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Metabolic control and sex: *A focus on inflammatory-linked mediators.*

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What is already known: -Men have a higher risk of metabolic disease than women until women progress through menopause.

What this review adds: -Information on how inflammatory pathways linked to metabolic homeostasis differs between males and females.

Clinical significance: -Gender is a factor contributing to differences in inflammatory pathways linked to metabolic phenotypes.

-Highlights the need to investigate male and female animals/participants in future research and therapy development.

Abstract

Background and Purpose: Men and women have many differing biological and physiological characteristics. Thus, it is no surprise, that the control of metabolic processes and the mechanisms underlying metabolic-related diseases have sex-specific components. There is a clear metabolic sexual dimorphism in that up until mid-life, men have a far greater likelihood of acquiring cardio-metabolic disease than women. Following menopause, however, this difference is reduced, suggestive of a protective role of the female sex hormones.

Experimental approach: Inflammatory processes have been implicated in the pathogenesis of cardio-metabolic disease with human studies correlating metabolic disease acquisition or risk with levels of various inflammatory markers. Rodent studies employing genetic modifications or novel pharmacological approaches have provided mechanistic insight into the role of these inflammatory mediators. Sex differences impact inflammatory processes and the subsequent biological response. As a consequence, this may affect how inflammation alters metabolic processes between the sexes.

Key Results: Recently, some of our work in the field of inflammatory genes and metabolic control identified a sexual dimorphism in a pre-clinical model and caused us to question the frequency and scale of such findings in the literature. This review concentrates on inflammatory-related signalling in relation to obesity, insulin resistance and type 2 diabetes and highlights the differences observed between males and females.

Conclusions & Implications: Differences in the activation and signalling of various inflammatory genes and proteins presents another reason why studying both male and female patients or animals is important in the context of understanding and finding therapeutics for metabolic-related disease.

Abbreviations

AMP-activated protein kinase (AMPK), adenosine triphosphate (ATP), brown adipose tissue (BAT), damage-associated molecular patterns (DAMPs), diethylnitrosamine (DEN), Estrogen receptor alpha (ERα), Estrogen receptor beta (ERβ), estradiol (E_2), estrone (E_1), Follicle-stimulating hormone (FSH), glial fibrillary acidic protein promoter driven (GFAP), G-protein coupled estrogen receptor (GPER), heat-shock proteins (HSPs), hepatocellular carcinoma (HCC), high fat diet(s) (HFD), High Mobility Group Box 1 (HMGB1), Inhibitor of κB (IκB) kinase (IKK), Insulin tolerance tests (ITT), Integrin (CD11c), Interleukin 1β **(**IL-1β), Interleukin 6 (IL-6), Interleukin 10 (IL-10), Interleukin-18 (IL-18), intramyocellular triacylglycerol (IMTG), knockout (KO), lipopolysaccharide (LPS), monocyte chemoattractant protein 1 (MCP1), non-alcoholic fatty liver disease (NAFLD), NOD (Nucleotide oliomerization domain), NOD-like receptor protein 1 (NLRP1), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), oral glucose tolerance (oGTT), ovariectomy (OVX), pathogen-associated molecular patterns (PAMPs), pattern recognition receptors (PPRs), respiratory exchange ratio (RER), signal transducer and activator of transcription 3 (STAT3), Toll-like receptor (TLR) 4, Tumor necrosis factor-α (TNF-α), very low density lipoprotein (VLDL), white adipose tissue (WAT).

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Introduction

Compared with premenopausal women, men have a higher risk of insulin resistance (S. H. Kim & Reaven, 2013; Lee, Ko et al., 2016), type 2 diabetes (Kautzky-Willer, Harreiter et al., 2016), and cardiovascular disease (Maas & Appelman, 2010). As women progress through menopause, the sex differences in the prevalence of metabolic disease are reduced (Janssen, Powell et al., 2008; Maas & Appelman, 2010). Even though obesity is more prevalent in women than men (Kanter & Caballero, 2012), a metabolically healthy but obese phenotype seems to be more evident in women (Pajunen, Kotronen et al., 2011). Thus, whereas gluteofemoral fat storage is favored in premenopausal women, both men and postmenopausal women have increased abdominal fat deposition (Abildgaard, Danielsen et al., 2018; Abildgaard, Pedersen et al., 2013; Karastergiou, Smith et al., 2012; Lovejoy, Champagne et al., 2008). Distribution of body fat is an important determinant for metabolic health (Karpe & Pinnick, 2015). Abdominal fat deposition contributes substantially to chronic low-grade inflammation (Schmidt, Weschenfelder et al., 2015) and is closely related to the development of type 2 diabetes and cardiovascular disease (Snijder, Zimmet et al., 2004; Yusuf, Hawken et al., 2005) whereas gluteofemoral fat deposition is associated with an improved metabolic profile (Karpe & Pinnick, 2015; Manolopoulos, Karpe et al., 2010) (see depiction in Figure 1 of differences between sexes). Thus, differences in fat deposition are believed to contribute substantially to the divergence in inflammatory mediators between sexes.

The metabolically healthy pear-shaped body composition seen in premenopausal women is believed to be partly mediated by the female sex hormone, estrogen. Estrogen has been shown to increase fat oxidation (Devries, Hamadeh et al., 2005), inhibit lipogenesis (Homma, Kurachi et al., 2000), and improve adipogenic potential in gluteofemoral adipocytes (Cox-York, Erickson et al., 2017) and studies in rodents, show that loss of ovarian function leads to a sustained diet-independent increase in fat mass (Rogers, Perfield et al., 2009; Stubbins, Holcomb et al., 2012; Stubbins, Najjar et al., 2012) that is prevented by estrogen supplementation (Gorzek, Hendrickson et al., 2007; Stubbins, Holcomb et al., 2012; Stubbins, Najjar et al., 2012). Furthermore, low serum estradiol (E_2) in middle aged women is associated with increased visceral fat mass and hepatic lipid deposition (Abildgaard, Danielsen et al., 2018). Interestingly, oophorectomy (the removal of ovaries and therefore the source of estrogen)-induced adiposity in mice seems to be highly dependent on decreased physical activity following oophorectomy (Gorzek, Hendrickson et al.,

2007; Yonezawa, Wada et al., 2012) – a finding that has yet to be replicated in humans. Besides improving metabolic function, estrogen seems to prevent low-grade inflammation through direct inhibition of leukocyte derived cytokine secretion (Harkonen & Vaananen, 2006; Kramer, Kramer et al., 2004; Kramer, Winger et al., 2007), and plasma levels of pro-inflammatory cytokines vary throughout the menstrual cycle, showing high levels when estrogen is low and low levels when estrogen is high (Bouman, Heineman et al., 2005). Taken together, these studies indicate that estrogen plays an important role in immunometabolism and could, in part, be responsible for the sex differences in regulation of inflammatory-linked mediators.

Estrogen and receptor signaling

Estrogen belongs to the family of steroid hormones and signaling is mediated through two intracellular estrogen receptors (ER), ERα and ERβ, both belonging to the nuclear receptor family of transcription factors (Heldring, Pike et al., 2007). Estrogen signaling is initiated by binding of estrogen to the ER and is followed by a cell specific transcriptional response depending on the composition of co-regulatory proteins (Katzenellenbogen & Katzenellenbogen, 2002). In recent years, a G-protein coupled estrogen receptor (GPER) mediating rapid non-genomic cell signaling has also been identified (Filardo, Quinn et al., 2000). Knock out of the ERα evokes impaired adipocyte function and profound insulin resistance - findings closely corresponding to oophorectomy (Heine, Taylor et al., 2000; Musatov, Chen et al., 2007), indicating that ERα likely mediates many of estradiol's effects on metabolism. In contrast, ERβ knock out mice show decreased ectopic lipid deposition and improved glucose metabolism (Foryst-Ludwig, Clemenz et al., 2008) suggesting that the ERα/ ERβ ratio in the specific tissue determines the metabolic effects of estrogen (Barros & Gustafsson, 2011). The specific impact of GPER on adipose tissue metabolism is more controversial (Barton & Prossnitz, 2015; Prossnitz, Arterburn et al., 2007) but GPER selective agonists might improve pancreatic beta-cell function and glucose homeostasis (Balhuizen, Kumar et al., 2010; Liu, Kilic et al., 2013). Different estrogen derivatives have been shown to have differing affinities to the ER with E_2 as the most potent derivative (Kuhl, 2005). In fertile, non-pregnant women, E_2 is the dominant estrogen primarily produced from the ovaries (Simpson, 2003). After menopause, the less potent estrogen derivative estrone (E_1) is the most abundant, produced from extragonadal sites such as adipose tissue (Hetemaki, Savolainen-Peltonen et al., 2017).

Given the known metabolic gender differences it is, therefore, an important consideration to include both genders in the testing of potential novel therapeutics to treat obesity and diabetes. Indeed, gender differences are observed in relation to a number of current on the market antidiabetic drugs. As reviewed by Franconi and Campesi (Franconi & Campesi, 2014), insulin, biguanides, sulfonylureas, thiazolidinediones, glucagon-like peptide-1 receptor agonists, dipeptidyl peptidase 4 inhibitors and α-Glucosidase inhibitors all have sex-specific differences in relation to either the effectiveness of the treatment, exposure or side-effects that can occur as a result of treatment. Quite often in pre-clinical studies of both metabolism and inflammation only the one gender is studied (with this gender most commonly being male). This is most commonly performed to reduce potential variance due to hormonal fluctuations in the females. Studying just the one sex also keeps costs down and as more males are utilized in studies, utilizing males in your own studies allows for comparisons between studies more easily. However, this approach may be short-sighted and may contribute to missed phenotypes and mechanistic insights.

Recently our work on the genetic alteration of the cytokine Interleukin-18 (IL-18) and its effect on metabolism has produced some further interesting findings in relation to sexual dimorphism. This further highlighted to us the important role sex has in inflammatory-linked metabolic studies and raised the question to us as to how prevalent metabolic sexual dimorphism is in metabolic studies of inflammatory-related cytokines. This review aims to highlight the differences observed between sexes in pre-clinical genetic models focussing on inflammatory-related processes.

Sex differences in immune function

Men have higher prevalence and severity of both viral and bacterial infections than women (Klein, 2000; Roberts, Walker et al., 2001). In contrast, many autoimmune diseases are more common in women (Whitacre, 2001). Sex differences in immune function are perceived as multi factorial. It is believed, that two X-chromosomes provide females with a more extensive repertoire of proteins and thus an increased diversity in the immune response compared to males (Fish, 2008). Furthermore, differences in sex hormones are thought to play a causal role. ERs are present on T-cells, B-cells, macrophages, neutrophils, and NK-cells, among others (Fish, 2008) and estrogen increases the number of regulatory T-cells (Arruvito, Sanz et al., 2007), decrease pro-inflammatory

cytokine secretion from leukocytes (Kramer, Kramer et al., 2004), and increase the anti-inflammatory activity of neutrophils (Garcia-Duran, de Frutos et al., 1999). Lastly, gender related behavioral aspects influence the prevalence of many infectious diseases. Both cultural, behavioral, and anatomical differences between the sexes play a prominent role in the exposure to pathogens and whereas women in sub-Saharan Africa are more than twice as likely to be infected with HIV-1 compared to men (Griesbeck, Scully et al., 2016), men are more likely to suffer from parasitic infections (Zuk & McKean, 1996). Altogether, these findings highly implicate the importance of considering sex-differences in immune based responses and disease.

Sex differences in metabolic syndrome

Environmental/lifestyle factors: High energy diets are a major contributing factor to the growing obesity and diabetes rates worldwide. Rodent models often use high fat diets (HFD) to simulate western diets and related metabolic conditions. In our hands, when using a HFD-intervention to induce obesity and glucose intolerance, we find male mice gain fat mass more rapidly than female mice but by the end of a 12-week dietary period both male and female mice, gain similar amounts of fat mass (Lancaster, Kraakman et al., 2014). Similar findings are observed with glucose tolerance. Male mice are clearly glucose intolerant and have hyperinsulinemia after 4 weeks of high fat feeding, whereas female mice are still protected at that timepoint. It is not until more chronic high fat feeding has taken place that glucose intolerance and hyperinsulinemia is observed in the female mice (Lancaster, Kraakman et al., 2014). Thus, although female mice eventually catch up to their male counterparts in terms of adiposity and glucose intolerance, this is delayed and there is a period of metabolic protection.

One of the major issues with westernised lifestyles with overindulgence in high caloric food that is high in fat and/or sugar and highly processed is that it can provide a source of inflammation to metabolic tissues and affect the immune system. Innate immune cells are able to sense, and are then activated by, pathogens through pattern recognition receptors (PPRs) which recognize exogenous pathogen-associated molecular patterns (PAMPs) (e.g., lipopolysaccharide (LPS)) and damage-associated molecular patterns (DAMPs) originating from compromised cells (e.g., adenosine triphosphate (ATP), uric acid, heat-shock proteins (HSPs)). This in turn, leads to the activation of innate immune cells (S. Sun, Ji et al., 2012). During the development of metabolic

disease, dietary factors could contribute to immune activation from both PAMPs and DAMPs. Over the last decade or so it has been recognised that alterations to the gut microbiota (dysbiosis) occurs with obesity and/or energy dense, poorly nutritious diets. Overproduction of LPS in this setting and increased mobility of this and other PAMPs from the gut through the intestinal barrier due to increased gut permeability can increase the exposure of metabolic tissues to inflammatory stimuli and initiate or potentiate inflammatory processes. Nutrient excess can be directly sensed by the pathogen receptors. Saturated long-chain fatty acids, deleterious lipids such as ceramides, as well as glucose and cholesterol have all been shown to activate components of these pathways. (Reviewed in (Jin, Henao-Mejia et al., 2013). Immune receptors can also be triggered by the production of endogenous DAMPs associated with alterations due to dysregulated metabolism. Such signals may include High Mobility Group Box 1 (HMGB1), fetuin, amyloid deposits, hyaluronan, and uric acid which are increased in metabolically compromised models and individuals and may contribute to the inflammatory milieu (Reviewed in (Jin, Henao-Mejia et al., 2013).

Sex differences in inflammatory-related metabolic syndrome

Closely linked to environmental risk factors for obesity and type 2 diabetes is the observation of chronic low-grade inflammation in metabolically compromised patients and animal models. Circulating and tissue-specific accumulation of pro-inflammatory cytokines has been investigated as contributing factors to dysfunctional metabolism and arise due to or a combination of genetic predisposition, high caloric intake and sedentary behavior.

Adipose tissue: The adipose tissue is an important organ in the regulation of metabolism via its actions in contributing as an energy storage sink, removing glucose and lipids from the blood-stream in the post-prandial state, as well as working as an active endocrine gland secreting adipokines. Infiltration of immune cells and activation of inflammatory cascades in adipose tissue has the potential to disrupt the metabolic actions of the adipose tissue beds and contribute to disruption in whole-body metabolic control (Exley, Hand et al., 2014). Interestingly, the induction of white adipose tissue expression of many inflammatory and immune related genes such as F4/80 (macrophage marker), Integrin (CD11c), monocyte chemoattractant protein 1 (MCP1) and tumor necrosis factor alpha (TNFα) in response to high fat feeding are markedly down-regulated in the

female mice compared to male counterparts (Lancaster, Kraakman et al., 2014). This reduced adipose tissue gene expression of inflammatory cytokines has been recapitulated by others (Singer, Maley et al., 2015). Accordingly, oophorectomy of female mice has been shown to induce severe adipose tissue inflammation including significant infiltration of macrophages and T-cells, and increased protein levels of TNF-α and interleukin 6 (IL-6) (Ludgero-Correia, Aguila et al., 2012). This is reflected in general systemic inflammation in the oophorectomized mouse including increased plasma levels of TNF-α, IL-6, and IL-18, and general leukocytosis (Cenci, Toraldo et al., 2003; Ludgero-Correia, Aguila et al., 2012; Stubelius, Andersson et al., 2017). Furthermore, LPS injections leads to increased adipose tissue and plasma IL-6 levels and a more evident fever response in oophorectomized mice compared to SHAM control mice (Iwasa, Matsuzaki et al., 2014), all in all suggesting that loss of ovarian function mediates an increased inflammatory response.

Liver: During obesity, lipid accumulation arises in the liver leading to non-alcoholic fatty liver disease (NAFLD). In humans, NAFLD more often affects men; and premenopausal women are equally protected from developing NAFLD (reviewed by Ballestri *et al* (Ballestri, Nascimbeni et al., 2017)). It has been suggested that pre-menopausal females are protected against lipid accumulation due to partition of fatty acids towards ketone body production rather than very low density lipoprotein (VLDL)-triacylglycerol. Equally, oophorectomy leads to lipid deposition and estrogen treatment after ovariectomy protects against fatty liver (Zhu, Brown et al., 2013). At the same time oophorectomy also leads to an upregulation of proinflammatory mediators (Inhibitor of κB (IκB) kinase (IKK), IL-6, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB)), in the liver, and this is counteracted by estrogen supplementation (Kireev, Tresguerres et al., 2010; Pighon, Gutkowska et al., 2011), suggesting that the loss of ovarian function also increases inflammation in the liver.

Skeletal muscle: Skeletal muscle is an insulin-sensitive tissue that plays a vital role in the disposal of glucose in the post-prandial state. Women have greater insulin-stimulated leg glucose uptake than matched men despite having higher intramyocellular triacylglycerol (IMTG) content (L. Hoeg, Roepstorff et al., 2009) while increasing lipid levels through lipid infusion results in less insulin resistance of skeletal muscle glucose uptake in women than men (L. D. Hoeg, Sjoberg et al., 2011). Interestingly, skeletal muscle cells from premenopausal women (high-estrogen

environment) showed a lower stress response after prolonged *in vitro* fatty acid (palmitate) treatment compared to skeletal muscle cells from postmenopausal women (low-estrogen environment) (Abildgaard, Henstridge et al., 2014). While Torres *et al.* recently showed, that estrogen has a direct impact on the mitochondrial membrane viscosity and improves bioenergetic function of the mitochondrion (Torres, Kew et al., 2018), suggesting that the sex mediated differences in lipid tolerability could be partly mediated by estrogen. Although not as highly studied, like other peripheral insulin-sensitive tissues, inflammation has been linked to skeletal muscle insulin resistance. Skeletal muscle can secrete cytokines and other factors and may become inflamed with disrupted metabolism. Likewise, circulating immune cells can infiltrate into the muscle bed and increase inflammatory processes potentially contributing to a decrease in insulin signaling processes. Despite these common findings, for the purposes of this review we have not been able to identify a paper that has made a side by side comparison of skeletal muscle inflammatory and immune markers between males and females of any species upon conditions of dietary or genetic obesity or other models of dysregulated metabolism. Chronic low-grade inflammation may also be important in other metabolic tissues such as the gut, pancreas and the brain.

Sex differences in pro-inflammatory-related mediators

Interleukins are a subgroup of cytokines that are immunomodulating proteins secreted by the immune system in response to stimuli such as infection, trauma and inflammation. They aid in cell to cell communication and act in an autocrine, paracrine and endocrine fashion relaying chemical messengers. Once produced and secreted from its cell of origin, interleukins travel to their target cell(s) and bind(s) to its receptor where they have been shown to play many physiological functions. They are designated numerically (there are 15 in total) and we will now discuss the main interleukins involved in metabolic control and known sex differences in these proteins.

***Interleukin 1β* (IL-1β):** IL-1β has been linked to disturbed metabolic homeostasis in both human and animal models. IL-1β concentrations in adipose tissue is increased in diet-induced and genetically-induced models of obesity (Stienstra, Joosten et al., 2010). Deletion of its receptor (IL-1R KO) is protective against HFD-induced insulin resistance (McGillicuddy, Harford et al., 2011) while administration of recombinant Il-1β leads to insulin resistance (Wen, Gris et al., 2011).

Furthermore, reductions in IL-1β expression is associated with improvement of insulin-sensitivity upon weight loss, while elimination of Nlrp3 signaling (the Nlrp3 inflammasome regulates caspase-1 activation that allows IL-1β to be released) is beneficial for insulin and glucose tolerance (Vandanmagsar, Youm et al., 2011). Deletion of the IL-1 receptor-associated kinase 1 (IRAK-1) another pro-inflammatory signaling mediator that acts via IL-1 receptor/Toll-like receptors also leads to improved insulin sensitivity (X. J. Sun, Kim et al., 2017). Apart from a calorie restriction study (Vandanmagsar, Youm et al., 2011), all these studies were conducted in male mice only and there is a scarcity of data in female models that we can identify. Anikinra is a recombinant human IL-1 receptor antagonist that has been shown in trials to be beneficial in patients with type 2 diabetes as it reduces glycated hemoglobin levels (Larsen, Faulenbach et al., 2007). Reduced markers of systemic inflammation (C-Reactive protein, and IL-6) were also observed in the patients with this treatment compared to placebo (Larsen, Faulenbach et al., 2007). In this trial, both male and female patients were enrolled for the study but with far more men (50 compared to 19). However, there is no breakdown of the treatment effect between sexes to delineate whether this IL-1 receptor antagonism was equally effective in both sexes. Even if there was, it is likely to be underpowered to determine any real effect due to the lack of female participants compared to male in the cohort.

The monoclonal antibody canakinumab which inhibits IL-1β is currently being tested in a large clinical trial named the Canakinumab Anti-inflammatory Thrombosis Outcomes Study (CANTOS). Despite large reductions in hsCRP and IL-6, canakinumab did not reduce the incidence of new-onset diabetes over a median follow up period of 3.7 years. Treatment did reduce HbA_{1c} levels compared to placebo during the first 6 to 9 months of the treatment, but no long-term benefits on HbA_{1c} or fasting plasma glucose were observed (Everett, Donath et al., 2018) So although canakinumab reduces inflammatory levels and lowers cardiovascular event rates in patients, it does not seem to lead to the prevention of diabetes among patients with pre-diabetes. The study was adjusted for sex in the analysis but no results are displayed to see if either gender responded better or worse to this treatment regime.

Toll-like receptor (TLR) 4 (a member of the IL-1 receptor superfamily) is a pattern-recognition receptor and an important component of the immune system in initiating inflammatory signaling via alterations to gene transcription. It plays a critical role in mediating the response to pathogens especially as the receptor for LPS. Metabolic sexual dimorphism is also present in $TLR4^{-/-}$ mice. Despite HFD-induced inflammatory gene expression being attenuated in $TLR4^{-/-}$ mice in both sexes, whole-body metabolic phenotypes were different. While male $TLR4^{-/-}$ mice were not different in comparison to WT mice in terms of body weight, food intake or insulin sensitivity in both chow fed and HFD-fed mice there was a distinct phenotype in female mice (Shi, Kokoeva et al., 2006). Female mice were fed chow or HFD for a period of 39 weeks. No matter which diet was utilised, female $TLR4^{-/-}$ mice were significantly heavier than WT mice with the $TLR4^{-/-}$ mice on the HFD almost 10 grams heavier by study end. This increase in weight was due solely to an increase in adiposity with lean mass remaining unchanged and was most likely due to an increase in food intake as energy expenditure measured in metabolic chambers was not different.

Despite the typical paradigm that obesity drives insulin resistance, the female $TLR4^{-/-}$ mice were more insulin sensitive on insulin tolerance testing (Shi, Kokoeva et al., 2006). Due to the food intake data, this study suggested a role for TLR4 in the regulation of food intake but specifically in female mice, quite a unique finding that a genetic model with impact food intake in a sex-dependent fashion. Interestingly, similar to this study, differences upon macrophage-specific deficiency of TLR4 were also only observed in female mice, suggesting a sexual dimorphism in the effects of macrophage TLR4 expression (Coenen, Gruen et al., 2009). Macrophages have been implicated in the development of both insulin resistance and atherosclerosis. Using a bone marrow transplantation procedure to delete TLR4 from macrophages (Mθ $TLR4^{-/-}$ mice), the authors demonstrated a decrease in macrophage and inflammatory markers in white adipose tissue of female chow-fed Mθ $TLR4^{-/-}$ mice compared to Mθ $TLR4^{+/+}$ mice but not in male mice. This was also associated with decreased atherosclerotic lesion area in the females. Despite this no difference was observed in body composition (Coenen, Gruen et al., 2009), nor when mice were challenged with a HFD. Thus, under certain metabolic and dietary conditions but only in female mice, TLR4 may at least in part mediate macrophage accumulation in adipose tissue and vessels.

Interleukin 6 (IL-6): IL-6 is a pro-inflammatory cytokine that is somewhat paradoxically found elevated in the plasma of obese individuals and linked to the induction of insulin resistance, while at the same time identified as a factor that is released from skeletal muscle and associated with many of the beneficial metabolic aspects of exercise (reviewed in (Pal, Febbraio et al., 2014)). Plasma IL-6 is increased in both rodents and humans with declined ovarian function compared to their fertile counterparts (Cioffi, Esposito et al., 2002; Stubelius, Andersson et al., 2017) but the specific role of IL-6 in relation to ovarian function is not clear. While traditionally IL-6 was seen as a secreted factor from immune cells involved in inflammatory processes, the findings that it is released from metabolic organs such as adipose tissue (as an adipokine) (Fried, Bunkin et al., 1998). and skeletal muscle (myokine) (Steensberg, van Hall et al., 2000) further linked its potential importance in metabolic control. To tease out its role and importance, many genetic mouse models of IL-6 have been produced. Whole-body deletion of IL-6 ($IL6^{-/-}$ mice) revealed an obesity phenotype and related insulin resistance (Matthews, Allen et al., 2010; Wallenius, Wallenius et al., 2002). In the initial study by Wallenius and colleagues (Wallenius, Wallenius et al., 2002), both sexes were assessed. An increase in adiposity with IL-6 deletion was consistently shown in both sexes as well as increased leptin levels and leptin insensitivity (leptin resistance) in older mice. $IL6^{-/-}$ female mice displayed an altered circulating lipid profile (increased triglyceride and very-low density lipoprotein) however, there was no such difference in the lipid profile of the male $IL6^{-/-}$ mice compared to WT. Decreased glucose tolerance was also observed in the female mice however, equivalent data was not provided for male mice (Wallenius, Wallenius et al., 2002). A later study did indeed indicate that male $IL6^{-/-}$ mice displayed insulin resistance on chow and HFD with concomitant liver inflammation observed (Matthews, Allen et al., 2010). Together, it is likely that IL-6 is necessary for the maintenance of weight and glucose homeostasis in both sexes, while complete loss of IL-6 plays a role in the regulation of the circulating lipid profile specifically in females.

To determine the impact of tissue-specific loss of IL-6 on metabolism the Hidalgo laboratory has conducted numerous studies utilizing cohorts of floxed IL-6 mice to investigate the metabolic effect of loss of IL-6 in a particular tissue/organ. Interestingly, many characteristics of these models occur in a sex- dependent manner. Deletion of IL-6 in the central nervous system (CNS) specifically in astrocytes (Ast-$IL-6^{-/-}$ mice, produced by crossing IL-6 floxed mice with glial fibrillary acidic protein promoter driven (GFAP)-Cre mice), led to an progressive increase in the

body weight of the Ast-IL-6$^{-/-}$ mice compared to floxed control mice after 8 weeks of age, but only in male mice (Quintana, Erta et al., 2013). This was potentially due to a decrease in the activity levels of the Ast-IL-6$^{-/-}$ mice. However, as the decrease in activity was seen in both sexes, why then the females were protected from weight gain is somewhat of an unsolved story. Associated with activity, the male floxed control mice had a decrease in exploratory behavior compared to females as measured by total number of head dips and time spent head dipping in a Hole-board apparatus test. This defect was completely rescued in the Ast-IL-6$^{-/-}$ male mice while head dipping measures remained unchanged in the females (Quintana, Erta et al., 2013). Interestingly there are also sex differences observed with transgenic upregulation of IL-6 in astrocytes (GFAP-IL-6 mice) (Hidalgo, Florit et al., 2010). Although both sexes were resistant to HFD-initiated obesity, the female GFAP-IL6 mice were much more resistant. This corresponded with a drop in the weight of the visceral adipose tissue depots in the females where there was no such effect in males. In fact the decrease in weight in the males appeared to have nothing to do at all with the prevention of the accumulation of fat mass but instead was due to a decrease in the size of the liver (Hidalgo, Florit et al., 2010). Consequently, both the deletion and up-regulation of IL-6 in these cells leads to sex-specific metabolic phenotypes.

Another tissue-specific model that has been investigated is the muscle-specific IL-6$^{-/-}$ mouse (mIL-6$^{-/-}$). In this instance there were marked differences in the response to the deletion between the sexes with opposing body weights observed (Ferrer, Navia et al., 2014). While male mice on both a chow control diet and a HFD were protected from weight gain, compared to floxed control mice, the female mice were in fact significantly heavier than their floxed controls (Ferrer, Navia et al., 2014). This was due to an increase in adiposity as the male mIL-6$^{-/-}$ mice had lower gonadal and subcutaneous white adipose tissue (WAT) mass as well as lower brown adipose tissue (BAT) mass. On the contrary, females displayed higher gonadal and subcutaneous WAT (Ferrer, Navia et al., 2014). As liver and *tibialis* muscle weights were not different, this is suggestive that alterations in fat pad weights were driving the body weight observation. Food intake was not different between the sexes suggesting energy intake was not driving the body composition findings, but in line with the body weight findings, male mIL-6$^{-/-}$ mice were more active and females less active than floxed controls of each sex in a hole-board test (Ferrer, Navia et al., 2014). From a glucose control perspective, male mIL-6$^{-/-}$ mice had decreased blood glucose and insulin levels, whereas females did not have differing glucose or insulin concentrations but did have an increase in circulating

leptin levels (Ferrer, Navia et al., 2014). To assess glucose control, oral glucose tolerance (oGTT) and insulin tolerance tests (ITT) were performed. In line with the body weight phenotype, male mIL-6$^{-/-}$ mice tended to have improved glucose excursions on a HFD but these values did not reach statistical significance.

In a follow up study, Molinero and colleagues identified that loss of IL6 in the muscle results in lower core body temperature and a higher respiratory exchange ratio (RER) in the light phase (the inactive rest phase for a mouse) (Molinero, Fernandez-Perez et al., 2017). Using indirect calorimetry it was found that the female mice but not the male mice had an increased energy expenditure (Molinero, Fernandez-Perez et al., 2017) which was somewhat conflicting with the previous finding of an increased body weight and adipose weight in these female mice. Also conflicting was the fact that no physical activity differences were observed in this analysis indicating the previous finding of increased activity on the hole-board test may indicate exploration rather than activity increases (Molinero, Fernandez-Perez et al., 2017). Nevertheless, these studies provide a clear indication of the important interaction between the expression of genes in the muscle and sex-specific characteristics and their overall impact on whole-body metabolism.

The adipose tissue is a vital metabolic organ in that it not only acts as an insulator, an energy storage sink and a contributor to post-prandial glucose disposal but is an important endocrine organ actively secreting adipokines such as leptin and adiponectin. Many cytokines including IL-6 are also adipokines and secreted from the adipose depots throughout the body. Consequently, the role of IL-6 specifically derived from the adipose in contributing to metabolic homeostasis is of interest. To investigate this, Navia and colleagues generated adipose specific IL-6$^{-/-}$ mice by breeding IL-6 floxed mice with aP2-cre mice (aP2-IL6$^{-/-}$mice) (Navia, Ferrer et al., 2014). Once again, in line with the astrocyte and muscle-specific models, sex differences were observed. The female aP2-IL6$^{-/-}$mice fed a HFD put on less weight than their control mice but this effect was not observed in the male aP2-IL6$^{-/-}$mice (Navia, Ferrer et al., 2014). The difference in weight was likely due to a decrease in the expansion of both the gonadal and subcutaneous WAT regions, although the liver weight was also shown to be lower (Navia, Ferrer et al., 2014). Deficiency of adipose IL-6 also decreased fasting circulating insulin and cholesterol levels specifically in the female mice. Reminiscent of the mIL-6$^{-/-}$ model, despite the body mass, adipose weight and plasma insulin findings (in the female mice), no difference in ITT and GTT's were observed for either sex

(Navia, Ferrer et al., 2014). Together studies into the role of IL-6 in various tissues demonstrates a robust sex effect on numerous metabolic parameters.

A cancer closely linked to compromised metabolic homeostasis and obesity is hepatocellular carcinoma (HCC), the most common form of liver cancer. Interestingly this cancer occurs more frequently in men, reported to be diagnosed at 3-5 times the rate in males as it is in females (Bosch, Thabut et al., 2004). This sexual dimorphism also holds true in animal models of this condition (Maeda, Kamata et al., 2005). The development of HCC follows a timeline whereby first there is the initial development of hepatic steatosis before the later activation of inflammatory pathways in the liver, resulting in an environment conducive to cancer growth. Experimental evidence suggest that IL-6 is a critical factor in both the initiation of the cancer and the sexual dimorphism. Administration of the chemical carcinogen, diethylnitrosamine (DEN) (which leads to HCC development) increases IL-6 levels to a higher degree in males than in females, while loss of IL-6 neutralises the gender disparity in HCC development (Naugler, Sakurai et al., 2007). Experiments designed to test the female sex hormone hypothesis by treating male mice with estrogen were successful in reducing IL-6 levels and suppressing liver injury (Naugler, Sakurai et al., 2007). Obesity caused by genetic alterations or dietary means also has the capacity to promote hepatic inflammation and drive tumorigenesis, a process that involves the induction of IL-6 and the activation of the transcription factor linked to cancer development, signal transducer and activator of transcription 3 (STAT3) (Park, Lee et al., 2010).

IL-18: IL-18, a member of the interleukin-1 (IL-1) family, is a cytokine that has been linked to alterations in metabolic homeostasis. Clinically, increased levels of IL-18 correlate with metabolic syndrome traits such as body mass index (BMI), waist circumference, plasma triglyceride and fasting glucose and insulin concentrations in both men and women (Hung, McQuillan et al., 2005), suggestive of a role of this cytokine in the pathogenesis of the metabolic syndrome. However, genetic manipulation of IL-18 in animal models has revealed the opposite. Whole-body deletion of IL-18 (IL-$18^{-/-}$ mice) or mice who have had the IL-18 receptor alpha knocked out (IL-$18r^{-/-}$), in fact, develop hyperphagia, obesity and insulin resistance (Netea, Joosten et al., 2006). Furthermore, mice transgenic for IL-18 binding protein (IL-18BP), a natural antagonist of IL-18 receptors, also developed insulin resistance (Netea, Joosten et al., 2006). Demonstrating that raising IL-18 levels may be metabolically protective, recombinant IL-18 (rIL-18) administered

intracerebrally or intraperitoneally inhibited food intake and reversed hyperglycemia (Netea, Joosten et al., 2006; Zorrilla & Conti, 2014; Zorrilla, Sanchez-Alavez et al., 2007). In genetically manipulating IL-18 signaling, IL-18 receptor alpha knocked was chosen, as IL-18Rα chain, is responsible for the extracellular binding of IL-18. Although, mice deficient in the IL-18Rβ chain exist; metabolic studies have not been performed. To our knowledge knock-out of both receptors does not exist.

Work from our laboratory has also substantiated these findings and provided further mechanistic insight. Treating myotubes in cell culture or skeletal muscle strips with IL-18 activated the critical metabolic regulator AMP-activated protein kinase (AMPK) and increased rates of fat oxidation, while *in vivo* electroporation of IL-18 into skeletal muscle to increase its expression also activated AMPK and markers of mitochondrial metabolism (Lindegaard, Matthews et al., 2013). Other work we were involved with, found that mice lacking the NOD (Nucleotide oligomerization domain)-like receptor protein 1 (NLRP1) inflammasome have an impaired capacity to produce IL-18 and as a consequence phenocopy mice lacking IL-18, with obesity and lipid accumulation observed (Murphy, Kraakman et al., 2016). Conversely, mice with an activating mutation in NLRP1, (and therefore increased IL-18), have decreased adiposity and metabolic dysfunction (Murphy, Kraakman et al., 2016).

In liver, administration of IL-18 to hepatocyte *in vitro* reduces the expression *of* PEPCK, and basal endogenous glucose production is increased in IL-18-/- mice in association to increased gluconeogenesis (PEPCK mRNA) (Netea, Joosten et al., 2006). Therefore, it has been suggested that IL-18 is involved in controlling basal hepatic glucose production (Netea, Joosten et al., 2006). In contrast, we did not find any differences in genes involved in gluconeogenesis (PEPCK or glucose-6-phosphat dehydrogenases mRNA) nor in intrahepatic triacylglycerol concentration when comparing IL-18R$^{-/-}$ with CON mice irrespective of diet (Lindegaard, Matthews et al., 2013). A finding which is reproduced by Pazos et al. (Pazos, Lima et al., 2015) Furthermore, even though the IL-18R$^{-/-}$ mice are insulin-resistant on an HFD relative to CON mice, there was no evidence of impaired insulin signaling or increased lipogenesis (mRNA expression of key fatty acid synthesis transcription factors (FAS)/enzyme sterol regulatory–element binding protein-1c *(SREBP)*) in the liver. Given that neither hepatic lipid deposition nor the expression of key enzymes involved in regulating hepatic glucose production is different when comparing IL-

$18R^{-/-}$ with CON mice, it is unlikely that changes in liver insulin sensitivity are responsible for the reduced whole-body insulin sensitivity observed in IL-18R-/- male mice. Although, studies still need to directly measure insulin sensitivity in the liver.

While this pre-clinical body of work implicated IL-18 as an important component of metabolic regulation, a caveat from these studies were that they were performed entirely on male mice. Research into IL-18 in other experimental settings had indicated the potential presence of sex divergence in the IL-18 physiological response. Injection of LPS results in significantly higher plasma levels of IL-18 in male mice (Aoyama, Kotani et al., 2009). Given this, and the known differences between the metabolism of males and females (described above), we set out to investigate whether we could repeat our findings in female mice. We studied female mice with a global deletion of the alpha isoform of the IL-18 receptor (IL-18R($^{-/-}$)) and their littermate wildtype (WT) control mice. Three studies were performed: 1) animals fed a HFD for 16 weeks to simulate our previous studies in males; 2) animals were fed a chow diet and aged for 72 weeks to simulate a mouse equivalent of post menopause (at this age female mice start to display some similar characteristics to postmenopausal women) and 3) animals (3 weeks-old) randomized to either bilateral ovariectomy (OVX) or control surgery (SHAM) and followed for 16 weeks to simulate depletion of sex hormones. In contrast to male mice, female IL-18R($^{-/-}$) mice gained less weight and under chow conditions tended to be more insulin sensitive than their WT littermates (Lindegaard, Abildgaard et al., 2018). However, with aging IL-18R($^{-/-}$) mice showed increases in both visceral and subcutaneous fat depots and glucose intolerance was now evident. While performing OVX did not affect body weight in IL-18R($^{-/-}$) mice, it did exacerbate glucose intolerance and impaired liver insulin signaling processes when compared with SHAM control mice (Lindegaard, Abildgaard et al., 2018). From these investigations we conclude, that female IL-18R$^{-/-}$ mice, only present with a similar metabolic phenotype as reported in male IL-18R($^{-/-}$) mice if they are exposed to conditions with reduced estrogen such as with aging or after undergoing OVX.

Tumor Necrosis Factor –alpha (TNF-α): TNF-α has been proposed to be an important cytokine in the pathogenesis of obesity and insulin resistance and is strongly linked to pro-inflammatory activation. In a study of 104 patients, the levels of circulating TNF-α were found to be raised in

male but not female patients with type 2 diabetes (Pfeiffer, Janott et al., 1997) and postmenopausal women show increased TNF-α levels compared to premenopausal women counterparts (Malutan, Dan et al., 2014). Animal studies have also identified sex differences regarding this cytokine. TNF-α mRNA levels in adipose tissue from genetically obese *ob/ob* male mice (obese due to hyperphagia) were threefold higher compared to female *ob/ob* mice (Neels, Pandey et al., 2006). Despite this, in this instance, there was no statistically significant difference in plasma TNF-α levels between the sexes. To further investigate the TNF-α pathway, the researchers investigated the receptors for TNF-α by genetically deleting them. Deletion of the TNF receptors p55 and p75 led to an 85% reduction in TNF-α mRNA levels in the adipose tissue of male mice (Neels, Pandey et al., 2006). Conversely, loss of the TNF receptors did not affect adipose tissue gene expression in female *ob/ob* mice suggesting that endogenous TNF-α signaling is a regulator of adipose tissue TNF-α expression levels in male but not female mice (Neels, Pandey et al., 2006). Further differences were observed upon administration of TNF-α to the mice. While this treatment increased TNF-α mRNA levels in both sexes, the response was blunted in the females by twofold (Neels, Pandey et al., 2006). Overall these data indicate a greater control of TNF-α expression and signaling in the female mice. In accordance with this, estrogen supplementation has been shown to blunt increases in TNF-α in response to artificially induced endotoxemia in rodents (Hassouna, Obaia et al., 2014), further suggesting that this female advantage in TNF-α control could be mediated by estrogen.

Leptin: Leptin is a circulating adipokine produced by adipose tissue. Leptin acts through binding to the long form of the leptin receptor (LEPR) which leads to many physiological functions including the regulation of appetite and food consumption as well as effects on metabolic rate, bone mass, reproductive system and insulin secretion. Along with these effects, leptin also is regarded as a proinflammatory factor and participates in innate immunity processes (Abella, Scotece et al., 2017). Serum leptin levels are sexually dimorphic in humans (Considine, Sinha et al., 1996) and rodents (Landt, Gingerich et al., 1998) however, these sexual dimorphisms are reversed between species with human women having higher levels than men, while male rodents have higher leptin levels than their female counterparts. The gender difference in humans is likely to be due to both a higher proportion of adipose tissue and increased production rate of leptin per unit mass of adipose tissue in women (Gutniak, Svartberg et al., 2001). One potential mechanism leading to this sexual dimorphism is the influence of steroid hormones on the synthesis of

transcripts that encode for leptin. Experiments in adipocyte-like cells (3T3-L1 murine adipocytes) have demonstrated that dihydrotestosterone reduces leptin transcript levels and as a consequence the amount of leptin found intracellularly and also the leptin that is secreted from the cell. In contrast, 17β-estradiol treatment significantly increases the abundance of transcripts encoding leptin and the amount of secreted leptin. Incubating cells with estrogen and androgen receptor antagonists had opposite effects on transcript abundance to steroid treatments (Jenks, Fairfield et al., 2017). Thus, while it remains to be determined, the sex hormones may play a regulatory role in leptin regulation and secretion. Given leptins' emerging link to inflammatory and immune processes, this adipokine may be yet another determinant of inflammatory-linked metabolic control and play sex-specific physiological roles.

Sex differences in anti-inflammatory-related mediators

***Interleukin 10* (IL-10):** Contrasting with the previously described pro-inflammatory cytokines, IL-10 is a cytokine with potent anti-inflammatory properties. Lower circulating IL-10 concentration is associated with being overweight or obese in young adolescents (Chang, Chang et al., 2013). In healthy adults there is a significant positive association between circulating IL-10 levels and whole-body insulin sensitivity (Straczkowski, Kowalska et al., 2005) and low IL-10 production capacity is associated with high plasma glucose, high HbA_{1c} levels, type 2 diabetes, and dyslipidemia (van Exel, Gussekloo et al., 2002). Genetic alterations of IL-10 (IL-10KO), IL10 receptor KO (IL-10RKO) and IL-10 transgenic (IL-10Tg) mice have been generated (Cintra, Pauli et al., 2008; Dagdeviren, Jung et al., 2016; Hong, Ko et al., 2009; Kowalski, Nicholls et al., 2011) along with studies by which IL-10 is administered as a treatment regime (H. J. Kim, Higashimori et al., 2004). Many, but not all, of these models have identified a protective role of IL-10 against metabolic disease, however from our analysis we cannot identify any study regarding IL-10 modulation that has been conducted in female mice and therefore the question remains as to whether or not it is of importance in female metabolic processes. Furthermore, both human and rodent studies find no changes in plasma IL-10 levels with declining ovarian function (Cioffi, Esposito et al., 2002; Ohtani, Garcia et al., 2007) indicating that female sex hormones are less likely to have direct impact on systemic IL-10 levels.

Adiponectin: Adiponectin is an adipocyte-derived secretory protein with profound anti-inflammatory and insulin-sensitizing functions. High plasma adiponectin levels are believed to

reflect healthy adipose tissue and closely reflects improved metabolic flexibility (Scherer, 2016; Turer & Scherer, 2012; Wang & Scherer, 2016). Adiponectin secretion from the adipose tissue is decreased with increasing central adiposity whereas lower extremity adiposity is associated with higher concentrations of adiponectin (Arita, Kihara et al., 1999; Turer, Browning et al., 2012). The anti-inflammatory effects of adiponectin are primarily driven through systemic M2 polarization of macrophages resulting in a decreased IL-6 and TNF-α release together with an increased secretion of IL-10 (Ajuwon & Spurlock, 2005; Kumada, Kihara et al., 2004; Ohashi, Parker et al., 2010).

Several human studies have shown that women have higher levels of circulating adiponectin compared to men, independent of differences in fat mass and insulin sensitivity (Saltevo, Kautiainen et al., 2009; Song, Oh et al., 2014). Interestingly, male mice fed a HFD showed decreased adiponectin expression in the adipose tissue, compared to chow fed male mice, whereas there were no differences in adiponectin expression between chow- and HFD fed female mice, despite similar increases in visceral fat mass across sexes (Nickelson, Stromsdorfer et al., 2012). This is in accordance with the general perception of healthier adipose tissue and better metabolic flexibility in females compared to males (Karastergiou, Smith et al., 2012; Lundsgaard & Kiens, 2014). A study in pubertal adolescents showed that as testosterone increased in pubertal boys, plasma adiponectin levels decreased, whereas plasma adiponectin in pubertal girls remained constant (Bottner, Kratzsch et al., 2004). Additional studies report stable plasma adiponectin levels during the menstrual cycle (Hall, White et al., 2009; Kleiblova, Springer et al., 2006; Wyskida, Franik et al., 2017), altogether suggesting that androgens but not estrogens could regulate plasma adiponectin levels.

Summary

Overall, this review highlights that metabolic response or control can be altered in a sex-dependent manner. Specifically, we have identified and described numerous instances in pre-clinical models whereby alterations in genes and proteins linked to inflammatory pathways leads to improved or disrupted metabolic control (See Figure 2 for a summary). However, whether phenotypes are observed, or the degree to which they are observed, can often be sex-dependent. While traditionally all sex differences in physiology were suggested to be due to different regulation in sex hormones, these differences could also be attributed to sex-specific behaviors or genetic and epigenetic effects caused by the inheritance and unequal dosage of genes located on the X- and Y-chromosomes

(Ratnu, Emami et al., 2017). Investigations of these underlying factors will continue to be an area of interest in the metabolic field over years to come. What is clear, is that this review underscores the importance of examining both sexes in metabolic studies. Recently, the Reue laboratory published a template encompassing considerations for the experimental design of pre-clinical studies regarding sex differences in metabolism (Mauvais-Jarvis, Arnold et al., 2017). Adoption of such approaches by laboratories will ensure research is undertaken in a way that is considerate to these differences and could lead to novel therapeutic targets and potentially even sex-specific treatments of metabolic disease in the future.

Nomenclature of Targets and Ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding, Sharman et al., 2018) and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18 (Alexander, Kelly et al, 2017)

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Declaration of transparency and scientific rigour

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the *BJP* guidelines for Design & Analysis, and as recommended by funding agencies, publishers and other organisations engaged with supporting research.

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Figure 1

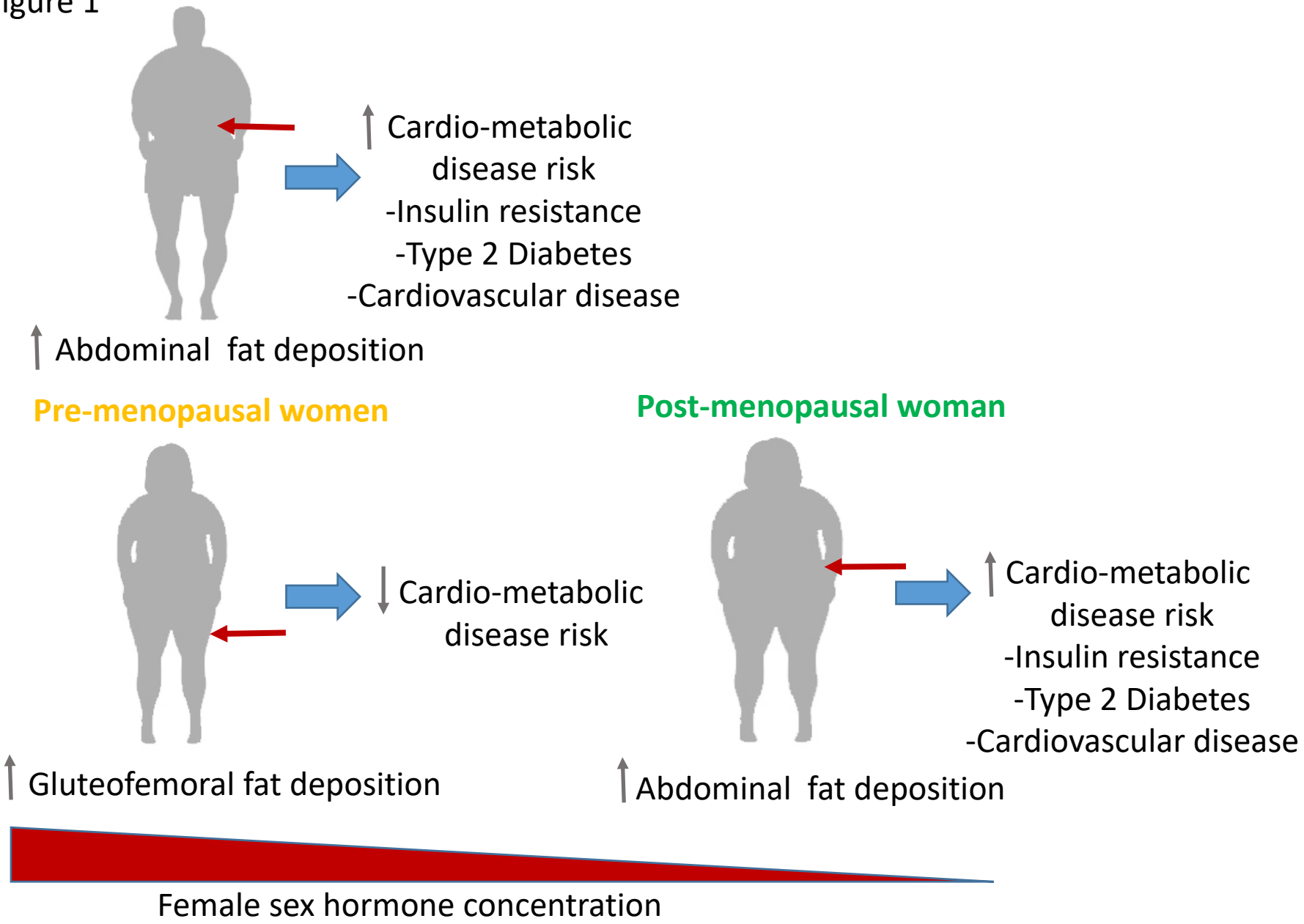

Figure 1: Despite obesity being more prevalent in women than men, prior to menopause, women have a reduced risk of metabolic disease. Following menopause and corresponding to an increase in deposition of abdominal fat and a decrease in the female sex hormones, metabolic disease risk is increased to similar rates as observed in men.

Figure 2

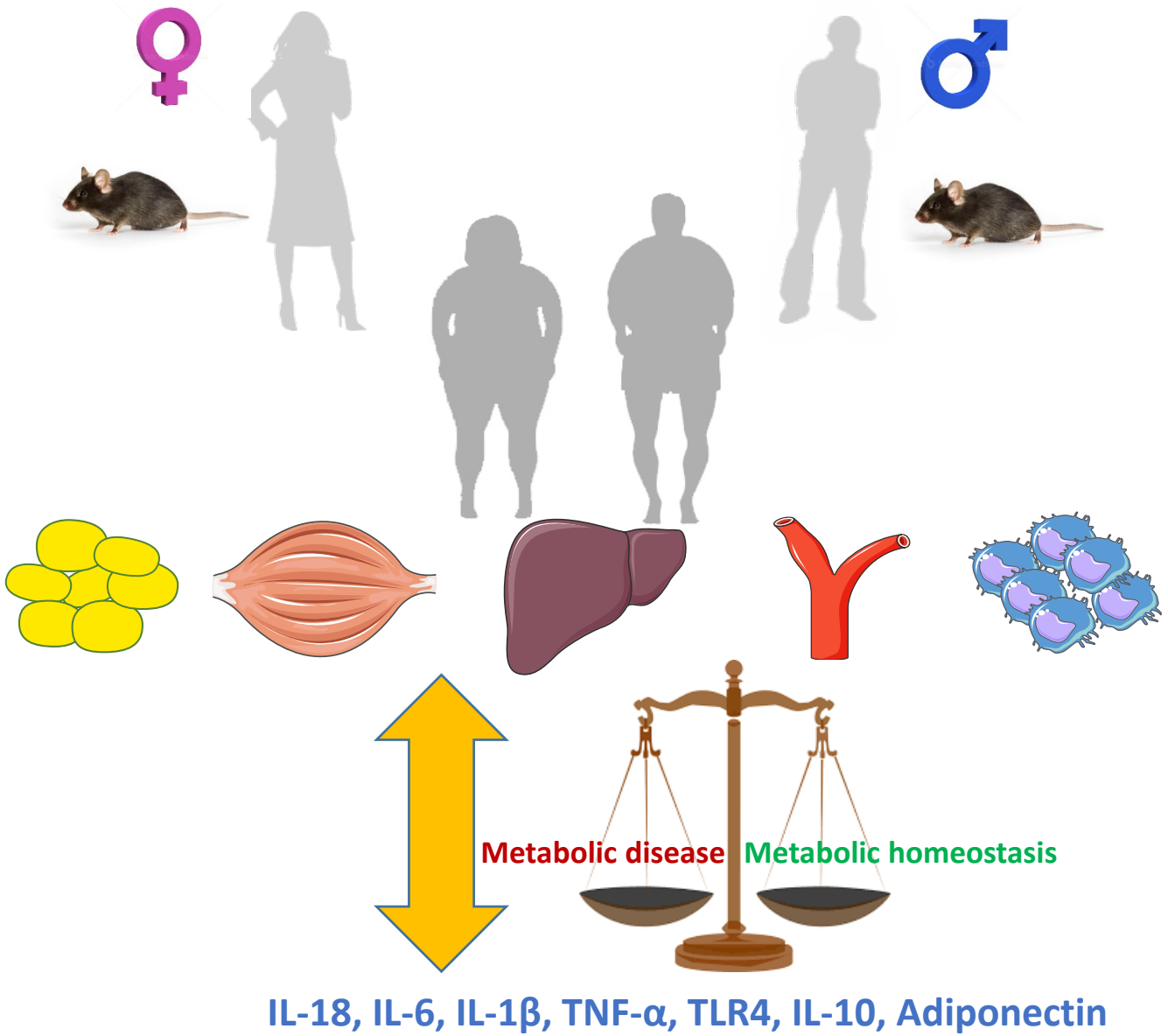

Figure 2: A potential contributor to gender-specific metabolic control is the impact of inflammatory processes. Mechanistic pre-clinical studies using a variety of genetic interventions have provided numerous examples of how the male and female metabolic response differs when inflammatory mediators are increased or decreased. Further, many models have not been tested in females limiting knowledge in this area.