

# openheart Does elevated bilirubin aid weight control by preventing development of hypothalamic leptin resistance?

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## GILBERT SYNDROME IS ASSOCIATED WITH LOWER GAIN IN FAT MASS DURING LATER LIFE

Gilbert syndrome (GS) is characterised by a lifelong genetically determined elevation of plasma unconjugated bilirubin levels.<sup>1</sup> This typically entails decreased hepatic expression of the enzyme that conjugates free bilirubin to glucuronic acid, uridine-diphosphoglucuronate glucuronosyltransferase 1A1 (UGT1A1); this decreased activity reflects homozygosity for variant alleles in which promoter mutations decrease but do not eliminate transcription. In addition, genetic upregulation of bilirubin production—reflecting upregulation of haem oxygenase activity or increased haem synthesis—also contributes to the elevation of unconjugated bilirubin observed in subjects with GS.<sup>1–3</sup> Diagnosis of GS generally requires a plasma bilirubin of 20 μM or above—alternatively, 1.2 mg/dL—but this diagnosis is not entirely objective, as non-genetic factors such as fasting status, gastrointestinal motility, enterohepatic bilirubin reabsorption/secretion and degree of light exposure can cause bilirubin levels to vary considerably over time. Moreover, many individuals carrying genetic variants which decrease hepatic UGT1A1 activity maintain plasma bilirubin levels below the cut-off limits for GS diagnosis; these variants alone are not sufficient to guarantee a GS diagnosis. In any case, the key point to recognise is that tissues of properly diagnosed subjects with GS are exposed to quite significantly elevated unconjugated bilirubin levels over the course of their lives.

A recent cross-sectional epidemiological study evaluating subjects with GS has achieved some remarkable findings.<sup>4</sup> This study enrolled 124 subjects with GS (average plasma unconjugated bilirubin 30.7 μM) and 124 age-matched and gender-matched controls (8.7 μM). This study was unique in

that it segregated the groups by age; subjects under and over age 35 were analysed separately. One of the most striking findings was this: whereas among the subjects under 35 body mass index (BMI) was only slightly but significantly lower in the GS group (22.5 vs 23.5,  $p < 0.05$ ), among those over 35 there was a large disparity: 23.8 vs 27.2 ( $p < 0.001$ ). The difference in body fat content in the over-35 group was even more stark: 21.8% in the subjects with GS vs 29.3% in the controls ( $p < 0.01$ ). These findings suggest that elevated unconjugated bilirubin—and/or possibly genetic upregulation of haem oxygenase activity—somehow prevents gain of body fat during ageing.

A recent study of diet-induced obesity in rats provides some confirmation of this idea.<sup>5</sup> Rats fed a diet high in fats and sugar were injected intraperitoneally with bilirubin or vehicle for 14 days. Bilirubin treatment prevented a deterioration in glucose tolerance and suppressed weight gain. In addition, compared with control mice, bilirubin-treated mice had reductions in total cholesterol and leptin and increases in adiponectin. A trend towards decreased calorie consumption in the bilirubin-treated rats also nearly achieved statistical significance ( $p = 0.06$ ).

Gunn rats, like humans with GS, are characterised by a genetically determined deficit of hepatic UGT1A1 activity and a lifelong elevation of unconjugated plasma bilirubin.<sup>6</sup> It therefore is pertinent to note that aged Gunn rats are characterised by a lower level of visceral fat than is seen in littermates with normal hepatic UGT1A1 activity.<sup>7</sup> Moreover, these rats enjoy better glucose tolerance, reduced oxidative stress and decreased serum levels of proinflammatory cytokines, suggesting that metabolic syndrome and systemic inflammation are ameliorated in such rats.



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## UNCONJUGATED BILIRUBIN FUNCTIONS PHYSIOLOGICALLY TO INHIBIT NADPH OXIDASE COMPLEXES

Physiological intracellular levels of unconjugated bilirubin have been shown to inhibit certain common isoforms of NADPH oxidase.<sup>8–12</sup> This phenomenon likely explains the profound antioxidant activity of haem oxygenase, which cleaves haem to yield biliverdin, carbon monoxide and free iron; biliverdin is then rapidly reduced by the ubiquitously expressed enzyme biliverdin reductase to yield bilirubin. Expression of inducible form of haem oxygenase, HO-1, can be boosted by oxidative stress—often derived from NADPH oxidase activity; the resultant production of bilirubin feeds back to quell this oxidative stress.<sup>11</sup> Although bilirubin can also act as a direct oxidant scavenger, its physiological intracellular level—in the low nanomolar range—is too low to compete in this regard with other intracellular scavengers (eg, glutathione, ascorbate) present in millimolar concentrations.<sup>13</sup>

Oxidative stress in adipocytes, stemming largely from NADPH oxidase activity, appears to play a key role in the induction of insulin resistance and the skewing of adipokine and cytokine production in hypertrophied adipocytes.<sup>14–19</sup> Hence, bilirubin and haem oxygenase activity could be expected to aid maintenance of adipocyte insulin sensitivity. Indeed, plasma levels of unconjugated bilirubin have been found to correlate inversely with risk for metabolic syndrome and diabetes in prospective epidemiological studies, as confirmed in a recent meta-analysis.<sup>20</sup> However, insulin induces uptake and retention of fatty acids by adipocytes; hence, bilirubin's presumed ability to aid maintenance of adipocyte insulin sensitivity would be expected to *boost* body fat content, not decrease it. Hence, we need to look elsewhere for an explanation of the decreased risk for body fat gain in subjects with GS.

## MICROGLIAL ACTIVATION AS A MEDIATOR OF HYPOTHALAMIC LEPTIN RESISTANCE: A TARGET FOR BILIRUBIN?

One of the phenomena that promote weight gain as people grow older is the development of hypothalamic leptin resistance.<sup>21</sup> The hormone leptin is produced primarily in adipocytes, and its plasma levels rise as body fat mass increases. Leptin functions to counteract inappropriate weight gain by acting on leptin-responsive neurons in the hypothalamus to suppress appetite while also boosting metabolic rate via sympathetic activation.<sup>22–24</sup> Of particular interest in this regard are leptin-responsive neurons in the arcuate nucleus of the mediobasal hypothalamus (MBH); the MBH has a poorly developed blood–brain barrier, and hence hormones, free fatty acids and other plasma components have ready access to it.<sup>25</sup> Leptin-responsive neurons in the arcuate nucleus boost anorexigenic signalling by increasing neuronal release of pro-opiomelanocortin, while suppressing release of the orexigenic hormones neuropeptide Y and agouti-related peptide within this nucleus. The

physiological importance of this mechanism, at least in mice, is confirmed by the fact that genetic strains of mice which are incapable of making either leptin (ob/ob) or functional leptin receptors (db/db) overeat and become obese and diabetic.<sup>26,27</sup>

Unfortunately, efforts to develop injectable leptin as an antiobesity drug have not been successful, as overweight subjects are resistant to its suppressive impact on appetite. Studies in rodents with diet-induced obesity suggest that this phenomenon reflects a loss of leptin responsiveness that is specific to the arcuate nucleus.<sup>21,28–30</sup> Activated leptin receptors trigger JAK2-mediated phosphorylation of STAT3, which then migrates as a homodimer to the nucleus to modulate gene transcription. In lean chow-fed rodents, a leptin injection rapidly boosts pSTAT3 levels in the arcuate nucleus and suppresses feeding; this response is substantially blunted in obese rodents. In contrast, leptin is able to raise pSTAT3 levels in other leptin-responsive regions of the brain in obese rodents.<sup>31</sup>

Although the molecular biology underlying hypothalamic leptin resistance in obesity is still somewhat obscure, studies focusing on high-fat/high-sugar diet-induced obesity in rodents have yielded some intriguing findings. In particular, activation and proliferation of microglia in the MBH are noted in rodents with diet-induced obesity.<sup>32–34</sup> The microglial activation noted in this situation appears to be mediated primarily by saturated fatty acids interacting with toll-like receptor-4 (TLR4) expressed by microglia.<sup>32,35,36</sup> (Plasma-derived fetuin-A forms a trimeric complex with fatty acids and TLR4, catalysing this interaction.<sup>37–39</sup>) Hence, TLR4 antagonists—but not TLR2 antagonists—prevent microglial activation and development of leptin resistance in rats fed a fatty diet.<sup>35</sup> Microglial proliferation is also noted in this circumstance, and measures which prevent microglial proliferation have likewise been found to prevent development of leptin resistance in rodents.<sup>32,33</sup> How activated microglia act to impair leptin responsiveness in the arcuate nucleus is still unclear.

A key role for saturated fatty acids in driving leptin resistance might help to explain why risk for obesity is lower in those who habitually consume plant-based or 'Mediterranean' diets in which saturated fats constitute a relatively low percentage of total fatty acids.<sup>40–47</sup> Risk for type 2 diabetes has been found to be markedly lower in individuals who follow a plant-based diet.<sup>48</sup> Increased hepatic production of fibroblast growth factor 21 may also contribute to the obesity prevention associated with plant-based diets of modest protein content.<sup>49–51</sup>

Activation of microglia via TLR4—as with lipopolysaccharides—has been shown to entail activation of Nox2-dependent NADPH oxidase.<sup>52–54</sup> Moreover, this activation is required for production of toxic oxidants such as peroxynitrite, and increased production of proinflammatory cytokines and prostanoids. Hence, it is straightforward to propose that bilirubin may have the ability to downregulate microglial activation by diminishing NADPH oxidase activation.<sup>55</sup> In light of the

foregoing discussion, a corollary of this is that elevated bilirubin—whether derived from plasma or from local haem oxygenase activity—may oppose the evolution of leptin resistance by inhibiting the activation (and likely proliferation) of microglia in the MBH. The ability of the HO-1 inducer haemin to alleviate hyperleptinaemia—a marker for leptin resistance—in fat-fed rats appears consistent with this possibility.<sup>56</sup>

With respect to bilirubin and microglia, it should be noted that, when unconjugated bilirubin exceeds its solubility limit (70 nM), it can disrupt membranes and promote microglial activation.<sup>57–58</sup> This explains the neural damage and microglial activation associated with perinatal bilirubin encephalopathy, which can occur in newborns whose livers have limited capacity to conjugate bilirubin at a time when the blood–brain barrier is poorly formed. Analogously, bilirubin neurotoxicity is seen in Crigler-Najjar syndrome, in which mutations of the UGT1A1 render it non-functional, and plasma bilirubin levels are roughly an order of magnitude higher than those seen in GS.<sup>59</sup> The concentrations of unconjugated bilirubin which result from haem oxygenase induction appear to be below its solubility limit, as such induction tends to suppress microglial activation and provide neuroprotection in rodent or cell culture models.<sup>60</sup> In endothelial cells, bilirubin's antioxidant activity has been found to be half-maximal at 11 nM; hence, bilirubin can function physiologically as an important intracellular antioxidant in concentrations far below its solubility limit.

One of the cytokines whose expression by microglia is contingent on Nox2 activity is tumour necrosis factor- $\alpha$  (TNF $\alpha$ ).<sup>52–53</sup> TNF $\alpha$ , via Nuclear factor-kappa beta (NF-kappaB) activation, provokes increased hypothalamic expression of phosphotyrosine phosphatase-1A (PTP1B), which functions as an antagonist of leptin signalling by reversing activating tyrosine phosphorylation of JAK2.<sup>61–64</sup> Hypothalamic PTP1B activity increases in response to high-fat diets in rodents, and neuronal PTP1B knockout mice fail to develop leptin resistance and obesity when fed such diets; a similar effect is seen when hypothalamic PTP1B activity is inhibited with antisense oligonucleotides.<sup>61–65–66</sup> Hence, the TNF $\alpha$  produced by microglia—and possibly other cytokines capable of inducing PTP1B in neurons—likely contributes to leptin resistance by boosting PTP1B expression.

Additionally, there is evidence that hypothalamic TNF $\alpha$  can oppose leptin resistance by additional mechanisms, likely including increased expression of suppressor of cytokine signalling-3 (SOCS-3).<sup>62</sup> This protein, via an inhibitory interaction with JAK2, blocks all known signalling pathways activated by the leptin receptor. It is elevated in the hypothalamus of fat-fed rodents, and mice that are heterozygous for SOCS-3 gene deletion are resistant to diet-induced obesity.<sup>67–68</sup> SOCS-3 is induced at the transcriptional level by leptin, and thus provides feedback regulation of leptin activity.<sup>69</sup> TNF $\alpha$  can increase its expression by boosting the half-life of its mRNA, thereby amplifying the efficacy of this negative feedback

mechanism.<sup>70</sup> In obese mice whose leptin is clamped at a lower level similar to that of lean mice, an injection of leptin causes a normal rise in arcuate pSTAT3; this might reflect the fact that their baseline level of SOCS-3 in leptin-responsive arcuate neurons is relatively low.<sup>71</sup>

If we presume, not unreasonably, that hypothalamic leptin resistance tends to evolve and worsen gradually over a lifetime—possibly reflecting proliferation of activated microglia in the arcuate nucleus—then the fact that body composition is only slightly modified in young subjects with GS relative to controls may simply reflect the fact that bilirubin cannot influence leptin activity until leptin resistance begins to develop.

### BILIRUBIN-MIMETIC STRATEGIES FOR OBESITY PREVENTION

These considerations may be of more than just theoretical interest. Although bilirubin is too insoluble to be useful as a nutraceutical, and its precursor biliverdin is quite expensive to synthesise, the biliverdin derivative and homologue phycocyanobilin (PhyCB) is a prominent light-harvesting chromophore in many cyanobacteria and blue-green algae. Spirulina, a cyanobacterium traditionally used as a food in certain cultures, can contain about 0.6% PhyCB by dry weight.<sup>72</sup> This likely explains why oral administration of spirulina—or of phycocyanin, the blue algal protein which carries PhyCB as a covalently attached chromophore—has been found to exert profound antioxidant and anti-inflammatory effects in rodent models of a wide range of health disorders.<sup>72–74</sup> Protective effects of oral spirulina in rodent models of neurodegeneration may indeed reflect, in part, diminished activation of microglia; in particular, spirulina is effective in rodent models of Parkinson's disease, in which activated microglia are suspected to play a key role in the destruction of dopaminergic neurons.<sup>55–75–78</sup> Although intakes of spirulina sufficiently high to exert important antioxidant activity are difficult to achieve in humans owing to the undesirable flavour and especially odour of spirulina, the development of PhyCB-enriched spirulina extracts suitable for nutraceutical use may make it far easier to achieve the benefits of PhyCB clinically.<sup>72</sup>

It may be noted that, in one of the very few controlled clinical studies in which ample doses of spirulina were administered—protease inhibitor-treated patients with HIV preselected for insulin resistance received 19 g daily of spirulina or soy protein—insulin sensitivity in the spirulina-treated subjects, assessed by a short intravenous insulin tolerance test, roughly tripled.<sup>79</sup> (The study was however marred by a high dropout rate in the spirulina group, as many of the subjects could not tolerate spirulina's flavour.)

Alternatively, it may prove feasible to induce an 'iatrogenic Gilbert syndrome' by administering drugs or nutraceuticals that inhibit UGT1A1 activity.<sup>13–80</sup>

If the hypothesis presented here is correct, the far lower body fat in older subjects with GS reflects the ability of bilirubin to suppress the activation and proliferation of

microglia in the MBH. The extent to which this expansion of activated microglia—and the associated impact on the function of leptin-responsive neurons—can be reversed by elevation of bilirubin (or administration of PhyCB) in patients who have already developed obesity with leptin resistance remains to be seen. Particularly because microglial mass increases, it may be rash to assume that this syndrome is fully reversible.<sup>81</sup> If PhyCB does prove to have utility for controlling hypothalamic inflammation, its greatest impact on obesity will likely be achieved by long-term administration in a preventive mode.

In any case, studies evaluating the impact of bilirubin or PhyCB administration on the development of hypothalamic leptin resistance in fat-fed rodents appear to be warranted. These studies could assess whether leptin's ability to amplify pSTAT3 levels in the arcuate nucleus of fat-fed rats—while suppressing feeding—is boosted by concurrent administration of bilirubin or PhyCB.

Two studies have been published very recently in which inclusion of spirulina in the diet has been shown to inhibit gain in body weight and fat in rats fed a high-fat diet; these appear to be the first studies to have evaluated spirulina's impact in this regard.<sup>82–83</sup> Although neither of these studies focused on leptin function, the fact that markers of adipose tissue browning were higher in rats receiving spirulina is consistent with effective leptin function in these rats. Moreover, a double-blind, placebo-controlled clinical trial has also emerged, in which spirulina supplementation (at only 2 g daily) was found to potentiate loss of body fat, body weight, waist circumference and BMI in overweight subjects placed on a calorie-restricted diet; reductions in triglycerides and C reactive protein were also greater in the spirulina group.<sup>84</sup>

### PRESERVING AND UPREGULATING HYPOTHALAMIC LEPTIN SIGNALLING AS A STRATEGY FOR METABOLIC HEALTH

Bilirubin mimesis may represent one example of a more general strategy for preventing or reversing inappropriate weight gain: counteracting hypothalamic leptin resistance or upregulating hypothalamic leptin signalling.<sup>59–85</sup>

For example, histamine, acting via H1 receptors, functions as a downstream mediator of hypothalamic leptin signalling, and this signalling can be upregulated by histidine supplementation.<sup>86–87</sup> Rodent studies indicate that an increase in dietary histidine can increase hypothalamic histamine levels, boost hypothalamic expression of histidine decarboxylase, inhibit food intake, increase sympathetic activity in fat tissue and decrease the size of the retroperitoneal fat pad.<sup>88–91</sup> In rats fed a high-fat diet, an increase in dietary histidine attenuated weight gain and decreased markers for inflammation in adipose tissue.<sup>92</sup> In a controlled clinical trial, overweight women with metabolic syndrome were supplemented with 4 g histidine daily or matching placebo for 12 weeks; the group receiving histidine lost 2.7 kg of body fat and achieved improvements in insulin sensitivity and other parameters related to metabolic syndrome,

changes that were significant relative to negligible changes in the placebo group.<sup>93</sup> Moreover, cross-sectional epidemiology has correlated increased dietary intake of histidine (absolutely or as a fraction of total protein) with lower daily calorie intake and decreased BMI and waist circumference.<sup>94–95</sup> Conversely, chronic use of prescription H1-antagonist antihistamines has been linked to increased risk for obesity.<sup>96</sup> Hence, histidine supplementation may emerge as an additional strategy for aiding weight control by optimising leptin signalling.<sup>97</sup>

Maintaining effective androgen activity may support hypothalamic leptin signalling in men. The activated androgen receptor in neurons functions to suppress NF-kappaB-mediated induction of PTP1B.<sup>98</sup> Hence, hypothalamic androgen activity may blunt the ability of activated microglia to induce leptin resistance. Reduction of testosterone levels associated with ageing (andropause), as well as androgen deprivation therapy in patients with prostate cancer, are known to increase the risk of weight gain and metabolic syndrome; conversely, restoration of youthful testosterone levels in ageing men has been found to be protective in these respects.<sup>99–103</sup>

Additional potential strategies for boosting hypothalamic leptin signalling have been proposed. Orally available selective inhibitors of PTP1B may have potential as pharmaceuticals for restoring leptin responsiveness.<sup>104–105</sup> In this regard, cinnamon, which contains a compound that can inhibit the rat homologue of PTP1B, has been shown to inhibit gain of body fat in rats fed a high-fat/high-fructose diet, and also has been found to reduce the BMI of women with polycystic ovary syndrome in a recent controlled study.<sup>106–108</sup>

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