

Unwrapping the mechanisms of ceramide and fatty acid-initiated signals leading to immune-inflammatory responses in obesity

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1 **Title:** Unwrapping the mechanisms of ceramide and fatty acid-initiated signals leading to immune-inflammatory
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3
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19
20 **Abstract**

21 Obesity is considered a global epidemic developed in part as a consequence of the overconsumption of a high
22 fat diet. One of the main negative outcomes of obesity is the development of low-grade chronic systemic
23 inflammation, induced by dysregulated immune responses, which can lead to multiple obesity-related diseases.
24 Ceramides are a group of bioactive lipids known to be elevated in obesity and obesity-associated conditions,
25 including cardiovascular disease and type II diabetes. Ceramides may be key players in promoting an obesity-
26 induced inflammatory environment due to their ability to activate key pathways such as TLR4 and Nlrp3, while
27 studies have shown that inhibition of ceramide synthesis gives rise to an anti-inflammatory environment. N-3
28 polyunsaturated fatty acids (n-3 PUFA) have been of interest due to their anti-inflammatory actions and shown
29 to have beneficial effects in obesity-related diseases. This review will highlight the impact of ceramides in
30 promoting an obesity-induced inflammatory microenvironment and discuss how n-3 PUFA could potentially
31 counteract these responses and have a regulatory effect promoting immune homeostasis.

32
33 **Keywords:**

34 Ceramides, obesity, inflammation, fatty acids, N-3 polyunsaturated fatty acids

35
36 **Abbreviations:**

37 Acid sphingomyelinase (ASM), cardiovascular diseases (CVD), dihydroceramide desaturase (DES),
38 dihydroceramide synthase (CerS), docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), fatty acid (FA),
39 high fat diet (HFD), obesity-induced inflammatory microenvironment (OIIM), serine palmitoyltransferase
40 (SPT), sphingomyelinases (SMase), Type II diabetes (T2D), western diet (WD), World Health organisation
41 (WHO), **ceramide synthase enzyme (CerS)**

44 **Introduction**

45 Obesity is one of the major worldwide health concerns of the 21st century. In 2016, 650 million adults were
46 classified as obese, 1.9 billion were classified as overweight and the current rising trend estimates that 1 billion
47 people would be obese by 2025 (WHO, 2016). Obesity-induced inflammatory microenvironment (OIIM) and
48 dysregulated immune responses have been shown to induce, escalate or be associated with most obesity-linked
49 diseases including cardiovascular disease (CVD), type II diabetes (T2D), cancer, osteoarthritis, depression and
50 autoimmune diseases (Hruby et al., 2016). Obesity can also impact upon many other conditions such as the
51 recent severe acute respiratory disease SARS-CoV-2. Meta-analysis of 400,000 SARS-CoV-2 patients has
52 shown that obesity increases infection risk by 46%, hospitalisation risk by 113% and death rate by 48%, while
53 there are concerns that vaccines might be less effective in obese individuals (Popkin et al., 2020). It is therefore
54 crucial to identify the main players driving OIIM and the molecular mechanisms underlying disease initiation.
55 The critical link between obesity and diet is well recognised (Bortolin et al., 2018; Almeida-Suhett et al., 2019).
56 Excessive lipid accumulation because of the overconsumption of high fat (HFD) or western (WD) diets, leads to
57 the expansion of white adipose tissue. Adipose tissue contains many immune cells, including macrophages and
58 T cells. Under lean conditions, adipose tissue maintains an anti-inflammatory environment, with M2 like
59 macrophage populations and regulatory T cells. However, in obese conditions the adipose tissue environment
60 changes, leading to the development of low-grade chronic systemic inflammation, due to shifts in the presence
61 immune cell populations (Donohoe et al., 2016).

62 Certain bioactive lipids have been shown to be elevated in obesity and modulate multiple critical immune cell
63 processes, affecting obesity-induced inflammation (Chaurasia et al., 2016; Hamada et al., 2014 and Chaurasia et
64 al., 2019). It is crucial to identify the main bioactive lipid species and explore their involvement into the
65 molecular mechanisms leading to OIIM. Sphingolipids, in particular ceramides, have attracted interest due to
66 their immunomodulatory effects. Here, we summarise the immunomodulatory effects of ceramides in the
67 establishment of OIIM and discuss the therapeutic potential of using n-3 polyunsaturated fatty acids (n-3 PUFA)
68 to promote an anti-inflammatory immune response.

70 **The establishment of obesity-induced inflammatory microenvironment**

71 The World Health Organisation (WHO) has defined obesity as abnormal or excessive fat accumulation that
72 presents a risk to health. Diets rich in high fat, sugar, salt and low fibre content such as HFD and WD have been
73 shown to induce obesity (Almeida-Suhett et al., 2019). Overconsumption of these diets with disrupted balance
74 in energy homeostasis leads to the accumulation of excessive lipids in adipose and non-adipose tissue of the
75 body (Donohoe et al., 2016).

76 The infiltration of macrophages and lymphocytes into adipose tissues leads to an immune tipping point, with the
77 production of inflammatory cytokines and lipid mediators giving rise to a chronic low-grade inflammatory
78 environment (Yang et al., 2010 & Christ et al., 2018). Murine models of diet-induced obesity have shown naïve
79 adipose-resident T cells decrease and give rise to effector-memory populations, in which the TCR-V β repertoire
80 were also impacted (Yang et al., 2010). Importantly, these mice had elevated levels of IFN- γ ⁺, granzyme and
81 other pro-inflammatory mediators which account for the obesity-induced chronic inflammation (Yang et al.,
82 2010). WD was shown to be misinterpreted as a threat to the host, in which innate immune system cells became
83 hyperactive through inflammasome activation, resulting in the production of pro-inflammatory cytokine
84 response via IL-1 β forming a hyperactive inflammatory environment (Christ et al., 2018). These studies
85 illustrated an extensive pro-inflammatory effect linked to the diet which ‘reprogram’ or ‘prime’ both innate and
86 adaptive immune cells towards a low-grade chronic inflammatory microenvironment observed in obesity.
87 Recent developments in lipidomic techniques have allowed lipid profiling of tissues from overweight and obese
88 individuals, identifying ceramides which could potentially act on immune cell processes and promote obesity-
89 induced inflammation.

91 **Ceramide Biosynthesis**

92 Ceramides are sphingolipids, synthesised in the endoplasmic reticulum (ER), that have been considered as key
93 players in promoting the OIIM. Ceramides are derivatives of long-chain bases (e.g. sphingosine) and FAs of
94 varying lengths (Hannun & Obeid, 2017). Not only are they a universal component of the cellular membrane,

95 they also play crucial roles in cell proliferation, apoptosis, migration, senescence, autophagy and inflammation
96 (Hannun & Obeid, 2017, Chaurasia & Summers, 2020).

97
98 Dysregulation in sphingolipid metabolism has been associated with various inflammatory diseases including
99 cancer, obesity and autoimmune conditions (Chaurasia & Summers, 2020). Elevated ceramide levels within
100 specific tissues have been associated with different pathological conditions (Table 1). Lipidomic analysis has
101 shown ceramides to accumulate in multiple tissues including liver, skeletal muscle, heart and adipose tissue in
102 obesity (Kien et al., 2013, Chaurasia & Summers, 2020).

103
104 Ceramide biosynthesis is regulated by three pathways; *de novo* synthesis, sphingomyelin hydrolysis and the
105 salvage pathway. The *de novo* pathway is the main biosynthetic route, starting with the condensation of
106 palmitate and serine, although other saturated fatty acids and amino acids can be involved (Hannun & Obeid,
107 2017). The main enzymes of the *de novo* pathway are serine palmitoyltransferase (SPT), that is also the rate
108 limiting step, 3-ketodihydrosphingosine reductase, dihydroceramide synthase (CerS) and dihydroceramide
109 desaturase (DES) (Figure 1). The other two pathways involved in ceramide biosynthesis mainly function as
110 recycling pathways which break down surplus complex sphingolipids via catabolic enzymes including various
111 sphingomyelinases (SMase). Acid sphingomyelinase (ASM) is one member of the hydrolase enzymes that
112 catalyses the breakdown of sphingomyelin to create ceramide (Xiong et al., 2016). Targeting the ceramide
113 biosynthetic pathways can be used to assess the impact of these bioactive lipids on specific immune cell
114 populations (Table 2).

115
116 Overconsumption of obesity promoting diets containing the key substrates, palmitoyl-CoA and serine, for
117 ceramide synthesis is likely to promote this elevation. In addition, inflammatory modulators, which are elevated
118 in the OIIM, can regulate cellular ceramide metabolism elevating cellular ceramide levels. Inflammatory
119 cytokines IL-1 β and TNF α have been shown to promote the accumulation of ceramides (Gill & Sattar, 2009,
120 Haus et al., 2009, Holland et al., 2011). TNF α has been shown to specifically induce genes involved in the
121 ceramide synthesis pathway, such as serine palmitoyltransferase (SPT) in murine fibroblast (L929) (Meyer & de
122 Groot, 2003). Sphingomyelinase (SMase), a key enzyme in the sphingomyelin hydrolysis pathway for ceramide
123 production has been shown to not only promote ceramide accumulation in bovine cerebral endothelial cells (Xu
124 et al., 1998) but recently, inhibition of neural sphingomyelinase-2 resulted in down regulation of the TNF α -
125 mediated expression of CD11c and secretion of inflammatory mediators IL1 β and MCP-1 in
126 monocytes/macrophages via the phosphorylation of JNK, p38 and NF- κ B/ NF- κ B/AP-1 pathways (Al-Rashed et
127 al., 2020). These *in vitro* observations are also supported by *in vivo* research where intratracheal administration
128 of TNF α boosted ceramide levels via inducing neutral sphingomyelinase in murine models (Mallampalli et al.,
129 1999). Additionally, it is known that TNF α can inhibit insulin signalling via stimulation of p55 TNF receptor
130 and sphingomyelinase activity, which plays a critical role in obesity-induced insulin resistance (Peraldi et al.,
131 1996). In addition, it is well documented that the accumulation of adipose tissue during obesity leads to decrease
132 in adiponectin, an important adipokine and their receptors (Nigro et al., 2014). Adiponectin has been described
133 to regulate ceramide metabolism via the adiponectin receptor, expressed endogenously by various cells
134 including T cells (Holland et al., 2011). Previous reports have shown that activation of the adiponectin receptor
135 leads to ceramidase activity and consequently to a decrease of ceramide levels, acting as a negative regulator in
136 cardiomyocyte and pancreatic β cell. As adiponectin levels are reduced in obesity, this may lead to increased
137 ceramide accumulation (Holland et al., 2017).

138
139 TLR-4-stimulated macrophages activate the transcription factor NF- κ B via I κ B kinase (IKK β). A knockout of
140 IKK β which inhibited the pro-inflammatory signalling mediated via TLR-4, led to a significant reduction in
141 ceramide synthesis (Holland et al., 2011). To further confirm the role of TLR-4-mediated ceramide synthesis,
142 macrophages were supplemented with palmitate but even the palmitate supplementation was not able to restore
143 ceramide levels. Signalling pathways modulated by ceramides to induce a pro-inflammatory response can also
144 enhance ceramide biosynthesis creating an inflammatory feed forward loop, which could be one of the critical
145 mechanisms maintaining a low-grade chronic inflammation.

147 **Immunomodulatory mechanisms of ceramide action**

148 Ceramides have been shown to modulate the function of immune system cells. Initially, studies have focused on
149 cells of the innate immune system while recent studies have suggested a link between ceramides and the
150 adaptive immune cell response (Chaurasia et al., 2016, Hamada et al., 2014, Turpin et al., 2014). **Although the
151 precise mechanisms are still not fully understood, below we will discuss the immunomodulatory roles ceramides
152 play in principal immune cells and how their modulation impacts their function and fate contributing to OIIM
153 (Figure 2).**

155 *Ceramide modulation on macrophages*

156 In obesity, macrophages are pivotal players in maintaining the tissue homeostatic microenvironment, but can
157 also promote the OIIM via polarising to a pro-inflammatory fate, producing inflammatory cytokines such as IL-
158 1 β and TNF α (Chaurasia et al., 2016 & Haus et al., 2009). **A clear association between these pro-inflammatory
159 cytokines and increased ceramides levels in obesity, particularly in adipose tissue and circulating serum has
160 been described (Haus et al., 2009).** In peripheral adipose tissue, both genetic and pharmacological inhibition (via
161 myriocin) of serine palmitoyltransferase (Sptlc) enzyme and ceramide synthesis pathway altered macrophage
162 populations, with a reduction of pro-inflammatory cytokines, including IL-6 and TNF α and an increase in the
163 M2 anti-inflammatory macrophage markers, such as IL-10 (Chaurasia et al., 2016).

164
165 Inhibition of ceramide biosynthesis may elicit, including improved glucose tolerance, increased insulin
166 sensitivity, amelioration of hepatic steatosis and reduction in adipocyte size (Chaurasia et al., 2019). Alterations
167 of the M1/M2 macrophage ratio with associated altered gene and cytokine expression, can push OIIM towards
168 an anti-inflammatory homeostatic environment (Chaurasia et al., 2016). **Injection of nanoliposome-loaded C6-
169 ceramide to mice models was able to enhance the M1 cytokine production (including IL12, IFN- γ and TNF- α)
170 and inhibit M2 cytokine production in bone marrow-derived macrophages. This observation is important to
171 conceive the flexibility of the immune response in relation to ceramides, which can be easily driven to pro/anti-
172 inflammatory states (Li et al., 2018).** Although ceramide synthase enzyme (CerS)-6 deficient mice present
173 reduced macrophage infiltration within gonadal white adipose tissue and improved insulin homeostasis,
174 macrophage-specific CerS6 deletion did not specifically reduce the body weight/adiposity nor improve insulin
175 sensitivity (Turpin et al., 2014). These observations highlight the crucial pathophysiological role of ceramides,
176 demonstrate immunomodulatory effects on OIIM and illustrate the complexity underlying these processes that
177 involve multiple tissues and immune cell types. In addition, reduced macrophage infiltration into white adipose
178 tissue of CerS5 knockout mice fed HFD was observed, as well as a reduction in pro-inflammatory gene
179 expression such as **Caspase1**, TNF α and IL-1 β (Gosejacob et al., 2016).

180
181 The Nod Like Receptor 3 (Nlrp3) has been shown to be a critical link between ceramide accumulation and
182 macrophage activation leading to OIIM. Nlrp3 can sense bioactive lipids including ceramides, which trigger the
183 macrophages activation and infiltration to the peripheral OIIMs and ablation of Nlrp3 prevented the obesity-
184 induced inflammasome activation in fat depots and liver together with enhanced insulin-signalling.
185 Additionally, when Nlrp3 ceramide sensing is interrupted, the levels of pro-inflammatory cytokines (IL-18,
186 IFN γ) are reduced alongside with the number of effector adipose tissue T cells. Importantly, the number of naïve
187 T lymphocytes (CD4⁺CD62L⁺CD44⁻, CD8⁺CD62L⁺CD44⁻) are increased suggesting an ameliorative anti-
188 inflammatory effect on OIIM and obesity-associated pathologies, also supporting the role of ceramides in both
189 innate and adaptive immune systems (Vandanmagsar et al. 2011).

191 *Ceramide modulation on Neutrophils*

192 In addition to macrophage infiltration into adipose tissue, other cells of the innate immune system have been
193 shown to infiltrate, promoting the OIIM. Neutrophils have been shown to be elevated in obese individuals (Xu
194 et al., 2015) and infiltrate into adipose tissue at the early stages of HFD consumption (Elgazar-Carmon et al.,
195 2008 & Hadad et al., 2013). Levels of basal superoxide production and also formyl-methionyl-leucyl-
196 phenylalanine (fMLP)-stimulated superoxides were found to be elevated in neutrophils isolated from obese
197 individuals (Brotfain et al., 2015). Both in stimulated and unstimulated conditions, neutrophils from obese

198 microenvironment were primed to increase superoxide production and chemotactic activity that can drive OIIM
199 (Brotfain et al., 2015). Early studies have shown *in vitro* priming of neutrophils with C2 ceramide lead to
200 enhanced superoxide levels followed by fMLP treatment (Richard et al., 1996). However, this response may be
201 concentration dependent, as in an additional study, *in vitro* activated neutrophils showed C2 ceramide to
202 increase (< 1 microM) and inhibit (> 1 microM) superoxide generation (Wong K., Li X. B., & Hunchuk N.,
203 1995). Neutrophil extracellular traps (NETs), DNAs components that are known to activate immune responses,
204 have been shown at elevated levels in obese individuals (D'Abbondanza et al., 2019). One study has shown how
205 the enhancement of NET production can occur via a ceramide/PKC ζ -mediated pathway, and treatment with
206 synthetic ceramide is sufficient to promote NET formation (Corriden et al., 2015). Furthermore, intracellular
207 C16 and C24 levels shown to contribute to spontaneous neutrophil apoptosis via caspase activation, *in vitro*.
208 Pharmacological inhibition of de novo pathway, reducing ceramide accumulation, created an anti-apoptotic
209 effect on neutrophils (Seumois et al., 2007).

210

211

212 *Ceramide modulation on T cells*

213 T cells have also shown to play a role in obesity and promotion of the OIIM. In HFD murine models, elevated
214 CD8⁺ effector T cells were found to infiltrate obese epididymal adipose tissue even before macrophage
215 accumulation, with reduced numbers of CD4⁺ helper and regulatory T cells (Nishimura et al., 2009). Genetic
216 depletion of CD8⁺ T cells inhibits OIIM cascade with reduced macrophage infiltration and improved systemic
217 insulin resistance. It is now clear that CD8⁺ T cells play crucial roles in macrophage differentiation, activation
218 and infiltration which contribute to the initiation and maintenance of OIIM (Nishimura et al., 2009).
219 Ceramide biosynthesis has been shown to impact effective TCR signalling, activation and effector responses. In
220 graft versus host murine models, acid sphingomyelinase (ASMase) deletion resulted in reduced CD8⁺ activation
221 and effector responses (Rotolo et al., 2009). Sofi et al. showed that CerS6 was as required for optimal T cell
222 response, with reduced allogeneic responses observed due to impaired C16 ceramide production (Sofi et al.,
223 2017). The ceramide biosynthesis pathways have also been shown to impact CD8 granule-mediated
224 cytotoxicity, in which sphingomyelinase (ASMase)-deficient mice are defective in exocytosis of cytolytic
225 effector molecules (Herz et al., 2009).

226 Other studies have suggested that ceramides can promote T cell differentiation towards a specific pro- or anti-
227 inflammatory fate, in a concentration and tissue-specific manner (Martín-Leal et al., 2020). Palmitate, a 16-
228 carbon saturated FA, is a main product of de novo fatty acid biosynthesis and common dietary component of
229 HFD. In both humans and rodents (including obese and insulin resistance murine models) elevated systemic FA
230 availability via acute infusion, can increase plasma ceramide concentrations (Watt et al., 2012 and Tran et al.,
231 2016). Palmitate has been shown to promote obesity related low-grade chronic inflammation and can directly
232 prime CD4⁺ T cell differentiation into a CD44^{hi}-CCR7^{lo}-CD62L^{lo}-CXCR3⁺-LFA1⁺ effector memory-like
233 phenotype (Mauro et al., 2017). This bias in CD4⁺ T cell differentiation leads to a preferential trafficking to non-
234 lymphoid periphery sites with an increased effector Th1/Th17 pro-inflammatory function, contributing to the
235 creation of OIIM. Palmitate was shown to modulate CD4⁺ T cells via enhanced activation of PI3K p110d-Akt-
236 dependent pathway (Mauro et al., 2017). Furthermore, ceramides can push homeostasis towards a pro-
237 inflammatory environment via reducing the Foxp3⁺ Treg population (Zhou et al., 2016 and Hollmann et al.,
238 2016). As mentioned, ASM is a major enzyme in the synthesis of ceramides from sphingomyelin. In ASM
239 specific knock out mice, *in vitro* and *in vivo* assays illustrated that ASM ablation leads to a higher number of
240 Tregs (Zhou et al., 2016). In addition to elevated Treg frequency, the suppressive activity of Tregs was also
241 enhanced both in the ASM knock out mice and in the presence of amitriptyline, a ASM pharmacological
242 inhibitor (Hollmann et al., 2016).

243 *Ceramide modulation on B cells*

244 Although changes in macrophages and T cells have been the main focus on the development on the OIIM, B
245 cells also contribute to the inflammatory environment. In diet-induced obese mice, Winer *et al* showed how B
246 cells accumulate in visceral adipose tissue. Depletion of B cells resulted in the suppression of M1 macrophage
247 activation, polarisation and CD8⁺ T cell activation with significant reduction in TNF- α and IFN- γ pro-
248 inflammatory cytokine levels in adipose tissue stromal vascular cells (Winer et al., 2011). Importantly, B cells

249 were shown to promote systemic as well as local (visceral adipose tissue) inflammation in obesity-associated
250 diseases such as insulin resistance and glucose intolerance. B^{null} mice showed improved glucose/insulin
251 sensitivity, adipose tissue inflammation, reduced adipose tissue hypertrophy and no impact on circulating
252 adiponectin levels (DeFuria et al., 2013). Consistent with the reduction observed in inflammatory B- and T-cell
253 cytokine levels, reduced inflammation in obese/insulin resistant B cell-null mice associated with an increased
254 percentage of anti-inflammatory Tregs (DeFuria et al., 2013). Additionally, ceramides were linked to B cell-
255 associated apoptotic program known as activation induced cell death triggered by B cell receptor. Specifically,
256 long chain ceramides C16, C18 and C24 synthesised via de novo pathway were shown to cross-link with B cell
257 receptor. This cross-linking triggers a specific sequence of biochemical events leading to caspase-dependent
258 apoptosis which potentially involves a mitochondrial damage and loss of function (Kroesen et al., 2001 &
259 Kroesen et al., 2003). Furthermore, ceramides (C2) are known to inactivate the anti-apoptotic protein B cell
260 lymphoma 2 (Bcl2) via dephosphorylating and reducing the mRNA levels of Bcl2 (Ruvolo et al., 1999 & Chen
261 et al., 1995).

262 **Therapeutic potential of n-3 PUFA on immune function and OIIM**

263 N-3 PUFA could be used to counteract the inflammatory response resulting from the over consumption of a
264 HFD. Although the extent of their effects is still debated, n-3 PUFA have been shown to have overall anti-
265 inflammatory effects, for example, n-3 PUFA supplementation has been shown to lower blood pressure and
266 reduce the levels of triglycerides, leading to a reduced risk of CVD (Hu et al., 2019). In inflammatory diseases,
267 such as type II diabetes, n-3 PUFA improved insulin sensitivity reduced the body fat ratio (Martins et al., 2018
268 and Gutiérrez et al., 2019).

269 The molecular mechanism of the anti-inflammatory action of n-3 PUFA remains of interest and is subject of on-
270 going investigations. Increasingly, evidence suggests that n-3 PUFA act in three major ways to help the
271 restoration of homeostasis in OIIM. Firstly, n-3 PUFA are recognised by distinct receptors such as TLR4, Nlrp3
272 and G protein-coupled receptors, mainly GPR120 expressed by immune cells (such as macrophages) leading to
273 reduced inflammatory cytokine and chemokine responses (Oh et al., 2010 and Mildenerger et al., 2017).
274 Secondly, n-3 PUFA can modulate the differentiation and motility of T lymphocytes (Cucchi et al., 2019).
275 Thirdly, n-3PUFA dilute the availability of pro-inflammatory n-6PUFA substrates (Nicolaou et al., 2014 and
276 Kendall et al., 2019)

277 As discussed, the saturated FA palmitate and ceramides can directly recognise and activate macrophages via
278 TLR4 receptor (Liu et al., 2013 and Eraky et al., 2018). This is a crucial point in which n-3 PUFA could act to
279 rebalance the immune response and promote an anti-inflammatory environment and potentially ameliorate
280 obesity-associated diseases such as T2D (Eraky et al., 2018). In a human clinical trial, overweight/obese
281 pregnant women were daily supplemented with n-3 PUFA which led to down-regulation of TLR4 expression
282 and reduction of IL-6, IL-8, and TNF α production in both adipose and placental tissues (Haghiac et al., 2015).
283 Further, *in vitro* assays on cells isolated from maternal subcutaneous adipose tissue and placenta illustrated that
284 palmitate was responsible for a 10-30-fold increase in the expression of TLR4, IL-6 and IL-8. Whereas n-3
285 PUFA supplementation in an ex-vivo cell culture, significantly reduced the transcription of TLR4, IL-6, IL-8
286 induced by palmitate, reducing its inflammatory effect by 70% (Haghiac et al., 2015). N-3 PUFA recognition by
287 GPR120 significantly reduced the production of pro-inflammatory cytokines such as IL-6, IL-8 and TNF- α
288 which is mediated by TLR4 (Haghiac et al., 2015 and Liu et al., 2013). Additionally, activation of GPR120 by
289 n3- PUFA reduced the levels of pro-inflammatory cytokines IL-1b, TNF α , MCP-1 and also lead to IL-10-
290 mediated promotion of M2 anti-inflammatory genes in adipose tissue (Yan et al., 2013 and Lee et al., 2019).
291 Consistently, GPR120 knockout in both murine model and *in vitro* monocytes and macrophages abrogated the
292 anti-inflammatory effects of n3- PUFA. After activation by n3-PUFA, the GPR120 coupled to β -arrestin2
293 leading to the inhibition of TAB1-mediated activation of TAK1, which may account for the inhibition of both
294 the TLR and TNF α pro-inflammatory signalling pathways (Oh et al., 2010).
295 Nlrp3 is another important innate immune cell receptor which can drive the anti-inflammatory effect of n-3
296 PUFA on OIIM, via inhibition of inflammasome activation (Kumar et al., 2016) and subsequent expression of
297 pro-inflammatory cytokines such as IL-1b, TNF α , MCP-1 (Yan et al. 2013). Importantly, n-3 PUFA-dependent

298 inhibition of Nlrp3 activation has been shown to exert beneficial effects on HFD-induced T2D murine models
299 via reconstitution of a homeostatic cellular microenvironment (Kumar et al., 2016 & Yan et al., 2013) and in
300 clinical trials via downregulation of the expression of various pro-inflammatory cytokines such as IL-18 and IL-
301 1 β in adipocyte and macrophages (Lee et al., 2019).
302 Furthermore, n-3 PUFA limit ceramide synthesis via diverting their major substrate palmitate (Tachtsis et al.,
303 2020) but also via directly inhibiting the ceramide biosynthetic enzyme genes serine palmitoyl transferase long
304 chain base (Sptlc) and degenerative spermatocyte homolog 2 (Des2), as shown in the liver of a murine model of
305 hepatic steatosis (Dong et al., 2017).

306 307 **Final remarks**

308 A growing body of evidence implicates various immune cells in sensing exogenous and endogenous metabolic
309 signals raised by metabolic dysregulation and obesogenic diets. Ceramides, potent signalling lipids involved in
310 energy homeostasis, are found to mediate a number of the cellular and pathophysiological process linked with
311 OIIM and other obesity-associated diseases. Increasing evidence suggests that n-3 PUFA can partly create an
312 anti-inflammatory environment acting via inflammatory receptors, such as TLR4 and Nlrp3 expressed on
313 macrophages. As these pathways are activated by ceramides, n-3PUFA could be a tool to counteract their pro-
314 inflammatory activities (Figure 3). The extensive modulatory effects ceramides have on various cells and
315 tissues, suggests the presence of many more mechanistic interactions still to be unrecovered.

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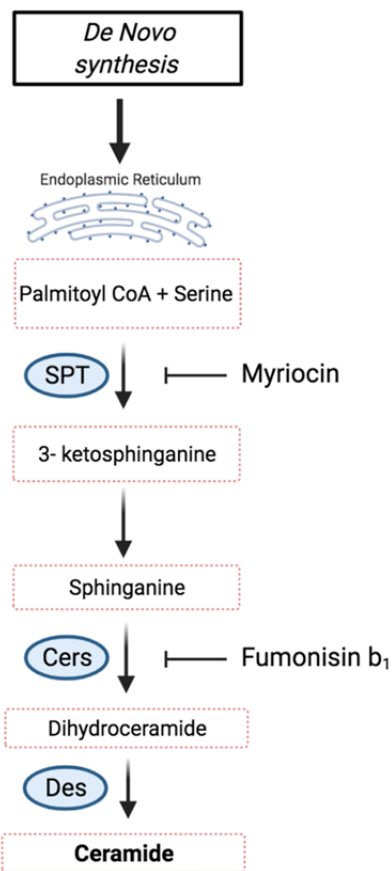


Figure 1. Ceramide *de novo* biosynthesis pathway. Biosynthesis of ceramides begins in the endoplasmic reticulum with condensation of palmitate and serine by serine palmitoyltransferase (SPT), followed by a series of reactions catalysed by 3-dehydrosphinganine reductase, dihydroceramide synthase (CerS) and dihydroceramide desaturase (Des). Highlighting Myriocin and Fumonisin b₁, commonly used inhibitors of SPT and CerS respectively.

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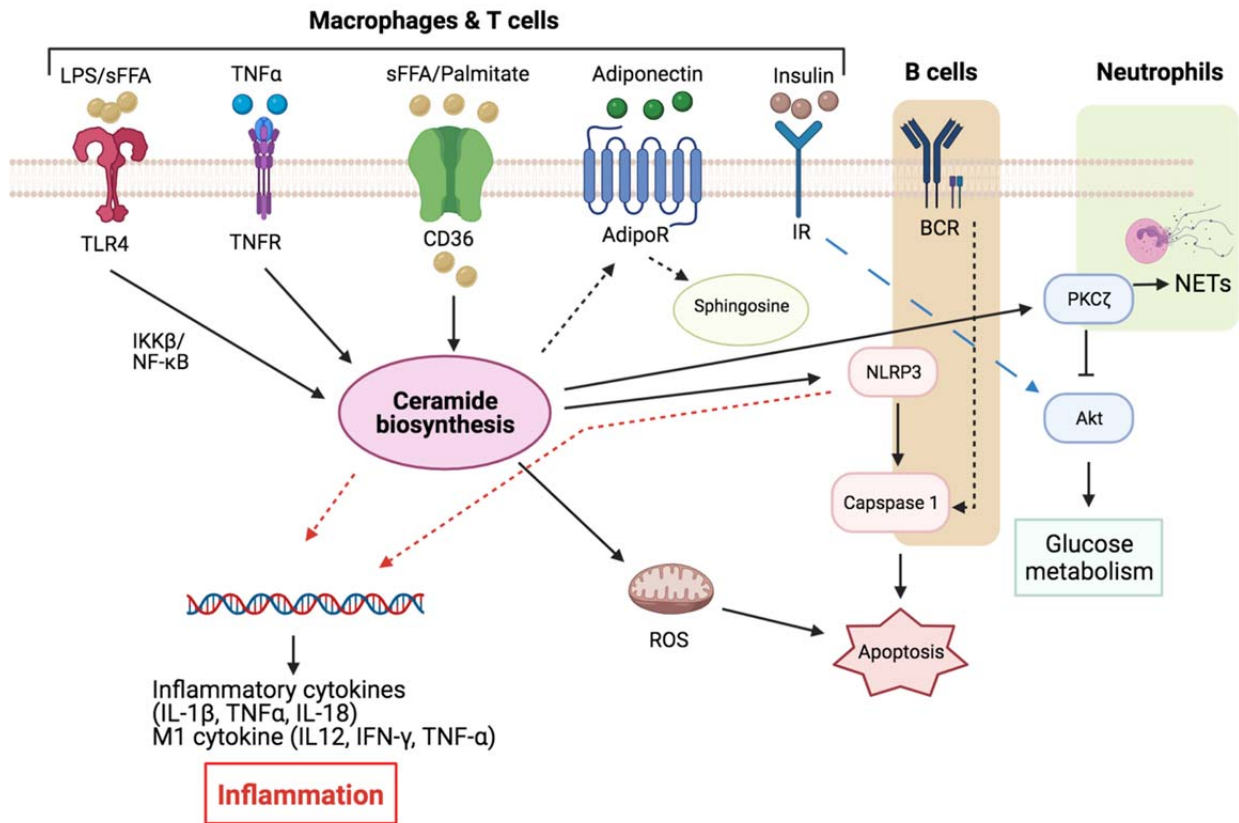


Figure 2. Schematic representation of interactions between ceramide biosynthesis and intracellular signalling in key immune cells. Ceramide overload triggered by key substrates such as sFFA, palmitate and LPS via TLR4, TNF and CD36 receptors leads to excessive ceramide biosynthesis. Ceramide elicits deleterious effects on insulin signalling and glucose metabolism via inhibiting Akt with PKC ζ . Ceramide also induces mitochondrial stress via blocking lipid oxidation and creating ROS that induce cellular apoptosis. Activation of Nlrp3 via ceramides also triggers apoptosis via caspase 1 in addition to its role in obesity-induced inflammatory action. (Akt, Protein Kinase B; CD-36, cluster of differentiation 36; AdipoR, Adiponection receptor; IKK, Ikappa kinase; IL, interleukin; IR, Insulin receptor; LPS, lipopolysaccharide, NF- κ B, Nuclear factor kappa-light-chain-enhancer of activated B cells; Net, Neutrophil extracellular traps; Nlrp3, NLR family, pyrin domain containing 3; PKC, protein kinase C; ROS, reactive oxygen species; sFFA, Saturated fatty acids; TLR4, Toll like receptor-4; TNF- α , Tumour necrosis factor alpha; Tumour necrosis factor alpha receptor).

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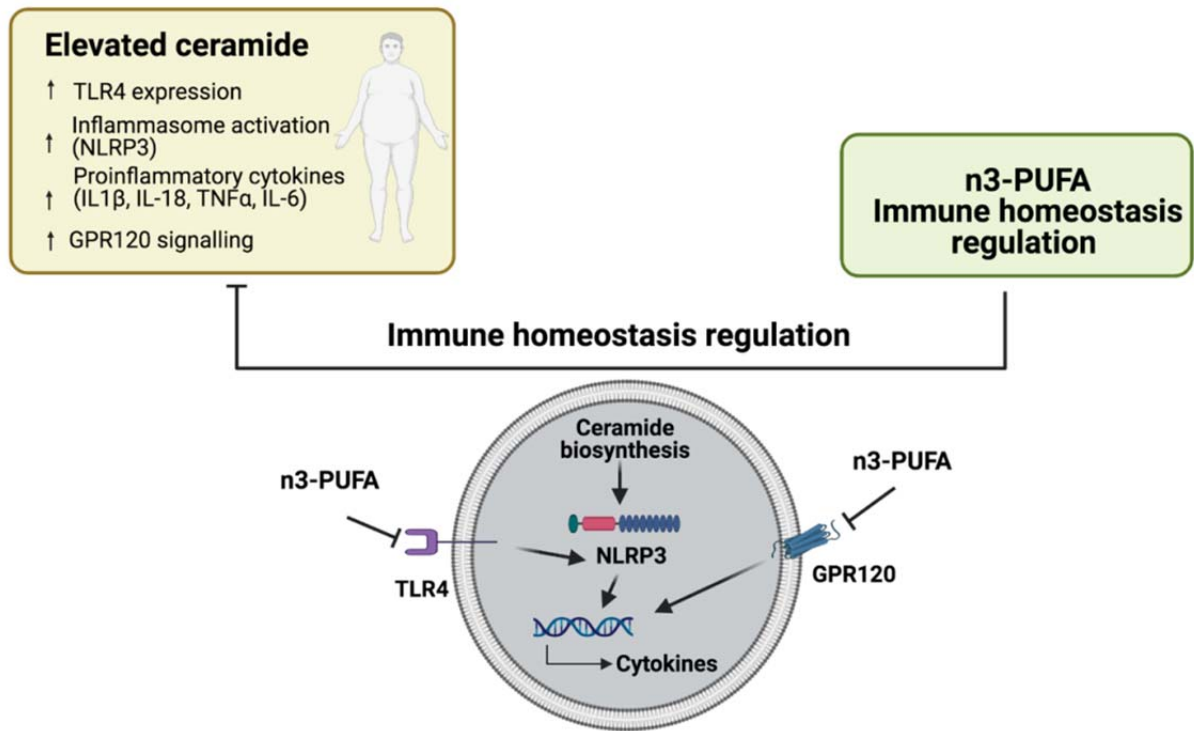


Figure 3. Schematic illustration highlighting the potential converge of ceramides and n-3 PUFA.

Ceramides, when elevated within an obese environment, promote inflammation through the activation of TLR4 and GPR120 receptors on immune cells. N-3 PUFA can regulate this environment, inducing an anti-inflammatory response via potentially blocking the corresponding receptors of ceramides and restore immune homeostasis.

TABLE 1: Recent publications highlighting the association between obesity- induced ceramide accumulation and various pathological conditions.

Tissue	Experimental model (in vivo/ in vitro)	Ceramide species	Ceramide concentration	Change in OIIM	Change in pathology	Ref.
Liver	<i>In vitro</i> -Human HepG2 liver cells, 0.5 mmol/l palmitate for 4-8 hrs.	Total intracellular and extracellular ceramide	Increased	NA	Obesity and diet-induced diabetes	Watt et al. 2012
	C57BL/6J mice on 60% HFD for 18 weeks	C16:0	Increased	NA	Glucose intolerance and obesity induced type II diabetes	Raichure et al. 2019
	C57BL/6J mice on 15% ethanol diet for 4 weeks and human VL17A cells	C14, C20, C20:1, C22, C24, and C26:1	Increased	NA	Promotes alcoholic steatosis	Williams et al. 2018
	Clinical trial- 980 free living human on mediterranean diet followed for 7.4 years	C16:0, C22:0, C24:0 and C24:1 in Plasma	Increased	NA	Higher risk for CVD	Wang et al. 2017
Cardiovascular	Clinical trial- 462 individuals with familial coronary artery disease	Total serum ceramides (30 out of 32 tested), C16:0, C18:1 and C24:1 most strongly associated	Increased	NA	Coronary artery disease risk	Poss et al. 2020
	<i>In vitro</i> Human AC16 cardiomyocytes, 0.5 mM palmitate and <i>In vivo</i> C57BL/6 mice on 60% for 8 weeks	C16:0, C24:0, C24:1	Increased	NA	Heart failure- Mitochondrial dysfunction, oxidative stress and cell death in cardiomyocytes.	Law et al. 2018
	Clinical Trial- Obese individuals (n=439)- white adipose tissue	C16:0, C18:0	Increased	NA	Obesity, insulin resistance, diet-induced diabetes	Turpin et al. 2014
Adipose tissue	<i>In Vitro</i> - Human 3T3-L1 adipocytes and RAW264.7 macrophages	Total ceramide and C2:0	Increased	Upregulation of IL6, IFN γ , TNF α and MCP1	Creation of OIIM	Hamada et al. 2014
	Wistar rats on 60% HFD for 8 weeks	C18:0, C20:0, and C24:0	Increased	Upregulation of TNF α	Insulin resistance and obesity-induced type II diabetes	Blachnio-Zabielska et al. 2018
	Clinical trial- Obese women (n=28) visceral adipose tissue	Total ceramide	Increased	Upregulation of TNF α	Metabolic syndrome	Choromańska et al. 2019
Plasma	Rhesus monkeys on high-fat and high-fructose (HFFD) 'western' diet for 8 months to 5 years	C14:0, C16:0, C22:0, C24:0	Increased	NA	Associated with obesity-induced type II diabetes	Brozinick et al. 2013
	C57BL/6J mice on 60% HFD for 18 weeks	C16:0	Increased	NA	Glucose intolerance and obesity induced type II diabetes	Raichure et al. 2019
	Obese children (n=80, aged 7–17 years)	Total ceramide including C14:0, C16:0, C16:1, C18:0, C18:1, C22:0, C24:0	Increased	NA	Nonalcoholic fatty liver disease	Wasilewska et al. 2018
	C57BL/6 mice on 55.2% HFD for 17 weeks	C14:0 and C18:0	Increased	NA	Contributes to obesity- associated insulin resistance	Turpin et al. 2019
	C57BL/6 mice on 60% HFD for 12 weeks	Total ceramide	Increased	NA	Associated with obesity-induced type II diabetes	Bandet et al. 2018
Skeletal muscle	Clinical trial-106 individuals with obesity and T2D	C18:1, C:20, C:22, C:24 and C:24:1	Increased (by two-fold)	NA	Associated with obesity-induced type II diabetes	Broskey et al. 2018
	<i>In Vitro</i> - Human C2C12 myotubes palmitate treatment (0.75 mmol/L) for 16 h	Total ceramide	Increased (60%)	NA	Associated with obesity-induced type II diabetes	Bandet et al. 2018
	<i>In Vitro</i> - Rat L6 myotubes and mouse C2C12 muscle cells, 0.5 mM palmitate	Total ceramide	Increased	Increased the expression of pro-inflammatory, Il6 and Ccl2	Associated with obesity-induced type II diabetes	Pillon et al. 2018
	Wistar rats on 60% HFD for 8 weeks	Total ceramide including C14:0, C18:0, C18:1, C24:1 and C24:0	Increased	NA	Associated with skeletal muscle insulin sensitivity	Blachnio-Zabielska et al. 2016

TABLE 2: Intervention methods to target the *de novo* ceramide biosynthesis pathway

Target enzyme	Experimental model (in vivo/ in vitro)	Ceramide species & Tissues	Intervention	Ceramide concentration	Change in OIIM	Change in pathology	Ref.
SPT (serine palmitoyltransferase)	C57BL/6J mice on HFD 12-20 weeks	Total ceramide including C24:0,C24:1, C22:0 in Adipose tissue, liver, muscle and serum	Pharmacological inhibition via Myriocin and Sptlc knockout model	Reduced	Reduced pro-inflammatory cytokines IL-6, MCP-1, and TNFα with increased anti-inflammatory cytokines (IL-10) More anti-inflammatory M2 macrophages Less macrophage infiltration to periphery	Improved insulin sensitivity and glucose homeostasis	Chaurasia et al. 2016
	In Vitro- Human 3T3-L1 adipocytes and RAW264.7 macrophages	Total ceramide and C2:0	Genetic Knockdown of SPT	Reduced	Reduced pro-inflammatory cytokines; IL-6, IFN-γ, TNFα and MCP-1	Restoring the homeostasis of the OIIM	Hamada et al. 2014
Ceramide synthases (CerS 1-6)	C57BL/6 mice on 55.2% HFD for 17 weeks	C16:0, C18:0- adipose tissue, liver	Cers6 genetic knockout	Reduced	No macrophage infiltration and reduced pro-inflammatory cytokines IL-6 and IL-1β	Protected from diet-induced obesity, glucose intolerance and type II diabetes	Turpin et al. 2014
	C57BL/6J mice on 60% HFD for 18 weeks	C16:0- liver and plasma	Cers6 knockdown via antisense oligonucleotides	Reduced (50%)	NA	Improved insulin sensitivity, glucose tolerance with reduced body fat content	Raichur et al. 2019
	In Vitro- Human 3T3-L1 adipocytes and RAW264.7 macrophages	Total ceramide and C2:0	Pharmacological inhibitor of CERs via Fumonisin B1	Reduced	Reduced pro-inflammatory cytokines; IL-6, IFN-γ, TNFα and MCP-1	Restoring the homeostasis of the OIIM	Hamada et al. 2014
	C57BL/6JCrI Cers5 KO mice on 60% HFD for 24	C16:0 and C18:0- lung, spleen, muscle, liver and white adipose tissue	Cers5 genetic knockout	Reduced	Reduction in pro-inflammatory gene expression such as NF-κB, TNFα and IL-1β	Reduced weight gain, ameliorate OIIM	Gosejacob et al. (2016)
	*Global knockdown of Cers1 in C57BL/6 mice on 55.2% HFD for 17 weeks	C18:0	Cers1 genetic knockout	Reduced (95%)	NA	Decreased body weight and fat content with improved insulin sensitivity and glucose tolerance	Turpin et al. 2019
Dihydroceramide Desaturase (DES)	C57BL/6 mice on 60% HFD for 12 weeks	Total ceramide- Serum, liver, white adipose tissue	Genetic Dergs1 knockout	Reduced	Reduced pro-inflammatory cytokines; IL-1 and TNFα	Reduced fat density and improved insulin sensitivity	Chaurasia et al. 2019

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