Perinatal PUFA Intake Affects Leptin and Oral Tolerance in Neonatal Rats and Possibly Immunoreactivity in Intrauterine Growth Retardation in Man

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Effects of Varied PUFA Intake by Pregnant and Lactating Rat Dams on the Neonatal Immune System

The importance of the essential fatty acids (EFAs) for early development has been illustrated in numerous studies. The essentiality of linoleic acid (C18:2n-6) and \( \alpha \)-linolenic acid (C18:3n-3) depends on the fact that they cannot be produced by animal cells and that they play a major role in numerous critical tissues and functions. These two fatty acids are also precursors for such vital long-chain polyunsaturated fatty acids (PUFAs) of the n-6 and n-3 series as arachidonic acid and docosahexaenoic acid [1]. The EFAs also play an important role in the immune system for its protective as well as tissue-damaging capacities. EFAs are important components in all cell membranes and modify membrane fluidity, function and microenvironment [2]. Thus they are important for the number and function of cellular receptors, their binding to ligands and the signal transduction process. Several cellular receptors form the basis for the function of the innate as well as the adaptive immune system.

The EFAs are also required for production of several components with important functions, such as prostaglandins and leukotrienes. They are mediators of tissue reactivity forming part of the inflammatory processes, e.g. bronchial
reactivity in asthma [3]. They also affect cells of the immune system, such as macrophages and lymphocytes, and may influence the production of cytokines and major effectors such as antibodies [4]. This forms the background to suggestions that dietary supplementation with α-linolenic acid may decrease the chemotactic response of neutrophils and monocytes and reduce their production of inflammatory mediators thereby dampening inflammation.

**Effects of EFAs on the Production of Leptin in Suckling Rat Offspring**

The hormone leptin is mainly produced by white adipose tissue (WAT), and also by placenta [5], mammary glands [6], and neonatal adipose tissue [7]. In addition to the regulation of food intake and energy expenditure, leptin is involved in several physiologic processes, including immune responses. Being structurally similar to IL-6 cytokines, it binds to receptors which belong to the class-I cytokine receptors. Leptin stimulates proliferation and differentiation of hematopoietic cells [8] and upregulates monocyte/macrophage functions [9]. It modifies T-cell responses with increasing T-helper-1 (Th1: IL-2, IFN-γ) and suppressing T-helper-2 (Th2: IL-4, IL-10) cytokine production [10]. A recent study showed that increased serum leptin in mice was related to enhanced metacholine responsiveness and IgE responses on sensitization with ovalbumin (OA) [11]. This was proposed to link obesity and allergy in man.

Thus, leptin might play an important role in the induction and maintenance of immune and inflammatory responses, especially vital in the perinatal period. Dietary fat quantity affects perinatal serum leptin levels. Increased maternal fat intake raises plasma leptin concentrations in neonatal rats and affects hypothalamus-pituitary-adrenal responsiveness in neonates and prepubertal rats [12].

Recently, we have shown that dietary fat quality modulates serum leptin levels in rat offspring during the suckling period [13, 14]. During late gestation and throughout lactation, rats were fed a control, or an EFA-deficient (EFAD) diet. The weight of inguinal WAT depots and the serum leptin levels of the EFAD offspring were significantly lower than in the control pups during the whole suckling period. In addition, leptin mRNA levels in inguinal WAT were reduced in the EFAD pups compared with the control pups at 3 weeks of age. Milk leptin levels were higher in the EFAD dams than in the control dams at 3 weeks of lactation.

We have also demonstrated the effects of dietary n-6/n-3 PUFA ratios on serum leptin levels in the postnatal period [15]. During late gestation and throughout lactation the rats were fed a diet containing linseed oil (n-3 diet), sunflower oil (n-6 diet), or soybean oil (n-6/n-3 diet). As a result the ratio of n-6/n-3 PUFAs in breast milk and in the serum phospholipids of the offspring were significantly different in the n-3, the n-6/n-3 and the n-6 dietary groups (table 1). Decreased serum leptin levels were observed in the offspring receiving
the n-3 diet compared with the n-6/n-3 group (fig. 1). Body weight, body length, inguinal fat pad weight, and adipocyte size of the offspring receiving the n-3 diet were also significantly lower during the whole suckling period compared with n-6/n-3 fed offspring. The mean serum leptin levels of the n-6 offspring were between the other two groups, but not different from either group. No differences were observed in the milk leptin content between the groups.

These results suggest the importance of the EFA intake and the n-6/n-3 PUFA ratios in the maternal diet for adipose tissue growth and for maintaining adequate serum leptin levels in the offspring.

Effect of EFAs on the Appearance of Oral Immunological Tolerance in Suckling Rat Offspring

During the neonatal period the gastrointestinal tract is exposed to a wide variety of microbial and food-related antigens. Usually, oral exposure to food antigens results in induction of oral tolerance, a state of specific immunological hyporesponsiveness upon further exposure to antigens [16]. Several
immunological mechanisms contribute to the induction and maintenance of oral tolerance such as anergy, clonal deletion and active suppression. In adult rats, active suppression is associated with the existence of regulatory suppressor cells (Treg/Th3) in the draining lymph nodes after immunization that are triggered by a specific antigen and responsible for the release of the antigen-non-specific suppressive cytokine TGF-β [17]. Consequently immune responses to other antigens in the close vicinity are diminished [18].

Failure to develop immunological tolerance may lead to an immune response resulting in allergic sensitization to food antigens [19]. Factors important for the induction or breakdown of oral tolerance in the neonatal period are poorly understood. In neonatal rodents oral exposure to antigen can induce tolerance or priming depending on antigen nature and dose, and on the maturity of the immune system [20].

Infant nutrition is one of the most powerful environmental factors that determine early growth and development. The breast milk contains numerous factors, including PUFAs, which may promote the development of the infant’s immune system [21] and affect the immune responsiveness to antigens [4, 22]. The levels of n-6 and n-3 PUFAs in the breast milk are determined to a large extent by the maternal diet [23]. Thus, variation in the PUFA intake in the maternal diet might significantly modulate neonatal development of immunological tolerance and gastrointestinal sensitization to food antigens.

Dietary intake of PUFA has been shown to influence the tolerance induction in adult mice [24]. Furthermore, different effects of dietary n-6 and n-3 PUFA on Th1- and Th2-like responses and the mechanisms of oral tolerance to OA have been demonstrated [25].

Recently we found that the dietary intake of EFAs had no effect on the induction of oral tolerance in adult rats, but influenced the development of

![Fig. 1](image-url)
tolerance in neonatal rats to a food antigen fed to their dams during lactation [26]. During late gestation and throughout lactation rats were fed either a diet supplemented with EFAs, or an EFAD diet. The rat offsprings were subsequently exposed to OA either via the milk at 10–16 days (neonatal rats), or as adults via the drinking water at 7–9 weeks of age.

In rats, which were only exposed to these diets as adults, oral exposure to OA, lead to antigen-specific suppression of the delayed-type hypersensitivity (DTH) response and IgG antibody response to OA. Tolerance to OA was observed in both the EFA-supplemented and EFAD groups, and was accompanied by a reduction in DTH and IgG antibody responses to an unrelated antigen due to bystander suppression [18]. Thus, the oral tolerance was maintained and mediated at least partly by an active suppression mechanism in the adult animals of both the dietary groups.

In the offspring of the dams fed the EFAD diet, antigen exposure via the milk resulted in suppression of the serum antibody levels and DTH response against OA indicating induction of oral tolerance. Higher TGF-β mRNA levels in the draining lymph nodes suggested mediation via Treg cells. In contrast, OA exposure of the dams fed the EFA-supplemented diet did not result in suppressed OA responses of their offspring. Interestingly, a markedly higher ratio of n-6/n-3 PUFA in serum phospholipids was detected in the offspring of the dams fed the EFA-supplemented diet. Since they did not develop oral tolerance to the OA fed their dams, it seems that the dietary n-6/n-3 PUFA ratio is one factor important for the induction, or failure, of oral tolerance.

In further studies we demonstrated the effects of n-6/n-3 PUFA ratios in the maternal diet on the induction of neonatal oral tolerance in the rat offspring [27]. During late gestation and throughout lactation rats were fed the n-3, n-6 or n-6/n-3 diets. At 10–16 days of age the rat offsprings were subsequently exposed, or not, to OA via the milk. In the offspring on the n-3 diet the exposure to OA via the milk resulted in lower DTH and antibody responses against both OA and human serum albumin, compared to those offsprings not exposed to OA, indicating induction of oral tolerance (fig. 2). The lymph nodes draining the immunization site were also less enlarged in the offspring exposed to OA via their dams, suggesting that in the offsprings on the n-3 diet the tolerance was mediated, at least partly, by an active suppression mechanism. In contrast, the offsprings on the n-6/n-3 diet did not show tolerance. A further increase in the n-6 PUFAs in the maternal diet was associated with the induction of oral tolerance in the n-6 group of the offsprings. However, bystander suppression was not observed in the offsprings receiving the n-6 diet, suggesting that oral tolerance may be mediated by anergy. These results suggest that the ratio of the n-6/n-3 PUFAs in the maternal diet might affect the mechanisms of neonatal oral tolerance and are in line with the data of Harbige et al. [25] demonstrating that dietary levels of the n-6/n-3 PUFAs influence the mechanism of oral tolerance in adult mice.
Thus the quality of fatty acid ingested by the mother may have effects on the development of immunological tolerance to dietary antigens in the offspring.

Possible Role of PUFA and Cytokine Aberrations in Intrauterine Growth Retardation

Intrauterine growth retardation (IUGR) occurs in about 1–4% of Swedish deliveries, but is much more common in developing countries like Pakistan [28]. There are many known risk factors like maternal undernutrition, infections, etc., but the etiological mechanisms have not been definitely defined. Impaired outgrowth of trophoblasts into the decidua after implantation of the egg, remodeling the spiral arteries, has been considered crucial. At the same time it is clear that the maternal immune response against the fetus plays a central role in directing implantation, trophoblast growth, hormone production, etc. [28]. On the other hand the maternal response must be modified, or it can destroy the fetus. The likely role of regulatory CD4+CD25+ T cells is suggested by their abundance in human decidua and capacity to suppress

Fig. 2. DTH responses against OA (a) and human serum albumin (HSA; b) in the offspring of dams fed the diet with different ratios of n-6/n-3 fatty acids and either exposed to OA orally (+) before immunization or not (−) (mean ± SE). *Results are significantly different in the group exposed to OA orally (p < 0.05) from the group not exposed to OA within the dietary group. Multiple comparisons made with Kruskal-Wallis and Dunn’s tests.
CD4+CD25− T cells by cell–cell contact [29]. The possible role during pregnancy of immunosuppressive cytokines from regulatory T cells such as IL-10 and/or TGF-β has not been defined.

**PUFAs in IUGR**

The transformation of PUFAs from mother to fetus starts early in pregnancy and is of great importance for normal development. In IUGR, abnormalities of PUFAs have been described, both as EFAD in 20% of the infants [30] and as significantly changed ratios between fetal and maternal fatty acids compared to normal pregnancies [31]. The linoleic acid ratio was increased and the PUFA ratio was decreased, but a higher concentration of n-6 than n-3 fatty acids was reported in fetal serum phospholipids. Since PUFAs from phospholipids are substrates for prostaglandin synthesis, it might be of interest that significantly higher amounts of PGE₂ have been found in human amniotic fluid at the time when atopic sensitization might occur [32]. It is still highly controversial how atopy develops but additional data support an important role of fatty acids.

**Immunological Aberrations in IUGR**

In the placentas of 34 normal Swedish mothers we found the expected predominance of Th2/Treg cytokines needed to balance the mother’s immune response against the placenta/fetus. In 20 IUGR placentas the mRNA for IL-10 in the decidua was low ($p < 0.05$) and for IL-8 high ($p < 0.05$; table 2) [33]. It was debated whether the low maternal IL-10 might result in an increased immune attack against the fetus thus impairing the proper infiltration of the spiral arteries by trophoblasts inhibiting the formation of the placenta and hampering the nutrition of the fetus. IL-8, which is proinflammatory and a strong chemotaxin for neutrophils, may add to damaging inflammation. In the presence of preeclampsia together with IUGR, mRNA for IL-10 in the decidua was still low ($p = 0.05$) and the proinflammatory IL-6 elevated ($p < 0.05$). Without preeclampsia the IL-8 was increased ($p < 0.01$). Combining the data on mRNA for IL-8/IL-10 in the trophoblasts and decidua from the 20 IUGR and 7 cases of small-for-date deliveries gave an increased ratio ($p < 0.01$).

In another study of cytokines in the decidua from 45 IUGR and 55 non-IUGR pregnancies of Pakistani mothers with multiple risk factors for IUGR, we found decreases in mRNA for IL-10 ($p < 0.0001$) and IL-12 ($p < 0.008$) comparing IUGR to non-IUGR placentas (table 2). In contrast, TGF-β was increased ($p < 0.009$) [28] a similar decrease in mRNA for IL-10 ($p < 0.03$), but increase in TGF-β ($p < 0.009$), was found compared to non-IUGR placentas.
At this time it is not clear whether it is possible to relate any or some of these changes in IUGR placentas to the poor outcome of these pregnancies. Previous studies have illustrated that TNF-α and IFN-γ may inhibit growth, differentiation and survival of trophoblasts [34] and IFN-γ; TNF-α and IL-2 may cause fetal resorption, whereas IL-3 and GM-CSF can increase fetal survival and weight [35].

Obviously pregnancy outcome is determined by a complex set of cytokines balancing each other. Our findings of clearly aberrant production in IUGR placentas of certain cytokines presumably originating from the mothers’ immune response against their fetuses/placentas may or may not be involved in the pathogenesis of IUGR. Maternal intake of PUFAs might be related to these aberrations, possibly impairing the normal development of sufficient maternal tolerance to her fetus.

### Table 2. Cytokine mRNA expression in IUGR placentas (with or without complications in pregnancy) and controls: summary of significant differences (p values)

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<th>Groups</th>
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<td>IUGR+PE vs. Controls</td>
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<td>IUGR+SGA vs. Controls</td>
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<td>PE vs. Controls</td>
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<td>IUGR–PE vs. Controls</td>
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IUGR = Intrauterine growth retardation; SGA = small for gestational age; PE = preeclampsia; NS = not significant; I<Control = IUGR<control; S<Control = IUGR+SGA<control; S+PE>C = IUGR+SGA+PE>control, etc.
Conclusions

Our studies indicate the importance of PUFA intake both for the fetus/placenta in relation to the maternal immune system and for the neonate’s immune capacity.

Giving lactating rats an EFAD diet decreased the weight of WAT and serum leptin in the pups during suckling compared to controls. At 3 weeks of age leptin-mRNA was still reduced in inguinal fat. Feeding an isocaloric diet with 7% linseed oil (n-3 diet) during late gestation and suckling, gave the offspring significantly lower serum leptin levels, body weight, inguinal fat pad weight and adipocyte size than those fed an isocaloric diet with soybean oil (n-6/n-3 diet). Leptin has cytokine structure and immunological effects.

An EFAD diet fed to rat dams during late gestation and lactation suppressed serum antibody and DTH in pups against OA given to the lactating rat dams. The increased TGF-β-mRNA in draining lymph nodes may explain the finding since bystander tolerance was also obtained in the pups against an unrelated antigen. No tolerance was noted in the control group. Using instead the n-3 diet mentioned above to the dams, oral tolerance both of DTH and serum antibody reactivity appeared against OA and an unrelated antigen. Feeding the n-6/n-3 diet to the dams, the pups did not become tolerant. Tolerance was also obtained on a diet with very high ratio of n-6/n-3, but seemingly via anergy. These data suggest that the ratio of n-6/n-3 in the maternal diet may be important for the appearance and form of oral tolerance in the neonate.

We measured mRNA for several cytokines in the decidua and trophoblasts from normal and IUGR placentas from Swedish mothers. The immunosuppressive cytokine IL-10 showed reduced expression in the decidua of the IUGR pregnancies (p < 0.05). Instead the proinflammatory cytokine IL-8 mRNA was increased (p < 0.05).

We performed a similar study in Pakistan where the prevalence of IUGR is 15–20%, with many maternal risk factors including undernutrition. A decrease was found in mRNA for IL-10 (p < 0.0001) and IL-12 (p < 0.008), but an increase in TGF-β (p < 0.009) compared to non-IUGR pregnancies. In the trophoblasts IL-10 mRNA was also lower (p < 0.03), but TGF-β mRNA was higher (p < 0.009). In the serum of IUGR newborns TGF-β levels were low (p < 0.05).

It may be considered that if maternal immunological tolerance against the fetus/placenta does not develop properly an increased risk of IUGR may follow. Our studies in rats suggest that the ratio of n-6/n-3 fatty acids may influence the capacity to develop immunological tolerance. A deficient capacity to develop tolerance may be followed by an increased risk of autoimmune and allergic diseases. Possibly IUGR should be included among these conditions since PUFAs seem to play an important role in the normal development and
function of the placenta, and seemingly in the development of tolerance to the fetus [28].

References

Perinatal PUFA Intake Affects Leptin and Oral Tolerance


Discussion

Dr. Sampson: In that Pakistani group, do you have any idea about the LCPUFA intake?
Dr. Hanson: There are no formal studies, we are trying to look into it but I would say that in general the fat intake is very heavy, but at the same time undernutrition or malnutrition is common. But I would expect that the ratios are high.
Dr. Björkstén: I was interested in all that you said but to start with the first part, actually in the first set of experiments you had a control diet with or without oral ovalbumin and PUFA. From the slides I read that by giving oral ovalbumin you induced oral tolerance, but there was no difference in the control group given ovalbumin and the PUFA group given ovalbumin. Did I miss anything there?
Dr. Hanson: The difference you see is really between the groups with different ratios given ovalbumin.
Dr. Björkstén: But this was the first experiment there with 4 groups, and ovalbumin was really the factor?
Dr. Hanson: Are you asking for the study with the 3 ratios, the diet groups?
Dr. Björkstén: No. The difference here, as I saw it, was between those fed ovalbumin orally or not fed, rather than between the diet groups. Does this indicate an effect of the diet because if you compare columns 2 and 4, to me this indicates that feeding orally actually reduces the immune response but at first it doesn’t look too convincing that it is actually the PUFA diet.
Dr. Hanson: If you compare, here you have the control diet and the response there and there, and there is no difference, and if you then look at the control diet with the ovalbumin you see no difference. If you look at the diet which is deficient then you see that both of these are reduced in responsiveness.
Dr. Björkstén: My point is, I am comparing panel 2 and panel 4, ovalbumin per-
orally in the two groups, and to me it would indicate that the diet may not be as impor-
tant.

Dr. Hanson: This is the only comparison which shows a difference.

Dr. Björkstén: I was also interested in the experiments where you had the 3 ratios, and you mentioned the possibility of regulatory T cells and TGF-β as explanatory. Did you measure them?

Dr. Hanson: You mean you would extract them and that would be very difficult, there would be very few. These are rat pups.

Dr. Björkstén: In principle pooling. I was just curious because if there is a pooled experiment for example of the group, then you could do it because it is a very interest-
ing observation.

Dr. Hanson: 10 pups per group, you could mince the 10 pups. I agree with you.

Dr. Haschke: In your fist part of the experiments when you mentioned the ratio between n-6 and n-3 and the group which had the ratio of 0.4, this induced tolerance if I got it correctly. Could you speculate on whether their human milk doesn't have a ratio of 0.4? But one might look at this in terms of human milk with high ratios and human milk with low ratios where the outcome in terms of non-tolerance would be dif-
ferent.

Dr. Hanson: Yes, and this, I am sure, is behind some of the confusion that we have because the mother’s diet will determine what is in her milk and that will have different effects on the baby. Perhaps we missed this, but we can now put it into the right perspective and then we can do more informatory experiments. This may include allergy studies with fish oil and others that may seem rather confusing presently. But if one determines the n-6/n-3 ratios used in these studies they may become easier to interpret. Those ratios may relate to the capacity to induce regulatory T cells.

Dr. Haschke: It would be logical to follow up with a trial perhaps where a low ratio of n-6/n-3 would be given to infants to induce oral tolerance.

Dr. Hanson: I would love to see that experiment, yes.

Dr. Björkstén: I would like to interact with what Dr. Hanson is saying. It is clear and we have shown that in breast milk from allergic and non-allergic mothers and also in the plasma of allergic children, so both in mothers, in their breast milk, in their serum, and in the babies, that the difference, related to allergy, is the ratios, not the absolute levels.

Dr. Jensen: By any chance have you looked at T-lymphocyte cell antigen markers in a preterm study in which at least longer chain PUFA intake seems to influence whether you have a more mature CD4+ lymphocyte population versus a more antigen naïve CD4+ lymphocyte population?

Dr. Hanson: It is a very relevant thing to do, but again the pups are very small and it would not be easy to get lymphocytes. But pooling as suggested might be a way to go. Certainly one needs to proceed from here on.