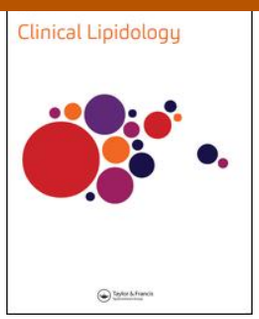

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Omega 3 fatty acids, inflammation and DNA methylation: an overview

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ABSTRACT

Omega-3 polyunsaturated fatty acids (n-3 PUFAs) are known to be anti-inflammatory and to alter gene expression within the cells. Emerging evidence indicates that one of the mechanisms for this process involves the alteration of epigenetic markers, such as DNA methylation. The focus of this overview is to document the current evidence for n-3 PUFA effects on DNA methylation and how these may impact on the inflammatory processes.

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Introduction

n-3 polyunsaturated fatty acids and inflammation

Populations with a diet rich in omega-3 (n-3) polyunsaturated fatty acids (PUFAs) have a lower incidence of chronic non-communicable diseases [1]. Evidence for n-3 PUFAs, such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), on their ability to modulate inflammation has come from *in vitro* work [2–4], animal models [5–8] and human studies [4,9,10]; although the latter evidence is weaker as a consequence of more complex environmental factors. The mechanisms through which n-3 PUFAs promote an anti-inflammatory environment within the body are multiple and complex [11] (Figure 1). Early evidence suggests that n-3 PUFAs exert these effects through changes in gene expression as a result of varied transcription factor activation [12,13], but which may also arise from changes in epigenetic markers, such as DNA methylation.

Epigenetics

Epigenetics investigates heritable chemical modifications to the genome which are independent of the DNA sequence [14–16]. These chemical changes, including DNA methylation, histone modifications, chromatin remodelling and non-coding RNAs [17–23], are vital for normal cellular development, cellular processes and cell-specific gene expression profiles [16,24]. Interactions between the environment, particularly diet, and the genome through epigenetic mechanisms lead to changes in phenotype and are implicated in many diseases [25–28]. They also provide a target for therapeutic interventions [29].

DNA methylation, a methyl group covalently attached to the fifth position of the pyrimidine ring to give 5-methylcytosine (5mC), is most commonly found on a cytosine positioned next to a guanine (CpG) [30]. DNA methylation levels within the genome are cell and tissue specific, with changes observed during the human lifespan [31–33]. Dysregulation of 5mC has been associated with diseases, including cancer and cardiovascular disease [24,34,35]. DNA methylation is the most accessible and widely studied epigenetic mark [36] and has been shown to vary with nutrition, disease and age [37–39]. Here, we will discuss the emerging evidence for the interaction of n-3 PUFAs and DNA methylation, specifically discussing their impact on inflammation.

Cross-sectional epigenome-wide association studies

Epigenome-wide associations studies (EWAS), which despite their name, focus mainly on DNA methylation, have linked n-3 PUFA consumption with differential DNA methylation. However, there is a lack of EWAS that use biochemical analysis to assess dietary n-3 PUFA, with a preference towards the readily available, but less accurate, food frequency questionnaire (FFQ).

One prominent EWAS within a distinct population, the *Yup'ik*, from Alaska (n = 185) utilised biochemical analysis to investigate associations with DNA methylation [40]. The

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Yup'ik have a traditional diet rich in fish which is changing with westernisation [41], allowing for stratification of this population into high and low PUFA consumption. Using red blood cell (RBC) nitrogen stable isotope (δ^{15} N), previously associated with EPA and DHA RBC levels [42], to separate into these high and low PUFA groups, associations with CpG methylation of biologically relevant targets, including genes involved in *T*-cell homeostasis were found [40]. Increased DNA methylation with high PUFA consumption was observed in 78% of the significant associations [40]; with genomic instability occurring with hypomethylation [43], the increased methylation observed may, therefore, be beneficial to the stability of the genome. A separate cohort, Greek pre-adolescents ($n = 69$), also found associations between dietary fats and DNA methylation, including sites within pathways linked to inflammation; nuclear factor kappa B (*NFκB*), peroxisome proliferator-activated receptor (*PPARα*), leptin (*LEP*) and interleukin (*IL*)-*6* [44]. However, the dietary fat intake for the cohort was assessed using FFQs, which did not directly measure the levels of n-3 PUFAs that have made it to the target tissue from the food.

Consistent with previous supplementation studies [45,46] and dietary salmon consumption [47], the *Yup'ik* cohort found individuals with higher n-3 PUFA intake had significantly lower levels of plasma triglyceride (TG), increased high-density lipoproteins (HDL), and higher but not significant levels of low-density lipoproteins (LDL) and total cholesterol [40]. However, Dekkers *et al*. [48] found that there was an effect of blood lipids (TG, HDL and LDL) on differential DNA methylation in the genes coding for six key regulators of lipid metabolism. It therefore, needs to be questioned if the differential methylation observed is due to the n-3 PUFAs in the diet, differences in blood lipid profiles, or both, and one method of addressing this is through closely controlled intervention studies.

Gestation and infant n-3 PUFA supplementation intervention studies

Epigenome-wide association studies

The impact of nutrition during early life is of increasing interest due to the plasticity of epigenetic regulation during development and the links to diseases in later life [49,50]. Supplementation during gestation or early infancy with n-3 PUFAs has therefore been studied to a greater extent than it has been in adults.

EWAS in combination pre-and post-intervention can be used to investigate the impact of n-3 PUFA supplementation on differentially methylated regions (DMRs) throughout the genome. Two intervention studies took slightly different approaches, the first supplemented the mothers with 3.7 g/day EPA + DHA (n = 36) or placebo (n = 34, placebo details not provided) from 20 weeks of gestation to delivery [51]. The second study supplemented

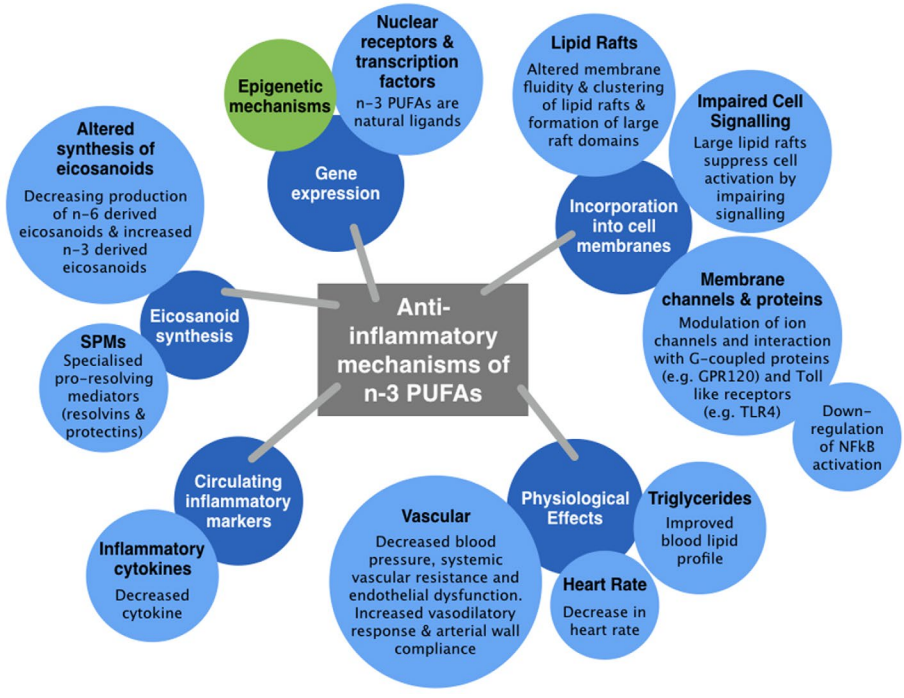

Figure 1. Mechanisms Omega-3 polyunsaturated fatty acids (n-3 PUFAs) action to promote an anti-inflammatory environment.
Notes: GPR120, G-coupled protein recptor 120; NFkB, nuclear factor kappa B; n-6 PUFAs, omega 6 polyunsaturated fatty acids; SPM, Specialised pro-resolving mediators; TLR4, toll like receptor 4.

9-month-old infants with either 1.6 g/day EPA + DHA (n = 6) or placebo 3.1 g/day linoleic acid (n = 6, LA in the form of sunflower oil) for nine months [52]. Neither investigation found a significant difference in DNA methylation between those taking the n-3 PUFA and those taking the placebo after adjusting for multiple testing [51,52].

However, a third more recent large randomised control trial (RCT) supplementing mothers during gestation with 0.8 g/day DHA + 0.1 g/day EPA (n = 190) or vegetable oil (n = 179), found that there were significant differences between DMRs, including those relating to immune function, between the two experimental groups [53]. The dose of n-3 PUFAs in this third study was much lower, in addition, due to the larger cohort it was possible to split the analysis between the sexes, with males being found to have a greater number of DMRs than females. The lack of findings in the first two studies referred to above may be due to the small, mixed sex samples used and future studies could be designed to eliminate this possibility. There are significant differences in the way males and females metabolise and store n-3 PUFAs [54–56] as well as differences in global methylation levels between the sexes [57]; as seen by the clear effect observed between sexes in the third study [53]. It is therefore, important to note that when using small sample sizes with n-3 PUFA and DNA methylation studies, a lack of separation of the sexes may result in critical results being overlooked.

Notably, all studies presented thus far have utilised the Infinium Human Methylation 450 k arrays. This technology measures DNA methylation at over 450,000 individual CpG sites throughout the genome. And although a powerful tool in explorative work in determining DMRs, there are limitations to the use of 450 k array data, including multiple testing, the need for complicated normalisation and the generation of artefactual data [58]. Therefore, targeted analysis of specific targets including candidate genes must be completed to confirm findings, and investigate differential methylation and biological relevance.

Specific targets and candidate genes

Long interspersed nucleotide element 1 (LINE1)

Measuring the levels of DNA methylation in repetitive elements, such as long interspersed nucleotide elements (LINEs) can provide a proxy for global DNA methylation [59]. It has been estimated that repetitive transposable elements account for 45% of the genome [60] and they contain one-third of the genomes DNA methylation [61,62] to repress their transcription [63]. Associations between *LINE1* methylation and lifestyle factors, including physical activity and nutrition [57,64–67] have been found and have also been shown to be associated with both disease [68–70] and ageing [71,72].

Lee *et al*. investigated the DNA methylation of *LINE1* in cord blood mononuclear cells (CBMCs) from mothers who received 0.4 g/day DHA (n = 131) or placebo (n = 130, olive oil) during gestation [73]. Neither this study or the third RCT above found any differences in *LINE1* methylation between supplementation and control groups [53,73]. However, Lee *et al*. did find an interaction between smoking status and DHA supplementation; CBMCs of mothers who smoked and took the DHA during gestation had significantly higher *LINE1* methylation levels than the CBMCs of smoking mothers in the control group [73]. This suggests a complex interaction between multiple environmental stimuli and the epigenome.

Cytokine genes

In addition to *LINE1* methylation, cytokine gene DNA methylation was also investigated by Lee *et al*., with no significant differences in methylation of *TNFa*, *IL13*, *GATA3*, *STAT3*, *IL10* and *FOXP3* being observed. However, the promotor methylation was lower (not significantly) for *IFNy* in those supplemented with DHA [73]. The study was novel in that the supplementation only contained DHA and was also at a low dose compared to many other studies. As observed in cell membranes where there is a dose-response increase in the incorporation of n-3 PUFAs, it is likely that an increase in the dose may have led to more significant changes in DNA methylation. *In vitro* studies provide evidence of differing effects of EPA and DHA on inflammation [74,75], it would be interesting to see if there are differing effects on the epigenome had a separate EPA supplementation group had been included. The comparison between EPA and DHA is often lacking in human studies due to the increased cost and a greater availability of mixed n-3 PUFA supplements commercially.

Adult n-3 PUFA supplementation intervention studies

Candidate targets

PUFAs are important structural components of cells membranes. Consumption of foods rich in n-3 PUFAs, such as salmon, result in increased incorporation and modification of the cell phospholipid membrane [47]. Composition of the membrane is altered by clustering of lipids rafts containing the n-3 PUFAs, resulting in the formation of large raft domains [76]. As shown in murine obesity models [77] and HeLa cells [78] large lipid rafts can suppress the cell activation by impaired signalling, ultimately affecting cell function through suppression of downstream pathways, including those involved in inflammation. Leptin is localised to these lipid rafts and its expression is reduced by n-3 PUFAs [79]. In humans, cross-sectional evidence indicates

differential methylation in the leptin pathway genes with varying n-3 PUFA status [44], and epigenetic control of *LEP* has been demonstrated in a murine model [80]. However, using a candidate gene approach, n-3 PUFA supplementation has been shown to have no effect on *LEP*, leptin receptor (*LEPR*) or pro-opiomelanocortin (*POMC*) promotor methylation in a murine model [81].

The membrane glycoprotein which promotes inflammation in monocytes and macrophages, cluster of differentiation 36 (CD36), has been found to be increased as the result of n-3 PUFA *in vitro* [82] and in animals [83]. *CD36* promotor methylation was significantly reduced, when adjusted for baseline body weight, in a weight loss and n-3 PUFA supplementation study in Spanish young adult overweight females [84]. However, changes in DNA methylation in cluster of differentiation (*CD14*), pyruvate dehydrogenase kinase 4 (*PDK4*) and fatty acid desaturase 1 (*FADS1*) was only significant as a result of the low-calorie diet and not the supplementation. Had the gene expression been studied we may have expected to see an increase in the mRNA for these genes. This effect is the counter to that expected from n-3 PUFAs where in human studies, decreased expression of *CD36*, *CD14* and *PDK4* mRNA is observed in peripheral blood mononuclear cells (PBMCs) isolated after supplementation [13]. It is possible that the low-calorie diet impacted on the DNA methylation to a greater extent than the n-3 PUFAs, which only attenuated the decrease in methylation of *CD36* once adjusted for baseline body weight [84].

There are relatively few studies that have investigated the effect of n-3 PUFA supplementation on DNA methylation in adults, and of these many have confounding factors. Addressing the confounding factors of this study [84], the participants were asked not to consume seafood and to follow a detailed diet plan during the intervention period to minimise variability. However, the Spanish diet is high in shellfish/fish, on average 88.6 g/person/day are consumed [85], and therefore, the researchers may have simply replaced the n-3 PUFAs that the participants obtained from their diets with the supplementation. Although no biochemical measurements for EPA and DHA were provided in this paper [84], earlier work from the same cohort found only a small difference in EPA and DHA after the intervention, with no data comparing baseline to endpoint [86]. Future studies should include measurement of n-3 PUFA incorporation in to the investigated cell membranes, for example, into PBMCs.

Endogenous metabolism of PUFAs occurs within a cell, the initial step in the metabolic pathway takes places in the endoplasmic reticulum where LA and alpha-linolenic acid (ALA) undergo elongation of the fatty acid carbon chain by fatty acid enlongase (ELOVL) and desaturation by insertion of double bonds by Δ6-desaturase and Δ5-desaturase [87]. The Δ6-desaturase and Δ5-desaturase enzymes, encoded by the *FADS2* and *FADS1* genes respectively, are rate-limiting steps in this metabolic pathway [88,89]. Genetic polymorphisms of these genes and others related to the PUFA metabolism can significantly alter the levels of PUFAs within the body [90] and differential methylation is likely to have a similar effect. The methylation of *FADS1* is altered by a low-calorie diet with a small interaction with n-3 PUFA supplementation [84]. When investigated alone, with a higher dose of n-3 PUFA supplementation, no significant changes in methylation were observed in *FADS1* or the elongation gene *ELOVL-2* [91]. However, the methylation of two other fatty acid metabolism genes, *FADS2* and *ELOVL-5*, increased with supplementation; with a larger number of CpG sites changing methylation in females than males and a negative relationship between the DNA methylation and the gene mRNA levels observed [91].

Future targets: inflammatory gene methylation

Modulation of inflammatory gene expression occurs through transcription factors, such as peroxisome proliferator-activated receptor gamma (PPARγ). Interactions between PPARγ and fatty acids result in a decrease in cytokine expression [92]. In a cancer cell line, treatment with EPA and DHA has been shown to increase expression of PPARγ gene, *PPARG* [93]. In murine models of diabetes, expression of *PPARG* is known to be modulated by DNA methylation within its promotor [94] and differential methylation is observed in type two diabetes mellitus (T2DM) [95], however the impact of n-3 PUFAs on *PPARG* DNA methylation has not been measured. Epigenetic regulation of the PPAR coactivator 1 alpha (PCG-1α) gene, *PPARGC1A,* has been implicated in T2DM in humans [96]. The regulation of the *PPARGC1A* appears to be influenced by changes in DNA methylation and has been shown to interact with PPARγ. In men, DNA methylation of the *PPARGC1A* gene promotor is significantly increased after a high-fat overfeeding diet [97]. Whereas, in obese patients fed a low-calorie diet, the methylation of *PPARGC1A* has been shown to decrease [98].

Inflammatory diseases are often characterised with chronic activation of NFkB transcription factor and release of inflammatory cytokines [99] and therefore, an important target for changes in DNA methylation by n-3 PUFAs. Associations between dietary fats and DNA methylation in the NFkB pathway, measured using the Infinium 450 k array, were observed in the Greek pre-adolescent cross-sectional cohort [44], and therefore, may provide valuable targets within intervention studies.

As previously described, there was no change in DNA methylation being observed in cytokine DNA methylation levels with gestational supplementation [73]. However, the study used low doses of DHA and it has not been investigated in adult intervention studies or with combined EPA

and DHA supplementation. Taking TNFa as an example, EPA treatment does not affect the stability of *TNFα* mRNA in cytoplasm and therefore, reductions in *TNFα* mRNA as a result of EPA treatment is likely to be the result of decreased formation rather than degradation of the cytokine [100]. The influence of n-3 PUFAs on *TNFα* expression may be the result of alterations in epigenetic mechanism, such as DNA methylation. Epigenetic regulation plays an important role in the control of *TNFα* expression [101–103]. Specifically, *TNFα* has been shown to be hypomethylated in Crohn's disease patients [104], is related to adipogenesis [105], and that CpG methylation interacts with histone methylation to silence *TNFα* expression during endotoxin tolerance [106]. Cross-sectional studies employing FFQ have found associations between *TNFα* methylation and dietary intake of certain nutrients including fats, however, no associations with n-3 PUFAs [107,108]. As seen with *TNFα*, studies examining DNA methylation must choose the tissue of interest carefully as more variability is observed between tissues from the same individual, than within tissue between individuals in the *TNFa* promotor [109].

Implications and considerations for future research

Most of evidence for the control of gene expression by CpG methylation comes from genes with CpG islands [30]. However, not all genes have CpG islands and it is not only the DNA methylation that occurs in the promotor region near the transcription start site (TSS) that plays an important role in regulation of gene expression, with evidence suggesting that 5mC in gene bodies and non-promotor regions plays an important role [110]. In genes which do not have a CpG island, including inflammatory genes *NOS2*, *IL-2*, *IFN-γ*, *MMP13*, *IL-1β* and *TNF-α*; CpG methylation proximal to the TSS has been shown to influence transcription [106,111–115]. Careful considerations must therefore be taken when designing assays to measure the DNA methylation of candidate genes.

As we have seen, investigations of the effects of n-3 PUFAs on DNA methylation are in their infancy and as such there are some points to consider when conducting this research. Within the n-3 PUFAs literature there is not yet a consensus on the required dose in humans required to produce an anti-inflammatory effect. Many of the existing supplementation studies so far investigating the epigenetic effect of n-3 PUFAs have used low doses with only small changes observed. In a review by Calder [11], it is suggested that a dose between 1.35 and 2.7 g/day of EPA is required for the anti-inflammatory effects. It is likely that there will be a dose-response interaction between n-3 PUFA supplementation and epigenetic mechanisms, as is seen with cytokine production [116]. There is also no consensus on the time frame required for epigenetic changes to occur. The largest incorporation of n-3 PUFAs into erythrocyte membranes is within the first 4 weeks of supplementation and it is not known if the epigenetic changes occur within the same time frame. There are fewer studies that measure the incorporation into PBMCs and none that consider CpG methylation alongside incorporation.

The majority of intervention studies investigating epigenetic changes do not account for the impact of the intervention on the cell population from which the DNA was extracted. This results in multiple complications, firstly if the influence of n-3 PUFAs is specific to one cell type it may not be observed if the cells are not separated prior to analysis. Secondly, changes may be falsely observed or masked with variations in cell populations. Differentiation into the cell subtypes found within blood requires changes within the epigenetic signatures and therefore, the observed changes in percentage methylation for an individual CpG may be the result of a change in the cell population rather than a result of the intervention. Data can be normalised to account for the proportion of different cells [52,91] and this is a consideration future studies should make.

Conclusion

Associations between n-3 PUFAs and differential DNA methylation have been demonstrated, with further evidence of n-3 PUFA impact on DNA methylation seen in supplementation interventions. A more targeted approach to the effects of n-3 PUFAs, and use of well-controlled supplementation studies, both *in vitro* and *in vivo*, could elucidate a mechanism through which n-3 PUFAs alter the DNA methylation within the inflammatory landscape.

Disclosure statement

No potential conflict of interest was reported by the authors.

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