sup>. Glucocorticoid treatment has been proven to be beneficial for subsets of TB patients, by increasing their survival<sup>83</sup> and body mass<sup>84</sup>. This is most likely due to the suppression of the severe inflammatory response in these patients, but the exact mechanism of glucocorticoid action during the disease remains unclear.

Besides the immune-suppressive effects, the treatment with glucocorticoids can result in multiple adverse effects such as glaucoma, hypertension, skeletal muscle atrophy, osteoporosis, obesity and diabetes<sup>85</sup>. In people with TB, a high dosage of glucocorticoids results in hyperglycemia, fluid retention and hypertension<sup>86,87</sup>. It is well established that prolonged administration of glucocorticoids affects glucose metabolism and causes metabolic changes. It has been shown that glucocorticoid treatment is followed by elevated leptin concentrations via transforming growth factor beta (TGF- $\beta$ ) induction of the ALK5-Smad2/3 pathway<sup>88</sup>, and if prolonged, can lead to leptin resistance and changes in fat metabolism<sup>89</sup>. Furthermore, glucocorticoids are well-known to interfere with the insulin pathway and glucose metabolism. Glucocorticoids affect cellular glucose uptake via glucose transporter type 4 (GLUT4) glucose transporters in skeletal muscles, which are responsible for the majority of insulin-mediated glucose uptake, and induce protein degradation and a decrease in protein synthesis via the PKB/Akt and mTOR pathways. As a result, prolonged glucocorticoid administration is a risk factor for insulin resistance, DM2 and muscle wasting (Fig.4)<sup>90,91</sup>.

Thus, glucocorticoids are on the one hand beneficial for TB patients since they suppress the inflammatory response, whereas on the other hand they may enhance the metabolic effects induced by TB and aggravate the TB-related insulin resistance and muscle wasting. A better understanding of the effects of glucocorticoid treatment on the progression of TB would not only improve TB treatment, but also could improve the quality of life of the patients after the infection has been cured.



**Figure 4. Molecular role of glucocorticoids in the insulin pathway and protein turnover.** GLUT4 enables glucose uptake into cell. Glucocorticoids impair the insulin-mediated glucose uptake by directly interfering with the insulin signaling pathway leading to an increase in protein degradation and a decrease in protein synthesis<sup>96</sup>.

## VII. Conclusion

There is undoubtedly a strong association between TB and DM. A recent study shows that DM increases the risk of TB development three-fold. Moreover, DM increases TB severity and mortality in infected patients, as well as the risk of failure of the treatment<sup>91</sup>. In turn, TB patients develop DM more frequently than healthy individuals. TB patients are shown to suffer from stress hyperglycemia, subclinical diabetes, pre-diabetes and clinical diabetes. This process, from normoglycemia towards diabetes, may progress very rapidly in some patients<sup>34</sup>. Both diseases are emerging worldwide epidemics. DM is a major health problem based on lifestyle, diet and genetic predispositions, and TB is globally the infectious disease with the highest mortality, and Co-infection with both TB and HIV causes a serious health problem in developing countries. Moreover, the infection, in most of the cases, is complicated with rapid weight loss, called wasting syndrome that contributes largely to the mortality of the infected patients<sup>92</sup>. Interestingly, treatment with glucocorticoids improves body weight, plasma albumin level and increases appetite in subsets of treated TB patients<sup>93</sup>.

Our immune system and metabolism are intricately intertwined. Production of pro-inflammatory cytokines such as leptin from adipose tissue contributes greatly to a cascade of metabolic changes such as hyperglycemia/insulin resistance, wasting syndrome, dyslipidemia, hypertension and microalbuminuria<sup>94</sup>. Leptin is a protein that plays a dual role in an organism both as a hormone that modulates energy homeostasis and as a cytokine that

promotes the inflammatory response. In monocytes/macrophages, leptin promotes the production of pro-inflammatory cytokines such as IL-6 and TNF- $\alpha$  that induce proliferation and phagocytosis<sup>95</sup>. Furthermore, leptin protects neutrophils from apoptosis and activation of caspases, whereas it induces chemotaxis and expression of several adhesion molecules<sup>40</sup>. An acute inflammatory stimulus, such as TB, results in increased leptin levels<sup>96</sup>, and this response has also been demonstrated in the zebrafish model<sup>97</sup>.

In conclusion, the association between TB and DM is widely recognized, but little is known about the exact mechanisms of the interaction between these two pathologies, providing a strong motivation to understand the underlying mechanisms of TB and DM synergy. Understanding of the bilateral interactions is necessary to develop effective therapeutic strategies to minimize the dual aspects of the diseases.

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