Vitamin A Induces Long-Term Epigenetic Modifications in the Innate Immune System

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Vitamin A supplementation

High-dose vitamin A supplementation (VAS) programs targeting 6–60-month-old children are carried out in more than 100 countries at risk of vitamin A deficiency (VAD). The aim is to provide VAS every 4–6 months; since vitamin A is a fat-soluble vitamin which is stored in the liver, it is believed that the high doses ameliorate existing VAD and also act as a depot of vitamin A to prevent VAD and its complications.

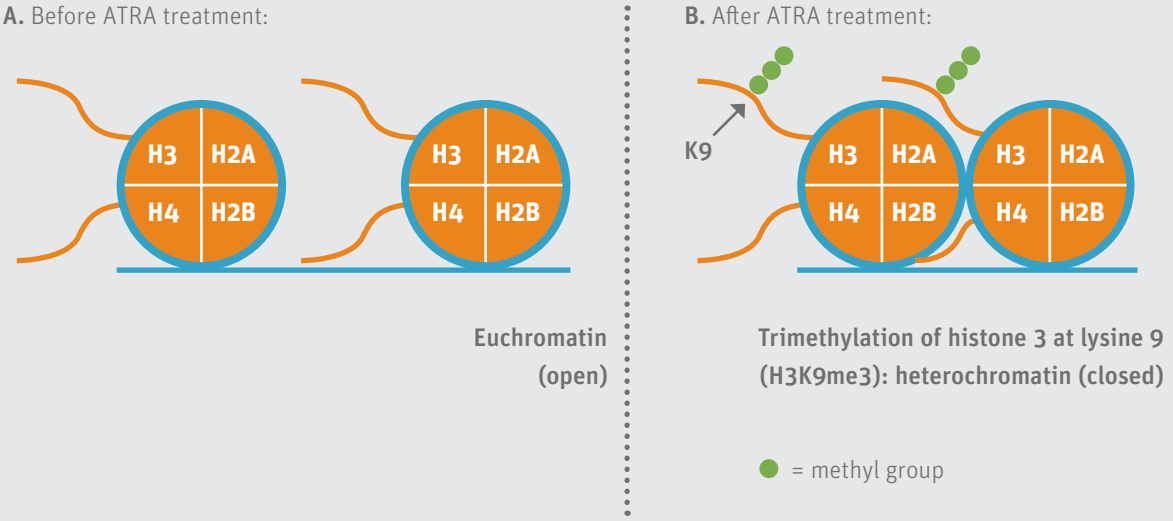
A child receiving a vitamin A supplement in Guinea-Bissau.

The VAS policy is based on randomized trials conducted in the late 1980s and early 1990s, before a vaccination program was widely implemented, showing that a high dose of vitamin A administered to children aged 6–60 months led to significantly lower mortality.[1] However, more recent trials have suggested that this may no longer be the case.[2,3] Intriguingly, one of these trials – an individually randomized placebo-controlled trial – suggested that the effect may differ by sex, being very beneficial in females, who had 55% (95% CI=13-76%) lower mortality after VAS compared with placebo, but not in males, who had 92% (-2-275%) higher mortality after VAS.[3] Since it is estimated that high-dose VAS would reduce overall child mortality by 24%,[4] the lack of overall effect in these recent trials is striking, and at a first attempt hard to explain.

Additionally, though some trials have indicated that neonatal vitamin A supplementation (NVAS) may lower overall mortality in infants up to 6 months of age,[5–7] other recent trials, many with longer follow-up, have indicated that NVAS may be associated with a tendency for increased mortality, especially in females, and with increasing length of follow-up.[8–13] A meta-analysis of trials with follow-up to 12 months of age showed that from 6–12 months of age NVAS versus placebo was associated with 20% (2%–42%) significantly increased mortality in females.[14]

We have proposed a common explanation for these findings: The high doses of vitamin A interact with the routine vaccines in a sex-differential manner, being beneficial for females who have a live vaccine such as BCG or measles vaccine as their most recent vaccine, but negative for females who have diphtheria-tetanus-pertussis (DTP)-containing vaccines as their most recent vaccine.[15–17] This would explain why VAS is beneficial for females from 6 months of age and onwards, where measles

FIGURE 1: When monocytes are incubated with all-*trans* retinoic acid (ATRA), promoters of important immunological mediators (e.g., cytokines such as TNF) become marked with three methyl groups at lysine 9 at the tail of histone 3 (H3K9me3), which is a repressive histone mark. This histone mark induces heterochromatin, which makes genes present at these locations less accessible and therefore less transcribed.



vaccine is the dominating vaccine, but associated with negative effects in females when given at birth and shortly after followed by DTP-containing vaccines.

> "We propose that high doses of vitamin A interact with the routine vaccines in a sex-differential manner"

To explain these effects we hypothesized that VAS not only restores vitamin A levels in the human body, but could also lead to long-lasting effects on the immune system and the way it handles vaccines and infectious disease challenges.

Vitamin A and the immune system

The short-term or direct effects of vitamin A on the immune system are well described. In general, vitamin A has an inhibitory effect on both the innate and adaptive immune system, by down-regulating pathogen recognition receptors and important intracellular signaling cascades, reducing the production of soluble signaling molecules such as cytokines and prostaglandins, and inhibiting the maturation and differentiation of certain immune cells.[18] However, these are all direct effects of vitamin A and its metabolites on the immune system. The question as to whether a high dose in children would lead to longer-lasting effects on the immune system, whether beneficial or negative, has not been addressed.

Potential long-term effects of vitamin A supplementation

The long-term immunological effects of a health intervention are not an unknown phenomenon. Comparisons with other large-scale prevention policies, such as vaccination regimes, might serve to raise hypotheses. For example, for BCG vaccine it has been shown that apart from preventing tuberculosis infection, the vaccine also has secondary so-called "non-specific" effects. In observational studies and later in two RCTs it was shown that BCG provided early in life prevents mortality from other non-related infectious diseases, especially from lower respiratory infections and sepsis.[19,20] The immunological mechanisms behind the non-specific effect of BCG have not been known. However, recently, in a proof-of-concept study in healthy volunteers, it was shown that the BCG vaccine induced long-term epigenetic changes in the monocytes after vaccination, so-called "trained innate immunity." These epigenetic changes made monocytes more capable of reacting to pathogens.[21,22] This provided a plausible mechanism for the reduction in all-cause mortality in BCG-vaccinated children. Indeed, in Guinean children, we found that those randomized to BCG at birth mounted stronger *in-vitro* cytokine responses upon unrelated innate stimulation than those who had not yet received BCG.[23]

A hypothesis for the potential long-term negative effects of vitamin A on the immune system could be adapted from the

effects observed from BCG vaccination. We therefore hypothesized that vitamin A could also induce epigenetic changes in monocytes, leading to a long-term decrease in the function of monocytes and therefore increased mortality from infections. We tested this in a series of experiments which were recently published in the *Journal of Leukocyte Biology*.[24]

> "We hypothesized that vitamin A could also lead to long-term decrease in the function of monocytes"

Vitamin A induces long-term epigenetic changes in monocytes

To address this hypothesis, we performed several *in-vitro* experiments on isolated human monocytes.[24] Incubation of monocytes with all-*trans* retinoic acid (ATRA), the most abundant metabolite of vitamin A, resulted in decreased cytokine production of monocytes a week after incubation with ATRA. Analysis of the epigenome of the cultured monocytes revealed epigenetic changes at the promoter sites of important signaling molecules of monocytes. Epigenetic changes can be considered as a long-term memory. The epigenetic modifications induced by ATRA in monocytes led to an epigenetic make-up of these monocytes that makes them less capable of reacting to pathogens effectively in our *in-vitro* system. Monocytes that had been primed with ATRA for 24 hours and were stimulated with a panel of different pathogens a week later were less capable of mounting a cytokine response (**Figure 1**). Interestingly, the addition of ATRA to BCG inhibited the epigenetic innate training effects of BCG, showing that these two interventions interacted with effects on the immune system.[24]

The translation from our *in-vitro* system to supplementation policies is of course still a big step, but an important one to make. Our *in-vitro* experiments support the hypothesis that vitamin A can induce long-lasting functional changes in monocytes and macrophages. Thus, apart from its benefit to vitamin A status, VAS may also have a detrimental effect on the innate immune system, which might lead to higher mortality rates. These findings might fit with the varying effect of VAS on mortality seen over time (with decreasing effects of VAS on overall mortality over time, as the vaccination program was widely implemented, and the prevalence of pathogens encountered may have changed too), and depending on vaccines received. In other words, depending on the challenges the immune system faces at the time of VAS, the effect of VAS may be beneficial, but it may sometimes also be harmful.

How to continue?

A very important question that needs to be answered is whether VAS also leads to a long-term down-regulation of the function of monocytes *in vivo*. This question could be addressed by performing functional analyses on white blood cells of, in the first instance, healthy volunteers, and possibly later of babies or children that have received VAS, to assess whether the epigenetic make-up and the function of the immune system has changed after supplementation. If this is indeed the case, then lowering the supplementation dose should be considered. Another important question that remains to be answered is why VAS should have divergent effects in males and females depending on vaccination status – a pattern seen for VAS but also for other micronutrients.[25]

In conclusion, large-scale VAS programs may no longer have the effect on mortality that they were hoped to have. The dampening effect on monocyte function observed in our human *in-vitro* model might also occur in the *in-vivo* situation. These long-lasting negative effects on the innate immune system could counteract the beneficial effects of restoring vitamin A levels.

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Vitamin A and Epigenetic Modifications
1. Observations by Keith P West, Jr.

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Arts and Benn hypothesize that early-life supplemental vitamin A may affect nutritional programming of the immune system, an established concept[1] being tested for vitamin A in animals,[2] *in-vitro* systems,[3] and randomized cohorts.[4] While an important scientific quest, Arts and Benn make an untenable leap to justify their vaccine-exposure hypothesis as relevant to policy by over-interpreting imprecise risk ratios (RR) from existing trials, evident by wide 95% confidence intervals (CIs) that should give pause about uncertainty.

For example, in a trial in Guinea-Bissau that the authors cite as providing evidence of decline in the impact of vitamin A on preschool child survival, there was a 9% reduction in mortality among 6–23-month-old children. While not significant (95% CI: -41% to 41%),[5] the estimate remains compatible with a long-established 24%–34% reduction in preschool child mortality that has been estimated across different vitamin A interventions.[6,7] Associated with this weak overall effect, there was a significant interaction by sex that revealed a clear reduction, and suggestive increase, in mortality among girls and boys, respectively. Despite a need for caution when interpreting subgroup effects and imprecise confidence intervals, the authors claim their results are strikingly different from previous trials.

Caution is similarly ignored in inferring that newborn vitamin A supplementation may increase infant mortality, especially in girls. Studies cited by Arts and Benn to support their claim all reported RRs of 0.90 to 1.12, with none having 95% CIs excluding 1.00.[5,8–12] Some estimates were suggestive of a higher RR from vitamin A receipt in boys,[5,11] another in girls,[9] and some in neither sex,[10,12] while all sex-specific 95% CIs except one (for girls in Guinea-Bissau[9]) included unity. At present, the most tenable explanation for variation in risk observed by sex across trials, given the evidence, is chance.

The above inference should not detract from Arts and Benn's pursuit of a vaccine-exposure hypothesis, but it should guide one's stand on policy. Newborn vitamin A trials in Southern Asia have all reported protective main effects (RRs) against infant mortality of 0.36 to 0.90, each with a 95% CI <1.00,[12–15] while across Africa effects have ranged from 0.98 to 1.16, all with 95% CIs that include unity.[8–11,16] Reasons for this "continental divide" may relate to regional differences in maternal vitamin A deficiency.[17] Thus, a policy implication also consistent with existing evidence would be to supplement newborns with vitamin A in Southern Asia, averting >150,000 infant deaths annually,[18] but not in Africa where, for reasons to be understood, it has, to date, shown no effect.

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Vitamin A and Epigenetic Modifications
2. Observations by Charles B Stephensen

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Cells of the immune system undergo epigenetic, transcriptional programming during normal development. Perhaps the best characterized example is the development of memory T-cells which develop both "inflammatory" phenotypes, such as Th1 cells that protect against infections including tuberculosis, and "regulatory" phenotypes, such as Treg cells, that dampen inflammation during the resolution phase of an immune response.[1]

Interestingly, the vitamin A metabolite retinoic acid can enhance the development of either cell type depending on experimental conditions.[2] Looking at either effect in isolation might lead to a prediction that vitamin A supplementation would only promote or only dampen inflammation, while the actual effect is more complex. Innate immune cells such as monocytes and macrophages also undergo transcriptional programming.[3] Retinoic acid again has apparently contradictory effects on these cells, both enhancing killing of tuberculosis bacilli by monocytes[4] but also dampening the production of cytokines by monocytes transcriptionally programmed by BCG to produce high levels of cytokines upon activation.[5]

The vitamin D metabolite calcitriol has similar effects, enhancing killing of tuberculosis bacilli by macrophages[6] but also programming a mechanism to dampen IL-6 production longer term.[7] When vitamin D is used to treat tuberculosis as adjunct therapy it can, at least in some settings, both speed bacterial clearance and dampen inflammation,[8] with both effects likely benefitting the patient.

In this issue Arts and Benn suggest that the ability of vitamin A to dampen BCG programming of monocytes to produce high levels of inflammatory cytokines will be a "negative" under circumstances where this programming may have non-specific protective effects for infants. This is an interesting and important hypothesis but, as the preceding examples show, making predictions from isolated *in-vitro* treatment effects for vitamin A is risky. It would be useful to test this prediction in an animal model to see if there is an *in vivo* correlate of the *in-vitro* observation. Even then, it is difficult to make concrete predictions for nutrients such as vitamin A and vitamin D, which have such varied effects on the immune system. Continuing to examine these questions will help us understand the mechanisms of action of these nutrients. Such advances in knowledge will help in the formulation of public health nutrition policies. It is important to re-evaluate such policies in the light of new research findings. This is particularly true of vitamin A at the moment, as such a re-evaluation is currently being discussed by the scientific community.[9,10]

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