Non-alcoholic fatty liver disease (NAFLD): a multi-system disease influenced by ageing and sex, and affected by adipose tissue and intestinal function.

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Abstract

In recent years, a wealth of factors are associated with increased risk of developing non-alcoholic fatty liver disease (NAFLD) and NAFLD is now thought to increase the risk of multiple extra-hepatic diseases. The aim of this review is firstly to focus on the role of ageing and sex as key, poorly understood risk factors in the development and progression of NAFLD. Secondly, we aim to discuss the roles of white adipose tissue (WAT) and intestinal dysfunction, as producers of extra-hepatic factors known to further contribute to the pathogenesis of NAFLD. Finally, we aim to summarise the role of NAFLD as a multi-system disease affecting other organ systems beyond the liver. Both increased age and male sex increase the risk of NAFLD and this may be partly driven by alterations in the distribution and function of WAT. Similarly, changes in gut microbiota (GM) composition and intestinal function with ageing and chronic overnutrition are likely to contribute to the development of NAFLD both directly (i.e. by affecting hepatic function) and indirectly via exacerbating WAT dysfunction. Consequently, the presence of NAFLD significantly increases the risk of various extra-hepatic diseases including cardiovascular disease, type 2 diabetes mellitus, chronic kidney disease and certain extra-hepatic cancers. Thus changes in WAT and intestinal function with ageing and chronic overnutrition contribute to the development of NAFLD - a multi-system disease that subsequently contributes to the development of other chronic cardiometabolic diseases.

Introduction

Current estimates indicate that around 30% of the global adult population are affected by non-alcoholic fatty liver disease (NAFLD) and the increasing prevalence of this disease has occurred in parallel with the global epidemic of obesity and type 2 diabetes mellitus (T2DM) [1, 2]. Considered to be the predominant cause of chronic liver disease in many parts of the world, NAFLD represents a spectrum of progressive hepatic disease phenotypes extending from hepatic steatosis to non-alcoholic steatohepatitis (NASH), liver fibrosis and cirrhosis [1, 3, 4]. Evidence now shows that NAFLD increases the risk of liver-related complications and is also a multi-system disease that increases the risk of cardiovascular disease (CVD) and cardiac disease [5, 6], chronic kidney disease (CKD) [7], T2DM [8, 9] and some extra-hepatic cancers [1]. Therefore, it is no surprise that the presence of NAFLD is strongly associated with an increased risk of all-cause mortality [3, 10]. Indeed, CVD is the main cause of mortality in patients with NAFLD, followed by extra-hepatic cancers and liver-related complications [10]. Additionally, recent evidence suggests that there may be an even greater cardiometabolic risk with the more advanced stages of liver disease, such as liver fibrosis, which is also a strong predictor of all-cause and disease-specific mortality [11-13].

In recent years, a wealth of factors have been shown to be associated with an increased risk of developing NAFLD. The aim of this review is firstly to focus on the role of ageing and sex as key, poorly understood risk factors in the development and progression of NAFLD. Secondly, we will discuss the roles of white adipose tissue (WAT) and intestinal dysfunction, as producers of extra-hepatic factors known to further contribute to the pathogenesis of NAFLD. Finally, we will summarise the role of NAFLD as a multi-system disease affecting other organ systems beyond the liver.

Sex and age as risk factors for NAFLD

The involvement of age and sex in the development of NAFLD have received increased attention in recent years yet the reasons why these are risk factors for NAFLD remains poorly understood. The prevalence of NAFLD is higher in men and is thought to increase into middle age and then decline after the age of 50-60 years [14]. In contrast, pre-menopausal women appear to be relatively protected

81   from NAFLD, however, this protective capacity is lost after the fifth decade of life when the

82   prevalence of NAFLD is thought to be similar in both sexes [14, 15]. The incidence of NASH and

83   cirrhosis is also thought to be greater in both men and women who are ≥ 50 years of age compared to

84   younger age groups [2]. Recent meta-analysis suggests that whilst pre-menopausal women may have a

85   lower risk of NAFLD, women ≥ 50 years of age may be at an increased risk of NAFLD progression,

86   compared to men of a similar age [16]. Specifically, among older age groups (≥ 50 years of age), the

87   relative risk of NASH and advanced liver fibrosis was found to be 17% and 56% higher respectively,

88   in women compared to men [16]. Conversely, the risk of NAFLD progression was not significantly

89   different between men and women in populations with an average age of ≤ 50 years [16]. Further work

90   is required to elucidate potential mechanisms underlying the apparent increased risk of NAFLD

91   progression in older women. For example, studies exploring sexual dimorphism in liver metabolism

92   have recently linked hepatic actions of estrogens to lipid metabolism and female reproductive

93   functions [17]. Whether these or other sexually dimorphic metabolic or endocrine factors are important

94   in NAFLD remains to be investigated [18].

95   Advancing age also increases the risk of hepatic and extra-hepatic complications of NAFLD [14]. Thus

96   it is expected that older patients with NAFLD will have a higher likelihood of overall and disease-

97   specific mortality [19, 20]. Whether the association between NAFLD and all-cause mortality is modified

98   by sex, is currently unclear. Previous studies suggest a worse outcome in men [20, 21], whilst others

99   have found trends suggesting that NAFLD is associated with an increased risk of all-cause mortality

100   in women but not men [22]. Thus, further large prospective cohort studies should explore whether the

101   direction and magnitude of the association between NAFLD and mortality are modified by sex.

102

103   **White adipose tissue mass and distribution in NAFLD**

104   A wealth of evidence indicates that obesity increases the risk of NAFLD [23-26]. Obesity is defined as

105   excess body fat and results from chronic overnutrition. For adults, it is most frequently classified as a

106   weight for height index or body mass index (BMI) and includes underweight or 'wasting' (< 18.5

107   kg/m^2), overweight (≥ 25 kg/m^2), obesity (≥ 30 kg/m^2) and morbid obesity (≥ 40 kg/m^2) [27]. In

108   contrast, waist circumference (WC) provides a simpler anthropometric measurement to diagnose

109   central obesity which is an important independent risk factor for NAFLD and an important component

110   of the metabolic syndrome (MetS). As previously described [28], MetS is defined as the presence of

111   three or more of the following criteria; increased WC, hypertriglyceridemia, reduced high-density

112   lipoprotein-cholesterol, hypertension and hyperglycaemia. It is worth highlighting that neither BMI

113   nor WC are considered reliable indicators of adiposity per se since they do not provide an assessment

114   of WAT mass nor volume [29]. Nonetheless, BMI and WC have proved to be extremely useful

115   measures for population-based studies and firmly established the importance of obesity as a risk factor

116   for NAFLD. Despite this, it is an oversimplification to consider NAFLD solely as a consequence of

117   obesity given the growing evidence indicating that NAFLD can also occur in individuals with a non-

118   obese BMI, or low WAT mass [30, 31]. It has been proposed that an increase in accumulation of central

119   WAT and a reduction in the functional capacity of WAT (particularly subcutaneous WAT (SAT)) to

120   store excess energy as triacylglycerol (TAG) are crucial factors that underpin the relationship between

121   obesity, systemic metabolic disease and NAFLD [31].

122   Studies utilising adipose tissue-targeted technologies coupled with histological assessment have

123   suggested that the hypertrophic expansion of adipocytes within visceral WAT (VAT) rather than SAT

124   is particularly associated with NAFLD. After approximately 4 years of follow up, a larger VAT area

125   was found to be associated with a higher risk of incident NAFLD, whereas larger areas of SAT were

126   associated with regression of NAFLD [32]. Moreover, several recent studies have demonstrated that

127   increased VAT, as opposed to SAT, increases the risk of, and predicts advanced liver fibrosis in

128   patients with NAFLD [33-35]. Similarly, evidence also indicates that VAT accumulation is an

129   independent risk factor for hepatocellular carcinoma (HCC) recurrence in patients with suspected

130   NASH [36]. Thus, this evidence supports a fundamental hypothesis that "the risk of developing

131   metabolic disease associated with obesity is governed by the regional distribution of WAT within the

132   individual, with the expansion of certain fat depots being more strongly associated with metabolic

133   dysfunction than others" [37]. Collectively, it is likely that the distribution and capacity of SAT to

134 effectively expand and store lipid, rather than the obesity per se, is a pivotal factor in the relationship

135 between increased adiposity and NAFLD risk.

136 The distribution of WAT is known to differ significantly between sexes, changes with increasing age

137 and has been hypothesised to be partly responsible for the increased prevalence of NAFLD in men

138 and older age groups, particularly post-menopausal women **(Figure 1)** [38-40]. Whilst the mechanisms

139 regulating the distribution of WAT remain largely elusive, evidence indicates that ageing and male

140 sex are associated with a restricted capacity to effectively expand so-called "metabolically protective"

141 SAT depots [41]. Whilst pre-menopausal women typically have greater total adiposity, men tend to

142 accumulate greater amounts of VAT with ageing and pre-menopausal women accumulate gluteal

143 femoral SAT which is associated with a lower risk of metabolic disease and NAFLD [42]. In both men

144 and women, older age (i.e. post-menopausal women and men > 50 years) is associated with a

145 reduction in the capacity of SAT to expand and an increase in VAT [43-45]. The limited capacity of

146 SAT to store TAG in men and with increasing age is likely to re-direct lipid accumulation ectopically

147 in non-adipose tissues, including the liver, leading to lipotoxicity, a chronic local and systemic pro-

148 inflammatory environment and eventually NAFLD development [46]. The importance of effective SAT

149 expansion can be seen in individuals with certain genetic or acquired lipodystrophies that are

150 characterised by the complete or partial absence of SAT [47]. In spite of their often lean appearance,

151 these individuals appear to exhibit much higher rates of NAFLD/NASH progression and other

152 cardiometabolic complications than would be expected based on their BMI alone [31, 47]. Given this, it

153 is likely that differences in WAT distribution between sexes and changes occurring with increasing

154 age are both important in the increased risk of NAFLD associated with ageing and with male sex.

155

156 **Adipose tissue dysfunction and NAFLD**

157 White adipose tissue is composed of mature unilocular adipocyte fraction and a stromal vascular

158 fraction, comprised of numerous cell types such as vascular, mesenchymal and immune cells. At a

159 cellular level, WAT expansion can be mediated by an enlargement of individual adipocytes

160 (hypertrophy), an increase in the number of adipocytes (hyperplasia) or a mixture of both. Adipocyte

161 hypertrophy, rather than hyperplasia, is more closely associated with WAT dysfunction and metabolic

162 disease [48]. Factors including hypoxia, low-grade chronic inflammation (i.e. metaflammation) and

163 improper extracellular matrix remodelling are thought to limit adipocyte differentiation and the healthy

164 expansion of adipose tissue (hyperplasia) [49, 50]. This limit can result in adipocyte hypertrophy,

165 dysfunction, stress and eventually death [51, 52]. In this context, WAT dysfunction refers to a reduction

166 in the tissues ability to effectively sense and respond to dynamic changes in nutrient availability (i.e.

167 metabolic inflexibility) and can coexist with adipose insulin resistance (IR) and metaflammation.

168 Specifically, this dysfunction is thought to affect WAT metabolism and in particular its ability to handle

169 lipids and increase the lipolytic rate of WAT due to a reduction in tissue insulin sensitivity, increasing

170 the flux of non-esterified fatty acids (NEFAs) to the liver and consequently increasing the risk of

171 NAFLD [31, 53-55].

172 Accompanying the changes in the distribution of WAT, ageing is associated with a marked reduction

173 in insulin, lipolytic and NEFA responsiveness in WAT. This metabolic inflexibility may underly the

174 known association between ageing and increased risk of NAFLD [43-45]. The reduction in SAT with

175 ageing in both men and women may in part be driven by a reduction in the adipogenic potential of

176 progenitor cells and the accumulation of senescent adipocytes in aged WAT. Preadipocytes isolated

177 from peripheral SAT in elderly individuals were found to have a reduced rate of replication compared

178 to those isolated from younger individuals [56]. Additionally, ageing is associated with an accumulation

179 of senescent adipocyte-derived stem cells within SAT which lack the ability to differentiate into

180 adipocytes in response to metabolic stress, consequently affecting the tissue's capacity to store TAG

181 [57]. Through their senescence-associated secretory phenotype, senescent adipocyte progenitor cells

182 within WAT are also likely to contribute to WAT inflammation and subsequent metabolic

183 complications [58, 59].

184 In addition to ageing, there are also sexually dimorphic differences in WAT function whereby WAT in

185 females is generally more insulin sensitive, more lipogenic and less susceptible to inflammation than

186 WAT from males. This phenomenon is also strongly associated with differences in sex hormone

187 concentrations [60, 61]. Menopause appears to associate with a preferential increase in VAT (rather than

188 SAT) in both obese and non-obese women [62-65], further supporting a role for sex hormones, such as

189 estrogen, in regulating the beneficial distribution and function of WAT. Circulating concentrations of

190 estrogen decrease markedly after menopause which is thought to lead to the redistribution of lipids into

191 VAT and the liver which, in combination with overnutrition, increases the risk of VAT accumulation

192 and NAFLD in post-menopausal women [66]. Pre-clinical studies utilising ovariectomised murine

193 models also support a causative relationship between reduced estrogen production, increased VAT mass

194 and the development of NASH [42, 66-68]. Whilst an in-depth discussion of the role of estrogen within

195 WAT is beyond the scope of this review (see other relevant reviews [42, 69, 70]), it is thought that the

196 increased expression of estrogen receptor alpha (ERα) in the gluteal femoral SAT of premenopausal

197 women promotes lipoprotein lipase activity and accumulation of TAG in adipocytes within this depot

198 [71]. Thus, it is likely that differences in WAT function (partly driven by differences in sex hormone

199 concentrations and the expression of functional target receptors) is an important factor underlying the

200 observed differences in NAFLD risk between men and women. Furthermore, changes in WAT with

201 ageing are likely to exacerbate WAT dysfunction associated with a state of chronic energy surplus and

202 are likely to have an important role in the increased risk of NAFLD associated with older age.

203

Adipokines and NAFLD

205 White adipose tissue is an endocrine tissue capable of secreting a wide range of adipokines which have

206 various roles in the regulation of whole-body energy homeostasis and inter-organ communication [72].

207 The aberrant production of these adipokines has been linked to multiple obesity-related metabolic

208 diseases. Amongst these adipokines, leptin and adiponectin are predominately produced by adipocytes.

209 In addition to its well-established role in regulating appetite and energy homeostasis [49, 73], leptin exerts

210 a dual action on hepatic function and NAFLD severity. Recent meta-analyses including an analysis of

211 over 30 studies indicated that circulating concentrations of leptin are elevated in patients with NAFLD

212 compared to healthy controls and supports a positive relationship between leptin and NAFLD [74]. As

213 recently highlighted [75, 76], under normoleptinemia conditions, leptin is thought to suppress hepatic

glucose production and hepatic lipogenesis thus providing an insulin sensitising anti-steatotic effect. Conversely, in the context of chronic hyperleptinemia as is common in obesity, a state of leptin resistance can result, which may also contribute to the NASH phenotype. It is suggested that in the liver, high concentrations of leptin can increase the expression of matrix remodelling enzymes via interacting with leptin receptors on Kupffer and sinusoidal endothelial cells, in turn activating hepatic stellate cells (HSCs), and possibly contributing to liver fibrosis [77].

Sexual dimorphism has also been reported for leptin expression [78]. Despite their lower risk of NAFLD, circulating concentrations of leptin are higher in pre-menopausal women compared to age-matched men and higher leptin levels are thought to be driven by both greater adiposity and an increased production rate of leptin per unit mass of WAT in women compared to men [79]. In both men and women, circulating concentrations of leptin are thought to gradually decline with ageing, with reductions being most noticeable in women compared to men whilst appearing to be independent of menopausal status [79, 80]. Despite these findings, it is currently unknown whether differences in circulating concentrations of leptin between sexes and age groups have an impact on the risk of NAFLD.

Similar to leptin, a wealth of studies indicate that the circulating concentrations of adiponectin, the most systemically abundant adipokine, are altered in patients with NAFLD (as reviewed in [81]). Adiponectin is a hepatoprotective adipokine that has well-established anti-inflammatory [82-84] and insulin sensitising effects [85] both systemically and within the liver. Meta-analysis indicates that adiponectin concentrations are significantly lower in patients with NAFLD compared to healthy controls, furthermore, NASH is associated with lower adiponectin when compared to simple steatosis [86]. Conversely, adiponectin concentrations are thought to increase in patients with NAFLD-cirrhosis potentially due to a reduction in the hepatic clearance of adiponectin and/or an increase in its production as a result of the tissue repair process associated with NAFLD-cirrhosis [87-89]. Along with its well-established role in promoting hepatic insulin sensitivity [90, 91], evidence indicates that adiponectin also has antifibrogenic effects via inhibiting the proliferation of HSCs [92]. Whilst the role of adiponectin in ageing remains uncertain, it is thought that circulating concentrations of adiponectin are paradoxically increased in older age and are positively associated with physical disability and mortality in elderly

241 individuals [93]. Furthermore, some evidence suggests that the association between adiponectin and

242 ageing may be modified by sex [94]. In addition to leptin and adiponectin, a wealth of other studies have

243 demonstrated that numerous other adipokines may be involved in the development and progression of

244 NAFLD **(Table 1)**. It should be noted that there is a substantial amount of conflicting evidence

245 regarding the changes in circulating concentrations of other adipokines in the context of NAFLD and

246 little is known about the potential pathological role of these adipokines in NAFLD (**Table 1**). Moreover,

247 further studies are required to elucidate whether the effects of age and sex on adipokine production

248 influences NAFLD risk.

249 White adipose tissue dysfunction and changes in adipokine secretion are also strongly associated with

250 increased low-grade chronic inflammation in WAT (metaflammation); characterised by the infiltration

251 of various leucocytes, an increase in the ratio of proinflammatory/anti-inflammatory macrophages and

252 leukocytes and the increased presence of crown-like structures (dying adipocytes surrounded by pro-

253 inflammatory macrophages) [95]. Consequently, metaflammation in WAT (particularly VAT

254 inflammation) is associated with an increased expression of pro-inflammatory cytokines such as

255 interleukin-6 (IL-6), interleukin-1 beta (IL-1β), tumour necrosis factor-alpha (TNF-α) and monocyte

256 chemoattractant protein-1 (MCP-1) [96-98]. Some of these have been shown to contribute to local IR,

257 elevated fatty acid (FA) lipolysis, anti-adipogenesis and pro-inflammatory macrophage infiltration [49,

258 97-101]. This in turn can impact both metabolic and endocrine functions of WAT. Evidence from murine

259 diet-induced obesity studies indicates that WAT inflammation and reductions in protective anti-

260 inflammatory lipokines such as palmitoleic acid may be important in the development of NASH [102-104].

261 By virtue of its anatomical links, via the portal vein, increased VAT inflammation is of particular

262 importance in NAFLD/NASH since VAT-derived inflammatory cytokines, (other adipokines, lipokines

263 and metabolites (e.g. NEFAs)) are initially transported to the liver and therefore may exacerbate

264 NAFLD severity. Consequently, this may in turn increase the associated risk of T2DM and CVD [105,

265 106]. Collectively, findings indicate that changes in the production of adipokines and increased WAT

266 inflammation may contribute to NAFLD via modulating local and hepatic function, inducing IR and

267 modulating the local and systemic pro-inflammatory conditions. Whether these changes in WAT

268   function contribute to the increased risk of extra-hepatic diseases associated with NAFLD

269   independently NAFLD requires further investigation.

270

Intestinal dysfunction, dysbiosis and NAFLD

272   Emerging evidence now suggests that changes in gut microbiota (GM) (i.e. dysbiosis) and intestinal

273   function may exacerbate WAT dysfunction which may indirectly contribute to metabolic dysfunction

274   and NAFLD [107]. The gastrointestinal (GI) tract is the first point of contact for ingested nutrients where

275   it has an integral role in nutrient breakdown and absorption, regulation of whole-body energy

276   homeostasis and is an important host defence barrier. Occupying the GI tract is an extensive number of

277   microorganisms, collectively known as the GM which are thought to modulate local and distal tissue

278   function via a range of complex mechanisms [108, 109]. The microbial organisms occupying the GI tract

279   mainly include bacteria, archaea, fungi and viruses (predominantly bacteriophages), however, studies

280   exploring the role of the GM in NAFLD have predominantly focused on bacteria [110, 111].

281   A plethora of studies have revealed that GM dysbiosis is associated with and is a contributing factor

282   to NAFLD [112-115]. The dominating phyla within human GM are Bacteroidetes and Firmicutes with a

283   significant inter-individual variation in the GM at lower taxonomical levels [116, 117]. Previous evidence

284   indicates that the relative abundance of Bacteroidetes is lower in patients with NASH compared to

285   those with hepatic steatosis and healthy controls [117]. More recently, *Bacteroides* abundance was

286   found to be significantly increased in patients with NASH and the abundance of *Ruminococcus* was

287   increased in patients with liver fibrosis [118]. As recently reviewed [113], this shifting in GM in relation

288   to NAFLD severity is supported by numerous other studies. Indeed, the presence of bacteria

289   belonging to the Proteobacteria phylum was increased significantly in patients with ≥F3 when

290   compared to patients with F0-F2 liver fibrosis (**Table 2**) [119]. Emerging evidence also supports a

291   strong link between the GM and NAFLD-cirrhosis indicating that the composition of the GM may be

292   a useful tool for the identification and staging of NAFLD. Utilising a unique twin and family study

293   design, one study identified a specific GM signature that had a robust diagnostic accuracy, with an

294   area under the reciever operating characteristic of 0.92, for the detection of NAFLD-cirrhosis [120].

295   Further work demonstrated the robustness and potential universal applicability of this microbiome

296   signature of NAFLD-cirrhosis in two independent cohorts across geographically and culturally

297   distinct populations [121]. However, given the impact of host genetics and environmental factors on the

298   composition of GM [122], it is unlikely that a single GM signature will be able to distinguish between

299   NAFLD phenotypes at an individual level.

300   Luca Miele and colleagues were the first to identify that patients with NAFLD generally have

301   increased intestinal permeability and alterations in intestinal tight junction integrity (observed as a

302   reduction in zonula occludens-1 within intestinal crypt cells), compared to healthy subjects [123].

303   Recent meta-analysis found that 39.1% of NAFLD patients had evidence of increased intestinal

304   permeability compared to 6.8% of healthy controls (OR 5.08, 95% CI 1.98 - 13.05) [124]. Furthermore,

305   subgroup analysis indicated that there was a higher incidence of increased intestinal permeability in

306   patients with NASH compared to patients with simple steatosis [124]. It is generally well-accepted that

307   the increased intestinal permeability commonly seen in NAFLD facilitates the translocation of GM-

308   derived metabolites and bacterial products (such as lipopolysaccharides (LPS) and ethanol) which

309   may in turn contribute to metaflammation and the pathogenesis of NAFLD [125].

310   In addition to altered GM and intestinal permeability, the abundance of GM-dependent metabolites is

311   thought to be altered in NAFLD, many of which may be detected in stool samples and may offer a

312   tool for the assessment of disease severity. For example, work comparing the abundance of distinct

313   stool metabolites in patients with NAFLD-cirrhosis vs healthy subjects revealed 17 metabolites

314   which, in combination, were able to accurately detect the presence of NAFLD-cirrhosis (AUROC

315   0.91, 95% CI 0.89 - 0.93) [121]. Thus, evidence is accumulating to suggest that accumulation of certain

316   microbial species, changes in intestinal function and increased intestinal permeability, are likely to

317   contribute not only to the pathogenesis of NAFLD but also to increased liver disease severity. Further

318   studies are required to elucidate the potential role of non-bacterial species within the GM on the

319   development and progression of NAFLD.

320   **Intestinal dysfunction, dysbiosis and links with WAT function in NAFLD**

321 Associated with WAT dysfunction are changes in intestinal function and GM dysbiosis, which have

322 also been proposed to be key factors contributing to NAFLD. Receiving around 70% of its blood supply

323 from intestinal vascularisation, the liver is constantly exposed to the metabolic products, toxins and

324 nutrients produced by the GM [126]. It has been suggested that when in a dysbiotic state, GM may

325 contribute to the development and progression of NAFLD via a range of pathways; including changes

326 in dietary energy harvest [127, 128], alterations in short-chain fatty acid (SCFA) production (particularly

327 butyrate) [129, 130], increased bacterial lipopolysaccharide (LPS) translocation [125, 131], alternations in bile

328 acid profiles [132] and increased endogenous ethanol production [133]. Indeed, the potential effects of these

329 factors on hepatic function and NAFLD have been discussed in various recent reviews [110, 112-114, 125, 134],

330 furthermore, alterations in appetite-regulating gut hormones are also likely to have an important role in

331 the development and progression of NAFLD, as recently reviewd [135-137].

332 Disruptions in intestinal permeability associated with obesity and NAFLD are likely to be accompanied

333 by a reduction in the integrity of intestinal tight junctions [123, 138]. Increased intestinal permeability in

334 the presence of GM dysbiosis is thought to facilitate the translocation of bacterial products including

335 pro-inflammatory endotoxins such as LPS. Circulating concentrations of LPS were found to be

336 significantly higher in patients with NAFLD compared to healthy controls [139, 140] and have been shown

337 to be positively associated with the expression of pro-inflammatory genes within both VAT and SAT

338 in individuals with obesity [141]. This is supported by evidence from pre-clinical murine studies

339 indicating that increased LPS may directly contribute to WAT inflammation and increase the release of

340 WAT-derived pro-inflammatory cytokines [142]. Accompanying these findings, various other studies

341 have proposed additional mechanisms by which changes in intestinal function and GM dysbiosis may

342 impact NAFLD development both directly and in-directly via detrimentally impacting WAT function

343 (**Table 3 and Figure 2**).

344 Evidence also suggests that the composition of the GM and intestinal function can differ between sexes

345 and such differences may partly explain differences in the risk of metabolic disease between sexes [143-

346 145]. Similarly, changes in GM composition and intestinal function are strongly associated with ageing

347 and are likely to contribute to the increased risk of NAFLD associated with older age both directly and

348   indirectly via exacerbating WAT dysfunction [146-148]. Similar to obesity, ageing is also associated with

349   disruptions in intestinal permeability subsequently facilitating the translocation of bacterial products

350   such as LPS which are known to contribute to both hepatic and WAT dysfunction (**Figure 2**) [149, 150].

351   Collectively, existing studies demonstrate the existence of a gut-WAT axis which, in addition to the

352   well-established gut-liver axis, may indirectly contribute to NAFLD pathogenesis. Furthermore,

353   differences in GM composition and intestinal function between men and women and with ageing may

354   contribute to both hepatic and WAT dysfunction and subsequently drive the development of NAFLD.

355

356   **NAFLD and extra-hepatic complications**

357

358   *NAFLD, T2DM and MetS*

359   Type 2 diabetes is both a risk factor for NAFLD and an extra-hepatic complication of NAFLD. The

360   association between T2DM and NAFLD is well-established and T2DM is considered to be one of the

361   most important risk factors for NAFLD. A meta-analysis of twenty-four studies found that the pooled

362   prevalence of NAFLD in patients with T2DM was 59.7% (95% CI: 54.3-64.9%), with the prevalence

363   of NAFLD being slightly higher in men (60.1%, 95% CI: 53.6-66.4%), compared to women (59.35%,

364   95% CI: 53.3-65.3%) [151]. Furthermore, the presence of obesity, hypertension and dyslipidemia, as

365   features of the MetS, were associated with an increased prevalence of NAFLD in patients with

366   T2DM, suggesting that these factors may act with T2DM to further increase the risk of NAFLD [151].

367   The presence of T2DM increases the risk of liver fibrosis by approximately 2-6 fold [152]. The

368   mechanism by which T2DM increases the risk of liver fibrosis is uncertain. However, numerous

369   factors have been proposed that could mediate the increase in risk of liver fibrosis in patients with

370   T2DM and these include insulin resistance, hyperglycaemia, hypoadiponectinemia, mitochondrial

371   dysfunction, increased reactive oxygen species, excess free cholesterol, increased proinflammatory

372   cytokines and endoplasmic reticulum stress [153]. Recently we have shown in patients with NAFLD

373   that increased circulating concentrations of growth-differentiation factor-15 (GDF-15), a stress-

374   inducible cytokine, are independently associated with the presence of ≥F3 and ≥F2 liver fibrosis

375   (**Table 2**) [154]. We also showed in this work that GDF-15 may be an important factor contributing to

376   the increased risk of liver fibrosis associated with T2DM, and that HbA1c levels explained ~30% of

377   the variance in GDF-15 concentrations [154]. However, further work is required to fully elucidate the

378   role of GDF-15 in the development and progression of NAFLD in patients with T2DM.

379   The estimated global prevalence of NAFLD among patients with T2DM is 55.5% (95% CI: 47.3-

380   63.7%) with prevalence estimates varying between geographical regions [155]. This study also found

381   that the estimated global prevalence of NASH and advanced fibrosis in patients with T2DM was

382   37.3% (95% CI: 24.7-50.0%) and 4.8% (95% CI: 0.0-17.5%) respectively [155]. The presence of

383   T2DM is also an important risk factor for the faster progression of NAFLD towards NASH, cirrhosis

384   or HCC [152, 156, 157]. Patients with NAFLD and coexisting T2DM are thought to have between a 2 and

385   6 fold increased risk of developing advanced fibrosis compared to patients with only NAFLD [152]. In

386   addition to T2DM, the presence of MetS is also recognised as an important NAFLD risk factor. The

387   presence of MetS in patients with NAFLD but without diabetes, is associated with more severe

388   NAFLD compared to patients without MetS [158]. Furthermore, this study suggested that a higher

389   number of MetS features was associated with a greater probability of NASH, with 70% of patients

390   diagnosed with NASH having three or more features of MetS. The presence of MetS has also recently

391   been shown to be associated with progression to advanced fibrosis in patients with NAFLD [159].

392   These findings support those of others which also show that NAFLD severity is positively associated

393   with the presence of MetS features, particularly the level of hypertension, hyperglycaemia and

394   hypertriglyceridemia [160].

395   It is important to highlight that the link between T2DM, MetS and NAFLD is complex and bi-

396   directional. Evidence from a recent large meta-analysis of over 500,000 individuals found that

397   NAFLD was associated with a ~2.2-fold increased risk of incident diabetes independently of age, sex,

398   adiposity and other common metabolic risk factors [161]. Interestingly, in this study, the risk of incident

399   diabetes was found to increase in relation to the underlying severity of NAFLD with a particularly

400   noticeable increase in risk according to the severity of liver fibrosis (n = 5 studies; random-effects HR

401   3.42, 95% CI 2.3-5.1) [161]. These findings support other evidence from meta-analyses and

observational studies which demonstrate that individuals with NAFLD had a higher risk for incident T2DM than individuals without NAFLD [8, 162]. Evidence collated from eight studies with a median follow-up period of 4.5 years indicated that NAFLD was associated with an increased risk of incident MetS with a pooled relative risk of 3.2 (95% CI: 3.1-3.4) when NAFLD was diagnosed via ultrasonography [163]. Collectively, this evidence suggests that a vicious cycle of worsening disease states is likely to exist between T2DM, MetS and NAFLD [152].

NAFLD and cardiovascular disease (CVD)

Evidence indicates that NAFLD is an important risk factor for various extra-hepatic diseases and the detrimental relationship between T2DM and NAFLD likely exacerbates this risk. Furthermore, given the strong associations with NAFLD and other cardiometabolic risk factors, including central obesity, atherogenic dyslipidaemia, and hypertension, it is no surprise that NAFLD is also associated with an increased risk of CVD [6, 164]. Recent evidence suggests that CVD is one of the most important causes of death among people with NAFLD [165], and patients with NAFLD are more likely to experience CVD-related death than a liver-related death [26, 164, 166]. Recent meta-analysis incorporating a total of 16 observational studies and over 34,000 individuals with a median follow-up of ~7 years, concluded that NAFLD conferred an odds ratio of 1.6 for fatal and/or non-fatal CVD events (random-effects OR of 1.6, 95% CI: 1.3-2.1) [167]. This is consistent with findings from others that suggest that the risk of incident CVD events increases further with greater severity of NAFLD even after adjusting for other established CVD risk factors [13]. Emerging data also supports the evolving notion that sex is an important modifier of NAFLD outcomes and suggest that the occurrence and prevalence of CVD related events and mortality are likely to differ between sexes. One study found that in ~108,000 individuals with NAFLD, cardiovascular events were 2 times higher in women compared to men (OR 2.1, 95% CI: 1.7-2.7) [168]. Women also had higher cardiovascular mortality with advancing age starting at age 42 years further highlighting the importance of both age and sex as important risk factors for both NAFLD and CVD [168].

NAFLD and Chronic kidney disease (CKD)

The risk of CKD is also increased in patients with NAFLD. Chronic kidney disease is a complex, progressive chronic condition that is defined by an abnormality in either the structure and/or function of the kidneys for ≥3 months with serious implications for health [7, 169]. Evidence from three meta-analyses demonstrates a higher incidence of CKD in patients with NAFLD [170-172]. The first of these studies, which included 33 observational (20 cross-sectional and 13 longitudinal) studies concluded that NAFLD was associated with a 2-fold increased prevalence of CKD (random-effects odds ratio 2.1, 95% CI: 1.7-2.7) and that NAFLD was associated with a nearly 80% increased risk of incident CKD (random-effects HR 1.8, 95% CI: 1.7-2.0) [7, 170]. Similarly, the second more recent meta-analysis confirmed that NAFLD was associated with a ~40% increase in the long-term risk of incident CKD (random-effects HR 1.4, 95% CI 1.2-1.5) [171]. Most recently, findings from a large updated meta-analysis indicate that NAFLD was significantly associated with a ~1.45 fold increased long-term risk of incident CKD and this association was independent of age, sex and conventional CKD risk factors [172]. Interestingly, these studies also support an association between increased NAFLD severity (particularly the presence of advanced fibrosis) and increased risk of CKD [170-172]. Another large database study in Germany also supports a strong link between NAFLD and increased risk of CKD that is independent of age, sex and the presence of additional cardiometabolic risk factors such as diabetes, obesity and hypertension [173].

NAFLD and non-hepatic cancers

In addition to increasing the risk of HCC, recent evidence suggests that NAFLD may also increase the risk of various non-hepatic cancers. Findings from a recent large population-based cohort study concluded that, compared to healthy controls, patients with biopsy-confirmed NAFLD had significantly increased overall cancer incidence over a median 13.8 years follow-up period (adjusted HR 1.3, 95% CI: 1.2-1.4) [174]. Whilst this increase was mostly driven by a higher HCC incidence, the presence of NAFLD was also associated with modestly increased rates of melanoma, pancreatic, and kidney/bladder cancers [174]. In support of these findings, recent meta-analysis of 10 cohort studies (> 180,000 individuals, 24.8% with NAFLD) found that NAFLD was significantly associated with a

456 nearly 1.5-fold to two-fold increased risk of developing gastrointestinal cancers (oesophagus,

457 stomach, pancreas or colorectal cancers) independently of confounding factors such as age, sex,

458 obesity, diabetes and smoking status [175]. There is currently very limited data on the severity of

459 NAFLD (particularly the severity of liver fibrosis) and the risk of developing extra-hepatic cancers.

460 One recent study found that more severe NAFLD was associated with significantly increased overall

461 mortality with most of the excess mortality observed being driven by extrahepatic cancer and liver

462 cirrhosis [176]. Whilst it is reasonable to assume that the risk of developing extra-hepatic cancers is

463 increased in relation to NAFLD severity, further large prospective studies are needed to confirm this

464 link. Such studies should account for the potential modifying effect of important genetic variants, age,

465 sex, and obesity along with other NAFLD-associated comorbidities when considering the relationship

466 between NAFLD severity and risk of specific extra-hepatic cancers. This latter consideration is

467 particularly important since it is not yet clear whether NAFLD is associated with an increased risk of

468 certain extra-hepatic cancers simply as a consequence of shared metabolic risk factors or whether

469 NAFLD itself directly contributes to an increased risk of developing extrahepatic cancers [175].

470

471 **Conclusion**

472 The risk of developing NAFLD differs between sexes, changes with age and is likely to be modulated

473 by complex interactions between genetic and environmental factors. Differences in WAT mass, its

474 distribution (VAT vs SAT) and functionality (metabolic & endocrine), are likely to be key drivers of

475 hepatic steatosis and NAFLD development. Similarly, differences in the regional distribution and

476 function of WAT between men and women and between age groups are likely to contribute to the

477 increased risk of NAFLD progression associated with sex and age. The development of GM dysbiosis

478 and intestinal dysfunction is likely to contribute to NAFLD both directly and indirectly via the

479 exacerbation of WAT inflammation and dysfunction through a range of GM-derived factors.

480 Collectively, in the presence of chronic nutritional surplus, both WAT and intestinal dysfunction act

481 in a synergistic manner to drive systemic metabolic dysfunction and the development of NAFLD and

482 are further influenced by sex and age. In turn, NAFLD increases the risk of chronic hepatic and extra-

483 hepatic metabolic diseases including T2DM, CVD, CKD, HCC and certain extra-hepatic cancers.

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493 **CONFLICTS OF INTEREST**

494 The authors declare no conflict of interest.

495 **AUTHORSHIP**
496 The authors had sole responsibility for all aspects of preparation of this paper.
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Figure 1. Age-related changes in SAT and WAT distribution in men and women associate with an increased risk NAFLD, MetS , T2D and CVD.

Sex and age are key factors that modify the risk of NAFLD and NAFLD progression. NAFLD risk is lower in younger women compared to younger men whereas the risk of NAFLD is similar in older men and women (i.e. post menopausal). Younger women have an increased capacity to preferential expand gluteal femoral SAT consequently protecting them from NAFLD. Age-associated changes in WAT leads to the redistribution of WAT which is typically characterised by a marked reduction in SAT and increased central metabolically-unfavourable VAT which may partly explain the increased risk of NAFLD associated with ageing in both men and women. WAT distribution is different between men and women, is heavily influenced by ageing and is strongly associated with NAFLD risk. *Abbreviations: MetS; Metabolic syndrome, CVD; Cardiovascular disease, SAT; Subcutaneous adipose tissue, VAT; Visceral adipose tissue.*

Figure 2. NAFLD is associated with changes in gut microbiota-derived factors that can alter hepatic and WAT function

Changes in GM in NAFLD result in alterations in the production of various metabolites/factors that are thought to contribute to NAFLD both directly (i.e. by directly impacting hepatic function) and indirectly through detrimentally influencing WAT function. As highlighted on the left – intestinal eubiosis and healthy gut function (such as that typically found in young individuals) promotes intestinal barrier integrity and homeostasis whilst restricting the production and dissemination of metabolically detrimental factors (such as LPS and endogenous ethanol) into circulation, the liver and WAT. Conversely, as highlighted on the right, intestinal dysbiosis (such as that often associated with older age) leads to alterations in various GM-derived factors/metabolites that impair the function of tight junction-associated proteins located within the intestinal epithelium. Consequently, these changes are thought to contribute to an increased risk of NAFLD both directly (via inducing hepatic mitochondrial function, inflammation and steatosis) and indirectly through detrimentally impacting WAT function (impairing WAT expansion, metabolic flexibility and increasing the production of pro-inflammatory cytokines). The increased production of inflammatory cytokines is thought to lead to a state of chronic low-grade inflammation which is likely to further disrupt the function of tight junction-associated proteins – thus forming a viscous cycle of worsening metabolic dysfunction and NAFLD disease severity. *Abbreviations: GM; gut microbiota, LPS; lipopolysaccharide, TMAO; Trimethylamine N-oxide, TAG; Triglyceride, FFA; Free fatty acid.*

552   **Table 1** - Changes in circulating concentrations of adipokines and their potential roles in NAFLD.

Adipokine	Association with NAFLD	Potential role in NAFLD
Leptin	Increased [74]	Anti-steatotic during normoleptinemia [77] Pro-fibrogenic during hyperleptinemia via increasing the expression of fibrogenic factors from activated HSCs [177, 178]
Adiponectin	Decreased [86]	Hypoadiponectinemia is thought to contribute to: increased hepatic steatosis, increased hepatic insulin resistance and an increased pro-inflammatory state [179, 180]
Resistin	No association [181-183], Increased [184-186]	Largely unknown, increased concentrations potentially promotes a pro-inflammatory environment [187]
RBP-4	Increased [188, 189]	Largely unknown, potentially induces hepatic mitochondrial dysfunction and promotes hepatic steatosis [190, 191]
Adipsin	Decreased [192], No association [193], Increased [194]	Largely unknown, low concentrations may impact hepatic function via a reduction in insulin production [195]
Chemerin	Increased [193, 196, 197]	Largely unknown, increased concentrations potentially protective via the suppression of pro-inflammatory cytokines [198]
Apelin	Not associated [199], Increased [200]	Largely unknown, increased concentrations are potentially profibrogenic via increasing the expression of profibrotic factors from HSCs [201, 202]

553   In contrast to classic adipocyte-derived adipokines leptin and adiponectin, studies investigating
554   changes in circulating concentrations of other adipokines in patients with NAFLD are largely
555   inconsistent. Similarly, whilst the role leptin and adiponectin in the development and progression of
556   NAFLD remain somewhat debated, there is currently very little known about the potential roles of
557   other adipokines on hepatic function and NAFLD. It should be noted that the expression of many
558   adipokines (e.g. Chemerin and RBP-4) is not restricted to WAT and also occurs within other tissues
559   including the liver. Consequently, whilst changes in the secretion of WAT-derived adipokines may
560   contribute to altered circulating concentrations, other sources (particularly hepatic) may also influence

561   circulating concentrations, hepatic function and NAFLD. *Abbreviations: HSCs; Hepatic stellate cells,*

562   *RBP-4; Retinol binding protein-4.*

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Table 2: Histological definitions of liver fibrosis stages and corresponding liver-biopsy validated liver VCTE cutoff values.

Liver fibrosis stage	Histological definition	Liver VTCE cutoff (kPa)
F0	None	
F1	Perisinusoidal or periportal	
F1A	Mild, zone 3 perisinusoidal	
F1B	Moderate, zone 3, perisinusoidal	
F1C	Portal/periportal	
F2	Perisinusoidal and portal/periportal	8.2
F3	Bridging fibrosis	9.7
F4	Cirrhosis	13.6

Liver fibrosis stages and corresponding histological definitions are based on the NASH clinical scoring network scoring system [203]. Liver VCTE cutoff values are based on the findings from a recent large validation study [204]. The liver VCTE threshold of 8.2 kPa was found to have a: sensitivity of 0.71 (0.64-0.77), specificity of 0.70 (0.62-0.77), PPV of 0.78 (0.71-0.83) and NPV of 0.61 (0.54-0.69) for the identification of ≥ F2 liver fibrosis. For the prediction of ≥ F3 liver fibrosis, 9.7 kPa was found to have a sensitivity of 0.71 (0.62-0.78), specificity of 0.75 (0.69-0.80), PPV of 0.63 (0.55-0.71) and NPV of 0.81 (0.74-0.85). For the prediction of ≥ F4 fibrosis, 13.6 kPa was found to have a sensitivity of 0.85 (0.69-0.95), specificity of 0.79 (0.74-0.83), PPV of 0.29 (0.24-0.57) and NPV of 0.98 (0.95-0.99). *Abbreviations: VCTE; vibration-controlled transient elastography, kPa; kilopascal, PPV; positive predictive value, NPV; negative predictive value*

Table 3 - Changes in GM derived factors/metabolites in NAFLD and their proposed effects in WAT
and the liver.

Factor/metabolite	Association with NAFLD	Proposed effect on WAT	Proposed effect on liver
LPS	Increased [139, 140, 205]	Increased inflammation and decreased insulin sensitivity [131, 206, 207].	Increased liver inflammation via the activation of hepatic macrophages and platelets [205]. Pro-fibrogenic via the activation of HSCs [208].
Endogenous ethanol	Increased [133, 209]	Largely unknown. Potentially induces oxidative stress and inflammation [210].	Increased hepatic mitochondrial dysfunction [211], increased hepatic steatosis and inflammation [133].
Butyrate	Decreased [212]	Decreased production is thought to contribute to increased inflammation and decreased fatty acid oxidation [213, 214].	Decreased production is thought to contribute to hepatic mitochondrial dysfunction [215], increased hepatic steatosis and inflammation [216].
TMAO	Increased [217, 218]	Increased inflammation and impaired expression of insulin signalling-related genes [219, 220].	Reduced insulin sensitivity and increased hepatic steatosis and inflammation [219, 221].
Indole	Decreased [222]	Reduced regulation of microRNA expression (specifically miR-181) leading to increased inflammation and decreased insulin sensitivity [223].	Decreased production is thought to contribute to increased hepatic steatosis and inflammation [222].

Changes in the production of various GM-derived metabolites/factors may contribute to the
development of NAFLD both directly (i.e. via a direct action within the liver) and indirectly via
affecting the function of WAT. Whilst evidence of altered circulating concentrations of certain factors
(namely LPS, endogenous ethanol and TMAO) are well-reported in patients with NAFLD, changes in
circulating concentrations of other factors (particularly SCFAs) require further investigation.
Furthermore, more research is required to elucidate the potential contribution of endogenously
produced ethanol on WAT dysfunction in the context of NAFLD. *Abbreviations: LPS;*
lipopolysaccharide, HSCs; Hepatic stellate cells, TMAO; Trimethylamine N-oxide.

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