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Research Article

Alpha Linolenic Acid Variability Influences the Positive Association between %Eicosapentaenoic Acid and % Arachidonic Acid in Chicken Lipids

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Abstract:

Body concentrations of Arachidonic Acid (AA, 20:4 n6) and Eicosapentaenoic Acid (EPA, 20:5 n 3) are influenced by diet. Previously, we reported that the concentration range of AA and EPA might explain that %AA and %EPA are positively associated, and that variability of OA (18:1 c9) influences this association. We now investigate whether also the range of ALA (18:3 n3) might influence the association between %AA and %EPA, using data from a diet trial in chickens. A broadening (narrowing) of ALA-variability made the %AA vs. %EPA scatterplot improve (be poorer), as observed both when calculating percentages of all fatty acids, and when using ALA, AA, and EPA only in the denominator.

Thus, the positive association between relative amounts of AA and EPA in breast muscle lipids of chickens is influenced by ALA variability. We raise the question of whether differences in concentration ranges between the many types of fatty acids (possibly acting via skewness) might serve as an evolutionary mechanism to ensure that percentages of fatty acids will be positively or negatively associated: a Distribution Dependent Regulation.

Keywords: alpha linolenic acid; arachidonic acid; eicosapentaenoic acid; chickens; random numbers

Definitions and abbreviations:

Variability: the width or spread of a distribution, measured e.g. by the range and standard deviation.
Range: showing the largest and smallest values.
Distribution: graph showing the frequency distribution of a scale variable within a particular range. In this article, we also use distribution when referring to a particular range, a – b, on the scale.
Uniform distribution: every value within the range is equally likely. In this article, we may write "Distribution was from a to b", or "Distributions of A, B, and C were a – b, c – d, and e - f, respectively".
"Low–number variables" have low numbers relative to "high-number variables".
ALA = Alpha Linolenic Acid (18:3 n3)
OA = Oleic Acid (18:1 c9)
LA = Linoleic Acid (18:2 n6)
ALA = Alpha Linolenic Acid (18:3 n3)
AA = Arachidonic Acid (20:4 n6)
EPA = Eicosapentaenoic Acid (20:5 n3)

Introduction

Fatty acids in blood and tissues are important in health and disease, and body amounts are influenced by diet [1, 2]. For example, ALA cannot be synthesized by mammals, and adequate dietary intake is essential for human health. Increased ALA intake may decrease proinflammatory cytokines [3].

Furthermore, it is well known that EPA (20:5 n3) and AA are metabolic antagonists [1, 4]. Eicosanoids derived from EPA may decrease inflammatory diseases [5, 6], improve coronary heart diseases [7, 8], and cancer [9]. However, in a systematic Cochrane Review of selected studies the beneficial effects of long-chain n3 fatty acids on all-cause and cardiovascular mortality was questioned [10].
AA is formed in the body from linoleic acid (LA 8:2 n6), a major constituent in many plant oils. This fatty acid is converted by cyclooxygenase and lipoxygenase into various eicosanoids, i.e. prostacyclines, thromboxanes and leukotrienes [1]. In contrast to the eicosanoids derived from EPA, those derived from AA, i.e. thromboxane A_2 (TXA_2) and leukotriene B_4 (LTB_4), have strong proinflammatory and prothrombotic properties [1, 11]. Furthermore, AA derived endocannabinoids may have a role in adiposity and inflammation [12].

We previously suggested that ALA, and possibly other fatty acids, might be involved in the regulation of AA metabolism [13]. Thus, the inverse relationship between relative amounts of AA and oleic acid (OA 18:1 c9)

in muscle lipids of chickens [13-15] might possibly be explained by inhibition by ALA of the synthesis of AA, and stimulation of OA formation [13]. Additionally, the fact that ALA is precursor of endogenous synthesis of EPA [1] may probably explain some of the health effects of ALA. Furthermore, it has been reported that a decreased level of the serum EPA/AA ratio may be a risk factor for cancer death [9]. Thus, when considering the beneficial health effects of foods rich in ALA, many of the positive effects would be anticipated if the fatty acid works to counteract effects of AA. It would appear, accordingly, that a coordinated regulation of the relative abundances of EPA and AA could be of physiological interest, so that an increase (decrease) in the percentage of one of these fatty acids would be accompanied by a concomitant increase (decrease) in percentage of the other. In accordance with these considerations, we reported that percentages of AA and EPA were indeed positively associated in breast muscle lipids of chickens [16-18].

Using random numbers in a computer experiment, we previously suggested [19] that, with 3 positive scale variables, two of which having low-number distribution, and low variability, as compared with the third variable, we might expect a positive association between percentages of the low-number variables, and a negative association between percentage of the high-number variable and percentage of each of the low-number variables. Furthermore, a decrease (increase) in the variability of either or both of the two low-number variables seemed to improve (make poorer) the association between their relative amounts. In contrast, a narrowing (broadening) of the distribution of the high-number variable seemed to make poorer (improve) the association between percentages of the low-number variables. These observations raise the question of whether the rules may apply for fatty acids. In support of this suggestion is our finding of a positive association between relative amounts of EPA and AA in breast muscle lipids of chickens [16, 17, 18], where these fatty acids are low-number variables with low variability (concentration range 0.13 – 0.24, and 0.25 – 0.42 g/kg for EPA and AA, respectively) relative to OA (range 1.04 – 8.56 g/kg), [20]. Alterations in the OA, EPA, and AA ranges strongly influenced the association between percentages of AA and EPA, in line with the general rules above [19]. For example, high OA variability improved the %EPA vs. %AA association appreciably. The finding that ALA has high variability (CV = 60%) relative to EPA and AA, raises the question of whether also ALA might influence the %EPA vs. %AA correlation. We reasoned that the particular concentration ranges of ALA, as well as those of AA, and EPA might possibly govern a balance between relative amounts of EPA and AA, and thereby ensure a balance between eicosanoids with opposing actions. We recently reported that also skewness of distributions might –in general- serve to explain some distribution dependent correlations; skewness was especially encountered with variables having greatly varying ranges [21]. The aim of the present work was accordingly to investigate whether alterations in the concentration range of ALA might influence 1) the association between percentages of EPA and AA, and 2) skewness of the %ALA distribution. Finally, we wanted to examine whether *skewness* of the %ALA distribution is related to the association between %EPA and %AA.

Materials and Methods

Chickens and Diet

We refer to our previous article [22] for details concerning the diet trial. In brief, from day 1 to 29 one-day-old Ross 308 broiler chickens from Samvirkekylling (Norway) were fed wheat-based diet containing 10 g fat per 100 g diet. ALA (18:3 n3), a precursor of EPA, provided 15% of the fatty acids, and LA (18:2 n6), a precursor of AA, provided 21%. The n6/n3 ratio was 1.4. Energy content of the feed was about 19 MJ/ kg. ALA provided 2.5% of the energy, and LA 4%. Other components in the feed were: Histidine 0.1%, choline chloride 0.13%, mono-calcium phosphate 1.4%, ground limestone 1.3%, sodium chloride 0.25%, sodium bicarbonate 0.2%, vitamin A, E, D, K, B 0.18%, L-lysine 0.4%, DL-methionine 0.2%, and L-threonine 0.2%.

Calculations and Statistical Analysis

We first reanalysed our previously reported association between %EPA and %AA [17], and next computed S, the sum (g/kg we weight) of all fatty acids, and R, the remaining sum when omitting EPA and AA. Thus, R= S – EPA - AA. To determine distributions of EPA, AA, and R, we made histograms. Random numbers were used to explore the effect of ALA variability upon the association between percentages of EPA and AA. The random numbers had either uniform distribution, or normal distribution (generated on the basis of mean value and SD). Since the diet trial had 163 chickens, for ALA we generated 163 random numbers with varying variability. The physiological distributions (ranges) were: for ALA 0.12 – 2.40, for AA 0.25 – 0.42, and for EPA 0.13 – 0.24. We computed percentages of the variables: %AA = (AA/S)*100; %EPA = (EPA/S)*100; %R= (R/S)*100, and made histograms to illustrate distributions of the percentages. Minimum and maximum values of the percentages were also calculated manually from the ranges. Dependency between percentages is shown by: %EPA + %AA + %R = 100. Thus, ALA is one of the fatty acids of R. With random numbers for ALA, we made a new R where the true ALA-values were replaced by the random numbers. Note that we use ALA also with random numbers to keep in mind that the aim of our analyses was to mimic results with real values of ALA, but upper case letters were used (RANDOM) in the figure texts to clarify. Using random numbers for ALA, generated within the physiological range, and the true, measured values for all other fatty acids, we made scatterplots of %AA vs. %EPA. Then we studied how alterations in the variability of ALA (as a component of R) might change the relationship between %AA and %EPA.

It occurred to us that there might be a lot of noise when using the total sum of fatty acids in the denominator when calculating percentages of the 3 fatty acids under investigation, i.e. ALA, AA, and EPA. We therefore did separate analyses with ALA, AA, and EPA only in the denominator. Thus, %ALA + %AA + %EPA = 100, i.e. %AA = - %EPA + (100 - %ALA). To obtain two unknown variables only, the equation was simplified by using hypothetical, random numbers, in two ways: 1) by making (100 - %ALA) approach zero (giving a positive %AA vs. %EPA association; further explained under Results and Discussion), and 2) by making %ALA approach zero (giving negative %AA vs. %EPA correlation; see below). We anticipated positive (negative) correlations also close to these conditions. Since %ALA seems to be crucial for the %AA vs. %EPA outcome, in the figures we show histogram of the %ALA distribution, and scatterplots of %AA vs. %EPA. Additionally, we present quartiles and skewness of the %ALA distribution. We made several repeats of the analyses, each with a new set of random ALA values. The outcome of the repeats was always the same, with scatterplots appearing unchanged, but corresponding correlation coefficients (Spearman's rho) varied slightly. Results are mainly presented as scatterplots and histograms. SPSS 25.0 was used for the analyses, and for making figures. The significance level was set at $p<0.05$. When appropriate, we present a more detailed description of the computer analyses under "Results and Discussion".

Authors' Contributions

The present study is a spin-off study of a previously published diet trial, conceived and conducted by AH. ATH conceived and designed the present study, analyzed and interpreted the data, conceived the hypothesis of Distribution Dependent Correlations/-Regulation, and wrote the article. ATH emphasizes that the excellent diet trial of AH - and the nice correlations observed - were crucial for the hypothesis. AH contributed substantially to the interpretation of data and revising the article critically for important intellectual content. Both authors read and approved the final manuscript.

Ethics Approval

The diet trial in chickens was performed in accordance with National and international guidelines concerning the use of animals in research (Norwegian Animal and Welfare Act, European Convention for the protection of Vertebrate Animals used for Experimental and other Scientific Purposes, CETS No.: 123 1986). The Regional Norwegian

Ethics Committee approved the trial, and the experimental research followed internationally recognized guidelines. There are no competing interests.

Results and Discussion

Descriptive Data

Descriptive data for the fatty acids under investigation are shown in Table 1. ALA, AA, and EPA contributed with 5.2, 3.4, and 2.0% respectively of all fatty acids. There was a striking difference in variability between ALA and the other fatty acids; ALA showed a 20-fold increase from lowest to highest value (CV 60.4%). In contrast to this, the variabilities of AA and EPA were low, with CV 9.7 and 11.1, respectively. Total weight of all fatty acids in breast muscle lipids of the chickens was 8.86 + 2.62 g/kg wet weight (mean + SD, n = 163).

Fatty acid	Minimum	Maximum	Mean	SD	%	CV
ALA	0.12	2.40	0.53	0.32	5.2	60.4
Arachidonic acid	0.25	0.42	0.31	0.03	3.4	9.7
EPA	0.13	0.24	0.18	0.02	2.0	11.1

Table 1. Descriptive statistics for the fatty acids under investigation (n= 163); minimum and maximum values, mean values (g/kg), with SD, % of total weight, and CV= (SD/Mean)*100.

Percentages calculated from total amount of fatty acids

Using the measured (physiological) values of ALL fatty acids, including ALA

We first investigate the association of %AA vs. %EPA when *all fatty acids, including ALA, have their physiological values (distributions/ranges)*, i.e. for EPA 0.13 – 0.24, for AA 0.25 – 0.42, and for ALA 0.12 – 2.4 g/kg. There was a positive relationship between %EPA and %AA, as illustrated in Figure 1, left panel; Spearman's rho = 0.750, p<0.001). Equation of the regression line: %AA = 1.23 (0.08)*%EPA + 1.01 (0.18). Figure 1, right panel shows the histogram of %R, i.e. sum of percentages of all fatty acids, minus AA and EPA. There seemed to be a fairly normal distribution of %R, Figure 1, right panel (and also of %EPA and %AA, not shown). Skewness of %EPA: -0.040; %AA: -0.083; %R: 0.203. %R-quartiles were *93.7, 94.5, and 95.1.* Absolute amounts of EPA and AA (g/kg) did not correlate significantly (r = -0.046, p = 0.563).

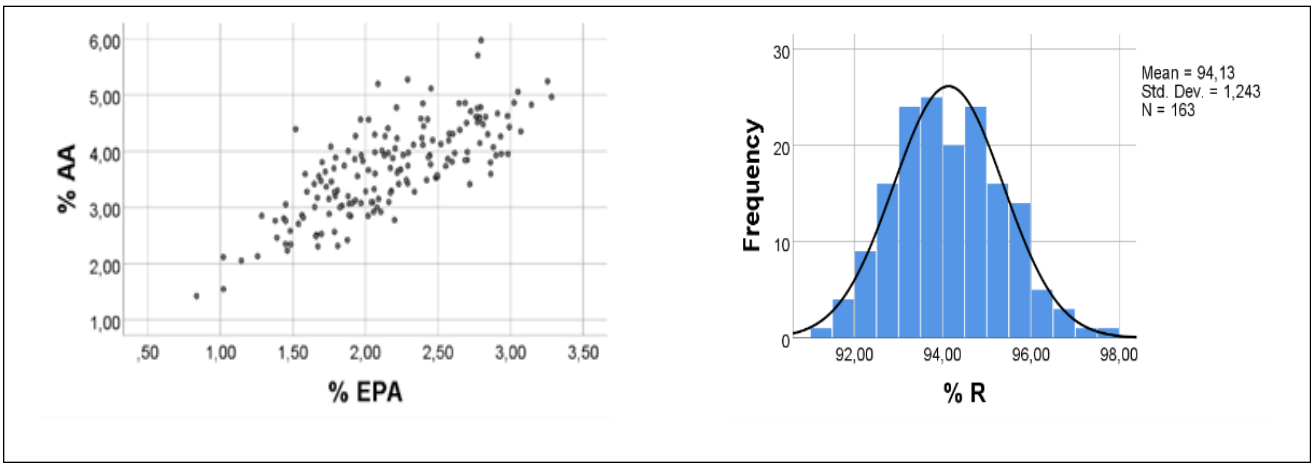

Figure 1. Left panel: Scatterplot of % EPA vs. % AA; Spearman's rho = 0.750 (p<0.001), n = 163; regression line: %AA = 1.23 (0.08)*%EPA + 1.01 (0.18). Right panel: histogram of %R (sum of percentages of all fatty acids, minus AA and EPA); cutoff values of quartiles: 93.7, 94.5, and 95.1%. Skewness of %EPA: -0.040; %AA: -0.083; %R: 0.203.

Using Random (Surrogate) Numbers for ALA, But Keeping the Physiological Values for All Other Fatty Acids

In the next experiment we used 163 *RANDOM numbers* for ALA, however generated with the physiological distribution: 0.12 – 2.40, but keeping all other fatty acids with their measured values. The outcome using random numbers with *Uniform* distribution is shown in Figure 2, upper panels, and random numbers with *Normal* distribution is shown in Figure 2, lower panels. The %EPA vs. %AA scatterplot with the true, physiological values, and those based upon random numbers did not differ much (Figure 2, compared with Figure 1), as was also verified by the equation of the regression lines. Using *uniform* distribution of the ALA surrogate numbers we found the following equation of the regression line: %AA = 1.16 (0.09)* %EPA + 1.07 (0.17); and Spearman's rho for % AA vs. % EPA: r = 0.695 (p<0.001). %R-quartiles were *93.9, 94.7, and 95.3%, respectively.* Skewness of %EPA: 0.044; %AA:-0. 0.095; %R: 0.140. When generating the random numbers with *normal* distribution of ALA, based upon mean (SD), i.e. 0.53 (0.32), the equation was: %AA = 1.17 (0.09)* %EPA + 1.12 (0.19); and rho for % AA vs. % EPA: r = 0.712 (p<0.001). %R-quartiles were: *93.3, 94.2, and 95.0%,* respectively; skewness of %EPA: 0.005; %AA: -0.105; %R: 0.196.

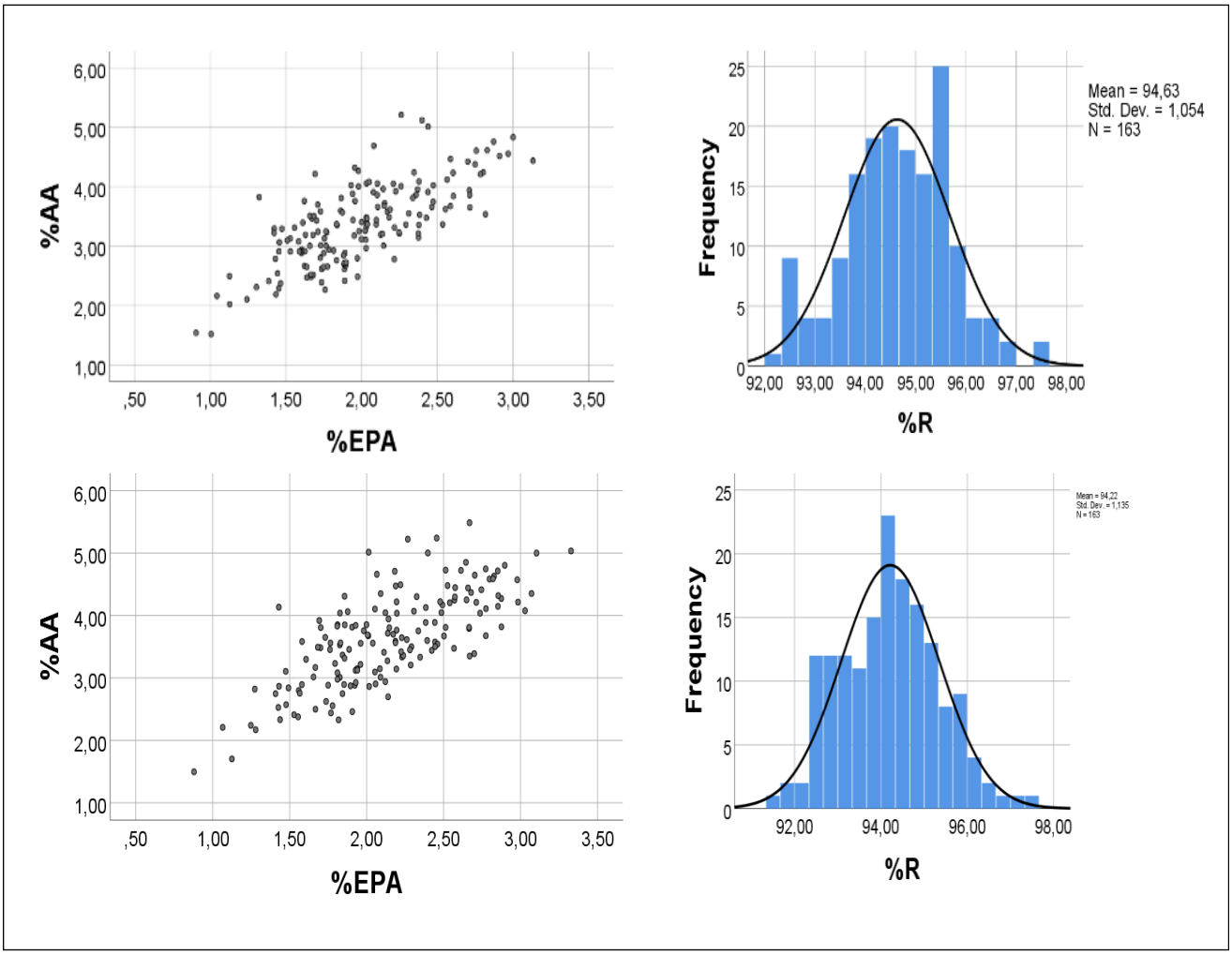

Figure 2. *Upper panels:* For ALA, RANDOM numbers with uniform distribution in the physiological range 0.12 – 2.40 were used. Scatterplot of %AA vs. %EPA (left), and histogram (right) of %R (sum of percentages of the remaining fatty acids, i.e. all except EPA and AA). % AA vs. % EPA: Spearman's rho = 0.695 (p<0.001, n=163); regression line: %AA = 1.17 (0.09)* %EPA + 1.12 (0.19). %R-quartiles: 93.9, 94.7, and 95.3. Skewness of %EPA: 0.044; %AA:-0. 0.095; %R: 0.140.
Lower panels: For ALA, RANDOM numbers with normal distribution were used, generated on the basis of the measured mean (SD) value = 0.53 (0.32); all other fatty acids were with the real, measured values. % AA vs. % EPA: rho = 0.699 (p<0.001, n=163); regression line: %AA = 1.14 (0.09)* %EPA + 1.20 (0.19). %R-quartiles were: 93.3, 94.1, and 94.9%, respectively. Skewness of %EPA: 0.005; %AA: -0.105; %R: 0.196.

Suggested Explanation of the Results, Using an Algebraic Approach

Some general considerations

Instead of thinking specifically about correlations between ALA, AA, and EPA, below we first consider - in general- three positive scale variables, A, B and C, giving %A + %B + %C = 100, i.e.% B = - % A + (100 - % C). This equation has three unknown variables, each of which with a particular distribution (range). It is therefore hard to predict whether or not there is a significant association between relative amounts of e.g. A and B. We may, however, simplify the equation by approximations, so as to involve two variables only. This may be achieved in two particular situations: 1) if the expression (100 -%C) approaches zero, or 2) if %C approaches zero. Thus, if %C consists of high values (close to 100) and the low-number, corresponding values of %A and %C are such that (100% - %C) > %A, then the equation would approach %B = %A, showing a linear positive association between %A and %B. The requirement (100 - %C) > %A is indeed satisfied, sine the remaining value when calculating (100 - %C) would have to be divided between %A and %B. For example, suppose that %C could reach 99%, then the remaining percentage is to be divided between %A and %B. Hence, the slope must be positive.

On the other hand, if %C consists of very small values, we should expect a negative %A vs. %B association, since the equation in this case would approach % B = - % A + 100. Additionally, we might anticipate positive or negative correlations between percentages of A and B also within a certain boundary around the above-mentioned conditions, but with poorer outcomes as the above-mentioned conditions are decreasingly complied with. This reasoning raises the question of how far from the "mathematically ideal", but "physiologically extreme" (if relating the A, B, and C variables to physiologically ones) conditions we may go and still

obtain a positive (negative) %B vs. %A association. Furthermore, this reasoning implies that there must be a **Turning Point** where a positive (negative) correlation between percentages of A and B turns to become negative (positive). Furthermore, around the Turning Point we should expect that there is no significant correlation between the percentages. We limit our analyses to mainly considering how the association between %B and % A responds to altering the C range. Below we first give a comment concerning the slope of the regression line.

Slope of the regression line

Above we argued that there might be a positive association between %B and %A, if %C values were very high so that the expression (100 - %C) approached zero. However, in this case it is inappropriate to write %B = %A, like Y = X. In the latter case, both the abscissa and the ordinate may have any value on the scale, and the Y vs. X graph would have slope = 1. In contrast to this, %B and %A – values are limited by the B and A distributions (ranges), respectively. A more general equation would be: $\%B_{(p-q)} = -\%A_{(r-s)} + (100 - \%C_{(t-u)})$ where the subscript parentheses indicate ranges of A, B, and C. The slope of the %B vs. %A regression line will accordingly be determined by the ranges of A(%A) and B(%B). Thus, if A- and also B - have the same distribution (range), then the slope should be close to 1. Indeed, in an experiment with range 0.10 – 0.15 for both A and B, and 1 – 10 for C, we did find slope =1, [19]. With *differing* ranges for A and B, e.g. for A 0.20 - 0.40, and for B 0.10 – 0.15, and for C 1 - 10, we found that the equation of the regression line was: %B = 0.38 (0.01)* %A + 0.22 (0.10).

Applying the above algebraic approach to explain the association between %EPA and %AA

We try to apply the above general consideration to understand the current outcome with fatty acids from breast muscle lipids of chickens. We use the special equation: %AA + %EPA + %R = 100, or %AA = -%EPA + (100 - %R), which is approaching %AA = %EPA, due to high %R values, i.e. there should be a positive association between percentages of AA and EPA, as was observed (rho about 0.7). Similarly, the negative association between %AA (EPA) and %R; rho =-0.951 (-0.887), p<0.001 for both, n =163 may be explained by approximations of the equations 1) %AA = -%R +(100 -%EPA) and 2) %EPA = -%R + (100 - %AA). Eq. 1) may be approximated to %AA = - %R + 100, since %EPA is small compared with %R. Similarly, eq. 2) May be approximated to % EPA = -%R + 100. Thus, %R should be negatively associated with both %AA and %EPA, as we observed.

The above experiment shows that the association between %EPA and %AA was not much disturbed by replacing the true values of ALA with random numbers, irrespective of whether the numbers had uniform or normal distribution. It would appear, accordingly, that the positive association between %EPA and %AA may not be caused by other type of biological regulation than regulation of concentration ranges. In the computer experiments below, we mainly use uniformly distributed, hypothetical (surrogate) random numbers for ALA.

Altering ALA – range (distribution, variability)

Below we present some experiments to show how the association between percentages of AA and EPA might change in response to changing the variability of ALA. Again, we consider the equation

%AA + %EPA + %R = 100, where R is sum of the remaining fatty acids when omitting AA and EPA. Thus, % AA = - %EPA + (100 - % R). Since ALA is included in R, we might expect that increasing values of ALA (%ALA) should make the expression (100 – %R) approach lower values, thereby possibly improving the positive association between %AA and %EPA. The opposite should be expected to happen with decreasing values of %ALA. Below we have tested how hypothetical alterations of the ALA range might influences the %AA vs. %EPA association. We emphasize that large variabilities were used, to better clarify main effects.

Decreasing and Increasing the Values of ALA (And %ALA)

We first narrowed the ALA distribution towards the lower limit of the physiological range, i.e. to 0.12 – 0.13 (instead of 0.12 – 2.40). We found no major change in the strength of %EPA vs. %AA correlation (rho = 0.676, p<0.001, n=163; scatterplot not shown. Quartiles of the %R distribution were: 93.0, 93.8, and 94.7%, against 93.9, 94.7, and 95.3 with the physiological ALA range. This small change in the %R distribution is in accordance with no major change in the correlation coefficient between percentages of AA and EPA. We then greatly increased the ALA range, to go from 0.1 to 10.0 (instead of from 0.12 – 2.40). In this case, %R-quartiles seemed to move towards higher values, being: 95.6, 96.3, and 97.0 %, respectively, against 93.9, 94.7, and 95.3 before broadening the ALA range. As suggested above, the correlation between %AA and %EPA seemed to improve: rho = 0.844, p<0.001, n=163. Skewness of the %EPA, %AA, and %R histograms became greatly increased, being 0.863, 1.179, and -1.038, respectively (against 0.044, -0. 0.095, and 0.140 with the physiological ALA range). Thus, there was an appreciably increased negative skewness of the %R distribution, and a concomitant increased positive skewness of the histograms of %AA and %EPA, in response to increasing the ALA variability. The apparent improved correlation between %AA and %EPA might accordingly be explained by a movement of the %R distribution towards higher values (see above), and possibly also by the observed alterations in skewness [21], see also below.

High amount of tissue ALA (and %ALA) is expected following high intake of foods rich in ALA, such as flaxseed oil. The results above suggest that increased intake of such oils might possibly improve the positive association between relative amounts of EPA and AA, thereby also improving the balance between eicosanoids derived from AA and EPA. We do not know, however, how rapid – and to which levels - blood and tissue ALA levels may go, in response to altering diet, physical activity, and other environmental changes.

Is the influence of ALA upon the %AA vs. EPA association caused by the precursor/product relationship between ALA and EPA?

Since ALA is a precursor of EPA [1] we might question whether this metabolic relationship influences the %AA vs. %EPA association. Hypothetically, we might raise the question of whether a “metabolic push” for any reason (rapid increase in ALA) might cause a positive association between ALA and EPA, whereas a “metabolic pull” (rapid decrease in EPA) might cause an inverse ALA vs. EPA relationship. A bivariate correlation analysis showed a weak positive association (Spearman’s rho = 0.242, p = 0.002, n =163) between absolute amounts (g/kg wet weight) of ALA and EPA; however with a poor scatterplot (Figure 3).

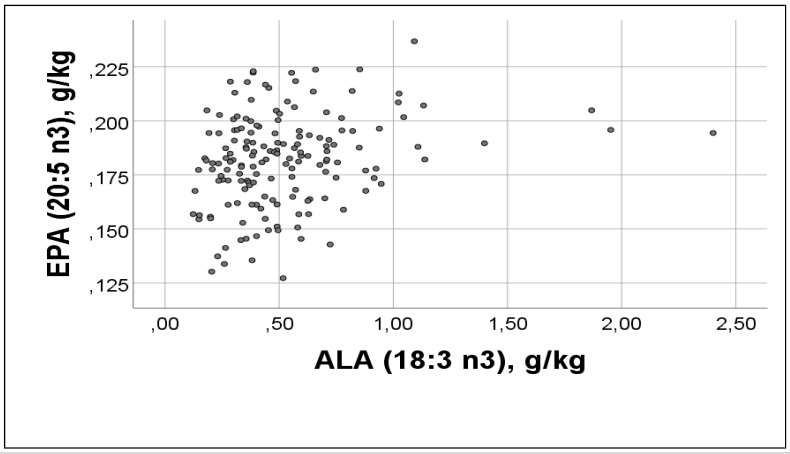

Figure 3. Association between absolute amounts (g/kg wet weight) of ALA and EPA in breast muscle lipids of chickens, see text; Spearman's rho = 0.242 (p= 0.002, n=163).

However, when controlling for total amount of fatty acids in a linear regression analysis there was no longer a significant association between ALA and EPA (t = 1.289, p = 0.199, n = 163). Thus, the weak positive ALA vs. EPA association is probably attributed to the general trend that absolute amounts of many of the fatty acids increase simultaneously, as we previously have observed (unpublished results).

To briefly summarize the previous analyses: When considering EPA and AA as percentages of total amount fatty acids, we find approximately the same positive association between %EPA and %AA, irrespective of using the true (measured) values of ALA, or random ALA numbers (with uniform or normal distribution), however sampled within the true concentration range. Increased variability of the random ALA numbers improved the %EPA vs. %AA association. Thus, the positive correlation between %EPA and %AA seems to be dependent on particular distributions of fatty acids, suggested to be an example of Distribution Dependent Correlations.

Percentages of the Sum of ALA, AA, and EPA Only

In the calculations above we used the sum of *all* fatty acids in the denominator when assessing the interplay between relative amounts of EPA, AA, and ALA. It occurred to us that this approach might possibly involve a lot of noise, since many fatty acids probably do not have specific regulatory functions in the current context. In an attempt to possibly "purify" the analyses, below we only include the fatty acids under investigation in the denominator, i.e. ALA, EPA, and AA. Thus, %AA and %EPA in the calculations below are percentages of the sum of AA, EPA, and ALA only, i.e. excluding other fatty acids. We first present the %AA vs. %EPA association with *true values* for *all* of the three variables under investigation. Next we show the same association when the true values of the fatty acids were replaced with RANDOM *numbers*, sampled within the true ranges, and how a change in variability (narrowing or broadening the ALA range) might influence the %AA vs. %EPA scatterplot and correlation coefficient.

Below we use the previous reasoning when studying the relationship between relative amounts of ALA, AA, and EPA, with reference to equations 1): %AA = - %EPA + (100 - %ALA); 2): %ALA = - %AA + (100 - %EPA), and 3) %ALA = - %EPA + (100 - %AA).

How will replacement of the true (measured) values of ALA, EPA, and AA with RANDOM numbers, sampled within the true ranges, influence the association between percentages of EPA and AA?

With true values of all of the 3 fatty acids, there was a significant positive %EPA vs. %AA association (Figure 4, left panel); Spearman's rho =0.777, p<0.001, n=163. In contrast, relative amounts of ALA and AA (EPA) correlated negatively: %ALA vs. %AA (EPA) rho = - 0.908(-0.966); eq. of the regression line for %AA vs. %EPA=1.32 (0.08) + 7.25(1.59).

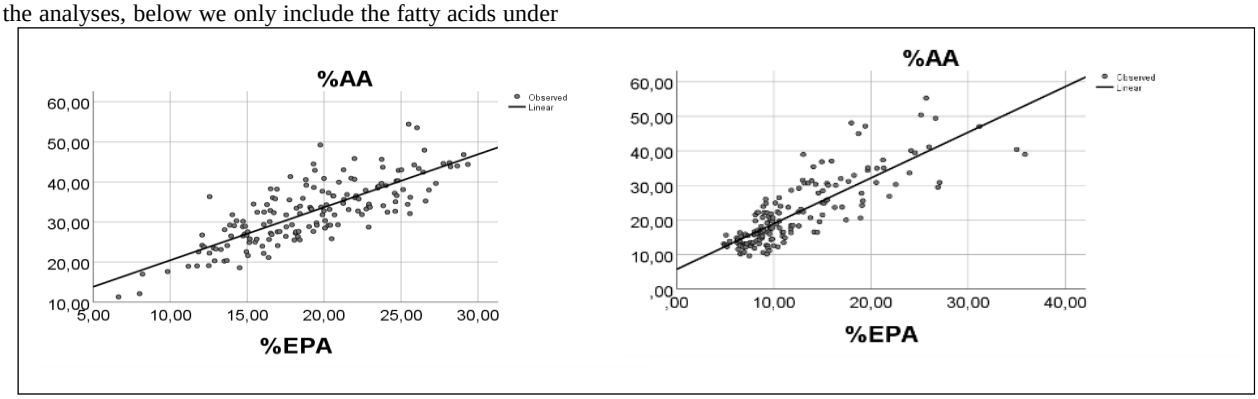

Figure 4. Scatterplot showing the association between %EPA and %AA. *Left panel:* the *true*, measured values of EPA, AA, and ALA were used. Percentages were calculated from the sum of EPA, AA, and ALA only, i.e. excluding all other fatty acids, see text. % AA vs. % EPA: Spearman's rho = 0.777, p<0.001, n=163. Eq. of the regression line: %AA = 1.32 (0.08) + 7.25(1.59). Cutoff-values for %ALA quartiles were 40.1, 47.6, and 56.4%. Skewness of %EPA, %AA, and %ALA: -0.006, 0.054, and 0.083.
Right panel: RANDOM numbers were used, however sampled within the true ranges for EPA, AA, and ALA. Spearman's rho = 0.826, p<0.001, n = 163. Eq. of the regression line: %AA= 1.32 (0.07)*%EPA + 5.73 (0.98). %ALA-quartiles: 56.5, *70.0, and 77.3.* Skewness of %EPA, %AA, and %ALA: 1.47; 1.15, and -1.159, respectively.

Cutoff-values for %ALA quartiles: *40.1, 47.6, and 56.4%.* Skewness of %EPA, %AA, and %ALA were *-0.006, 0.054, and 0.083*, respectively. Corresponding skewness values of the *absolute* amounts (g/kg) were -0.159, 0.657, and 2.502; i.e. skewness of %ALA distribution had moved to the *left* compared with skewness of absolute ALA values (Figure 5).

In contrast to this, distribution of %EPA had moved to the *right* compared with that of EPA (histogram not presented). Thus, there was a similarity between skewness movements observed with true and random values of the fatty acids (further commented below

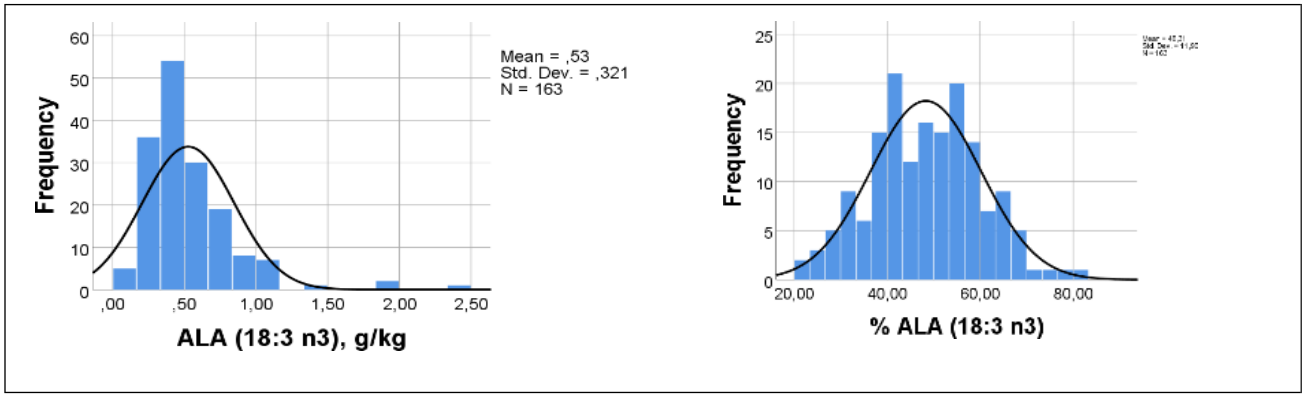

Figure 5. Histogram of absolute and percentage values of ALA (computed from EPA + AA + ALA), n=163. Skewness of ALA: 2.502, and of %ALA: 0.083.

We next replaced the true (measured) values of EPA, AA, and ALA with uniform RANDOM numbers for the 3 fatty acids (Figure 4, right panel), however sampled within their physiological ranges (i.e. for EPA 0.13 - 0.24; for AA 0.25 – 0.42; and for ALA 0.12 – 2.40). Spearman's rho for the %AA vs. %EPA association was 0.826, p<0.001, n = 163; eq. of the regression line: %AA= 1.32 (0.07)*%EPA + 5.73 (0.98). Thus, the regression lines with true and random values did not differ much. With random numbers, there was a negatively skewed distribution of %ALA (i.e. with a tail to the left), and positively skewed histograms of %EPA and %AA. Skewness of %EPA, %AA, and %ALA were 1.473, 1.147, and -1.159, respectively. %ALA quartiles were 56.5, 70.0, and 77.3%, respectively. As explained earlier [19, 21], the observed skewness may be attributed to the combination of variables (EPA and AA) having low numbers/low-variability relative to ALA [20].

Using an algebraic approach to understand how ALA-variability might influence the association between percentages of EPA and AA

When considering the equation %AA = -%EPA + (100 - %ALA), the actual values of the fatty acids do not seem to justify a nullification of the expression (100 - %ALA). This approximation would have been necessary to approach a condition where %AA = %EPA, required to obtain a positive association between %AA and %EPA. Neither is it justified to approximate the equation to

%AA = -%EPA + 100, necessary to obtain a negative relationship between %EPA and %AA. However, we might expect to find positive (negative) correlations also around these conditions. This reasoning raises the question of where a negative (positive) correlation between hypothetical values for %AA and %EPA turns to become (positive (negative), i.e. when passing through the previously suggested Turning Point between positive and negative correlations [19,23]. To test this hypothesis, and possibly finding the Turning Point, below we show results of experiments where the range of ALA was gradually altered by using hypothetical ALA ranges. Somewhere in-between ALA (%ALA) values giving the positive or negative %EPA vs. %AA correlations, we might expect to find the Turning Point. We accordingly decreased the ALA range stepwise (keeping the true ranges for EPA and AA). The following 12 hypothetical ALA-ranges were used: 0.1 – 10.0; 0.1 – 5.0; 0.1 – 3; 0.1 – 2.0; 0.1 – 1.5; 0.1 – 1.0; 0.1 – 0.5; 0.1 – 0.4; 0.1 – 0. 3, 0.1 – 0. 2; 0.1- 0.15 1, 5; 0.1 – 0.11. The corresponding values of Spearman's rho for %EPA vs. %AA were: 0.921/ 0.891/0.879/0.779/0.802/0.679/ 0.330/ 0.142 (p=0.071)/-0.207(p=0.008)/ -0.716/-0.847/-0.903. These values show that the positive association between percentages of EPA and AA is gradually attenuated as the ALA variability (expressed as maximum divided by minimum value) decreases, and collapses when ALA variability is approximately 4.0 (Figure 6). When further decreasing variability of ALA (by narrowing the ALA range), the positive correlation between %AA and %EPA turns to become negative. Furthermore, the negative association between percentages of EPA and AA is rapidly improved in response to further narrowing of the C range (to 0.10 – 0.11, Figure 6). Alternatively, we may say that a strong negative association between percentages of EPA and AA, observed when the hypothetical ALA variability is very low, is rapidly attenuated by slightly increasing variability; the negative association between %EPA and %AA turns to become positive by further increasing ALA variability. Interestingly, when ALA variability reaches the upper end of the physiological ALA range (i.e. 2.4 g/kg), then the correlation between %EPA and %AA is approaching its maximal value (Figure 6).

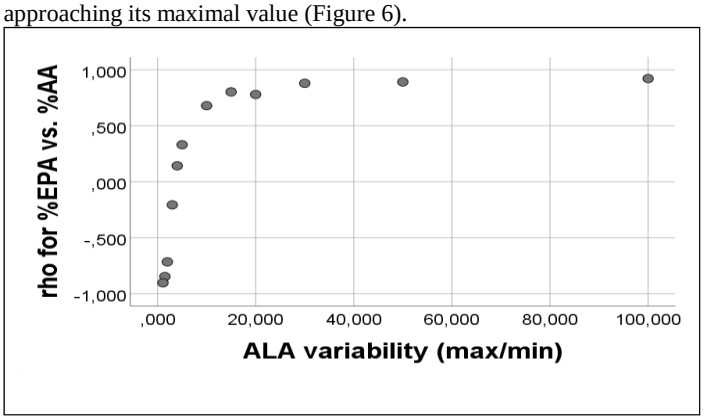

Figure 6. Scatterplot showing the association between ALA variability (expressed as the maximum value divided by the minimum value) and Spearman's rho for the association between %EPA and %AA. The figure relates to the eq. % EPA + %AA + %ALA =100, or %AA = - %EPA + (100 - %ALA), see text. For all points on the figure, we generated 163 RANDOM numbers with uniform distribution, sampled within the true (measured) concentration range, i.e. for EPA 0.13 – 0.24 g/kg, and for AA 0.25 – 0.42 g/kg. For ALA we used the following 12 *hypothetical* ranges: 0.1 – 10.0; 0.1 – 5.0; 0.1 – 3; 0.1 – 2.0; 0.1 – 1.5; 0.1 – 1.0, 0.1 – 0.5; 0.1 – 0.4; 0.1 – 0. 3, 0.1 – 0. 2; 0.1- 0.15 1, 5; and 0.1 – 0.11. p<0.001 for all correlation coefficients, except for those close to the *Turning Point* between positive and negative rho – values.

From this experiment, it would appear that the Turning Point is achieved when ALA range is between 0.1 – 0.4 and 0.1 – 0.3 g/kg. These results with hypothetical random numbers for ALA raises the question of whether true ALA levels may attain so low levels that the positive association between relative amounts of EPA and AA becomes seriously disturbed, eventually leading to a negative relationship between %EPA and %AA. If so, we might possibly expect metabolic disturbances related to an imbalance between eicosanoids derived from AA and EPA. We do not know, however, whether such conditions do exist. In this context we should recall that no major influences upon the %EPA vs. %AA association was observed with low ALA levels, when all of the measured fatty acids were included in the denominator. In any instance, the above calculations illustrate a potentially strong effect of ALA variability upon the relationship between relative amounts of EPA and AA.

How will a change in ALA variability influence quartiles of %ALA, and the correlation between %EPA and %AA?

With reference to the equation %AA = -%EPA + (100 - %ALA), we might expect an attenuation of a hypothetical negative %AA vs. %EPA association, in response to increasing values of %ALA, since the expression (100 - %ALA) in that case would move towards increasingly smaller values. Conversely, decreasing %ALA levels should move the correlation towards negative correlations between percentages of AA and EPA, since the above equation then would move towards %AA = -%EPA + 100. Increased (decreased) %ALA values are obtained by hypothetically increasing (decreasing) the ALA range/variability.

ALA Variability and Quartiles of %ALA

In line with the above reasoning, increased (decreased) ALA variability was indeed accompanied by a movement of the %ALA distribution towards higher (lower) values, as judged by Q1, Q2, and Q3 values of the %ALA distribution (Figure 7). Q1, Q2, and Q3 of %ALA in the 12 conditions presented above were: 82.8/90.1/93.2; 73.1/83.7/88.4; 61.2/74.0/81.2;54.1/70.3/75.3;46.7/58.3/67.8;33.5/47.8/57.5;26.6/37.5/44.0;27.4/34.2/39.8;23.1/28.7/34.3;19.4/21.7/24.8;17.4/19.6/21.4;15.6/16.7/17.9.

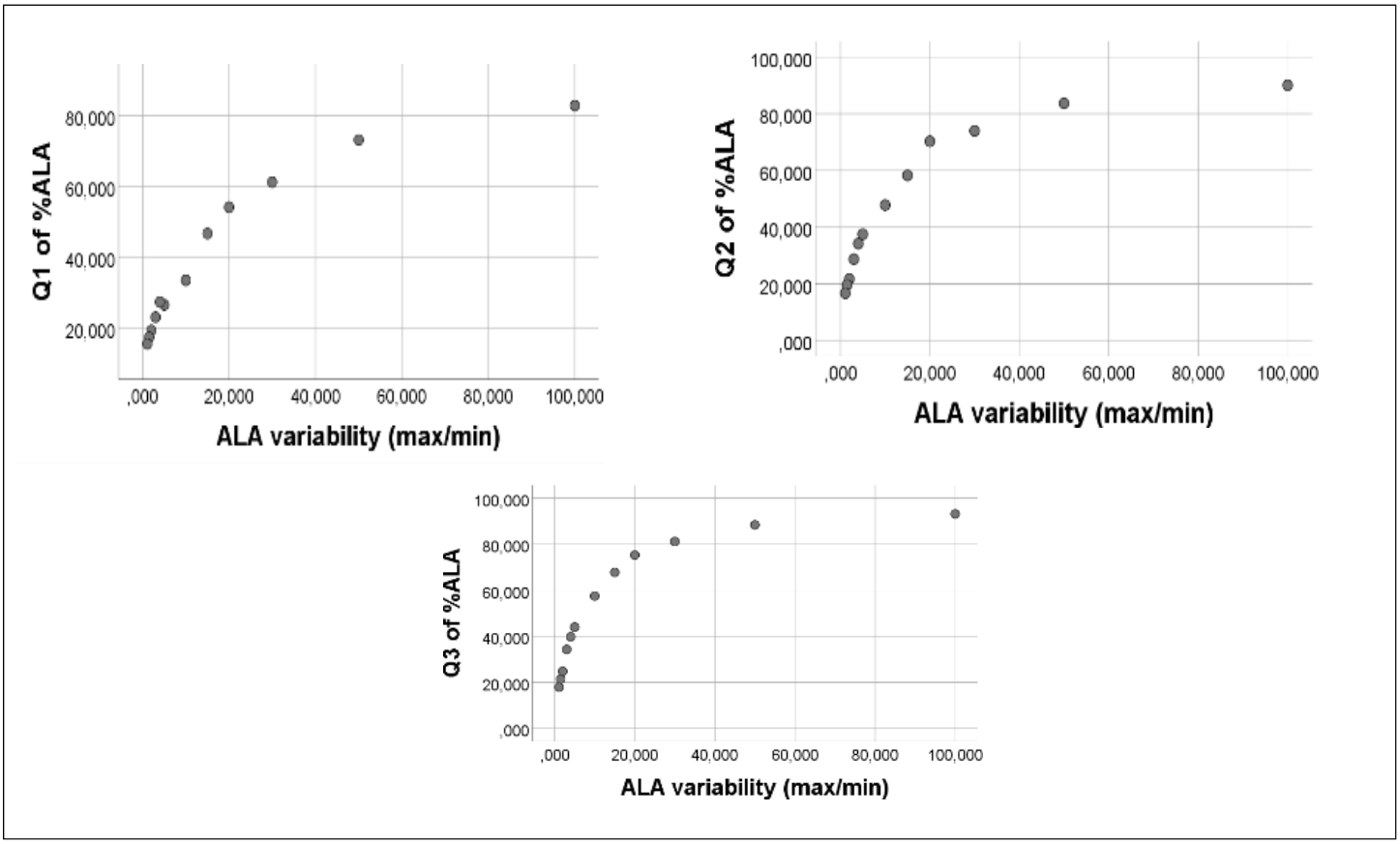

Figure 7. Scatterplot showing the association between ALA variability (expressed as max/min) and Q1 (upper panel), Q2 (middle panel), and Q3 (lower panel) of the %ALA distribution; the figure relates to the eq.
% EPA + %AA + %ALA =100, or %AA = - %EPA + (100 - %ALA), see text. For all points on the figure, we generated 163 RANDOM numbers with uniform distribution, sampled within the true (measured) concentration range, i.e. for EPA 0.13 – 0.24 g/kg, and for AA 0.25 – 0.42 g/kg. For ALA we used the following 12 *hypothetical* ranges: 0.1 – 10.0; 0.1 – 5.0; 0.1 – 3; 0.1 – 2.0; 0.1 – 1.5; 0.1 – 1.0, 0.1 – 0.5; 0.1 – 0.4; 0.1 – 0. 3, 0.1 – 0. 2; 0.1- 0.15 1, 5; and 0.1 – 0.11.

These results seem to substantiate the suggested movements of the %ALA distribution in response to altering ALA variability.

%ALA quartiles and correlation between %AA and %EPA

Above we argued that the equation %AA = -%EPA + (100 - %ALA) may possibly be used to predict whether percentages of EPA and AA will be positively or negatively correlated. We showed that increase (decrease) in ALA variability seemed to move the %ALA distribution towards higher (lower) values, thereby probably favoring (making poorer) a positive %AA vs. %AA relationship.
To experimentally test this hypothesis, we studied the association between quartiles of the above hypothetical %ALA values and Spearman's rho for %AA vs. %EPA. As anticipated, we observed (Figure 8) that moving

%ALA quartiles towards higher values was accompanied by gradually attenuating negative rho – values; the rho – values then turned to become increasingly positive, by continuing to move the quartiles towards higher values (as effected by increasing the ALA variability). The Turning Point between positive and negative %EPA vs. %AA correlations was attained when the 1st, 2nd, and 3rd quartiles of the %ALA distribution were approximately 28, 30, and 38%, respectively (Figure 8).

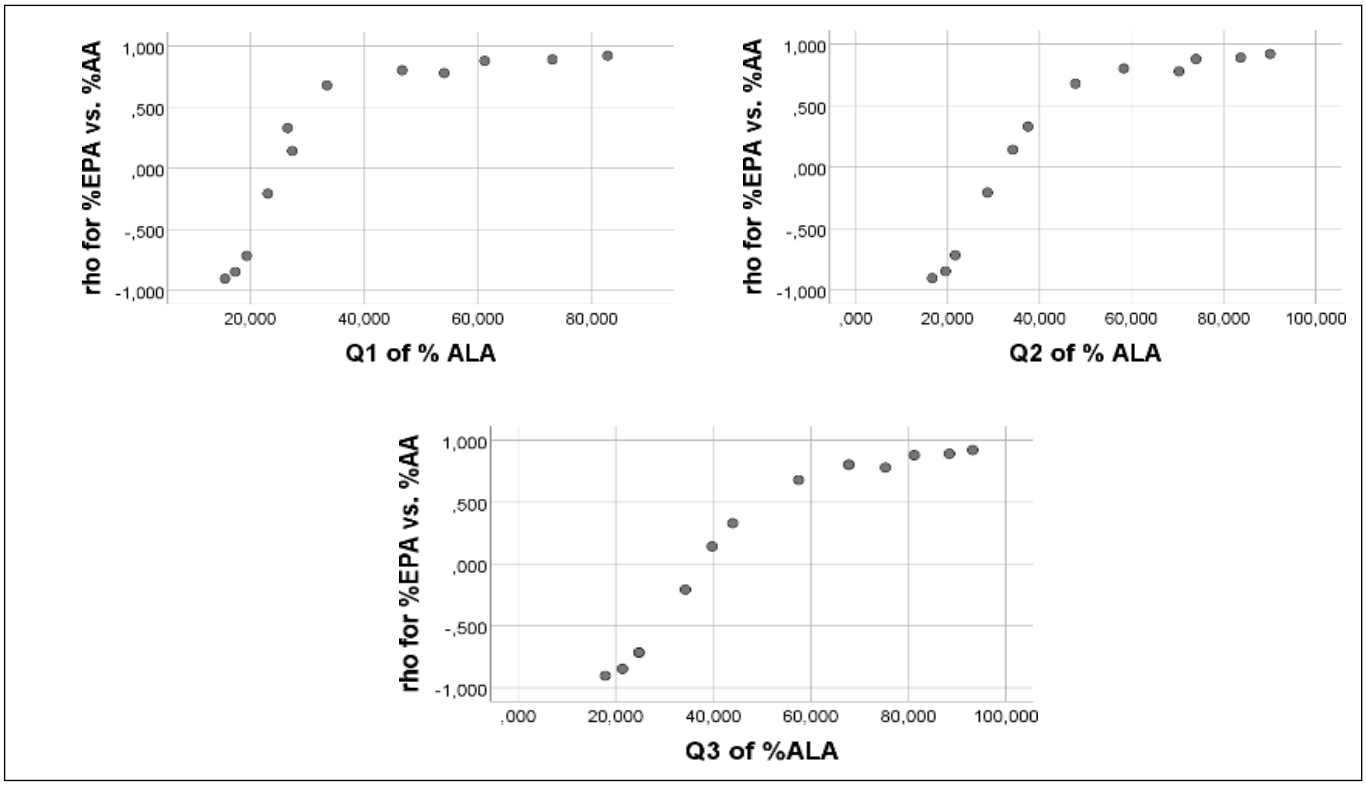

Figure 8. Scatterplot showing the association between Q1 (upper panel, left), Q2 (upper panel, right), and Q3 (lower panel) of the % ALA distribution and corresponding Spearman's rho values for %AA vs. %EPA; the figure relates to the eq. % EPA + %AA + %ALA =100, or %AA = - %EPA + (100 - %ALA), see text. For all points on the figure, we generated 163 RANDOM numbers with uniform distribution, sampled within the true (measured) concentration range, i.e. for EPA 0.13 – 0.24 g/kg, and for AA 0.25 – 0.42 g/kg. For ALA we used the following 12 *hypothetical* ranges: 0.1 – 10.0; 0.1 – 5.0; 0.1 – 3; 0.1 – 2.0; 0.1 – 1.5; 0.1 – 1.0, 0.1 – 0.5; 0.1 – 0.4; 0.1 – 0. 3, 0.1 – 0. 2; 0.1- 0.155; and 0.1 – 0.11. $p<0.001$ for all correlation coefficients, except for those close to the *Turning Point* between positive and negative rho – values.

These results show that we (*hypothetically)* may have strong positive or negative correlations between percentages of EPA and AA even far from the "mathematically ideal" conditions, with reference to the equation above. To briefly summarize the preceding paragraphs: Increased range/variability of ALA will cause a movement of the %ALA distribution towards higher values and thereby strongly influence the association between percentages of EPA and AA. We hypothesize that this sensitive mathematical phenomenon is possibly an evolutionary regulatory mechanism.

Using skewness of the %ALA distribution to understand correlation between %EPA and %AA

Previously [21], we suggested that skewness of relative amounts of variables was encountered with variables having different ranges, and skewness increased with increasing difference between ranges. We furthermore hypothesized that, with 2 low-number/narrow-range variables (A, B) relative to a third variable (C), we might expect (the arrow means "leads to"):

1) High C variability → High skewness of %C (A, B) → Strong %A vs. %B correlation.

2) Low C variability → Low skewness of %C (A, B) →Poor %A vs. %B correlation.

ALA has high variability (CV = 60.4%) relative to EPA and AA (CV about 10%). If the above suggestion is correct, we would expect 1) a relationship between ALA variability and skewness of %ALA, and 2) a relationship between skewness of %ALA and correlation between %EPA and %AA. Thus, skewness of %ALA might possibly serve as an intermediate between ALA range and %EPA vs. %AA correlation. Above we showed the association between ALA variability and correlation between %EPA and %AA. Below we investigate the association between 1) ALA variability and skewness of %ALA, and 2) skewness of %ALA and correlation between %EPA and %AA.

ALA variability and skewness of the %ALA distribution

In response to increasing the ALA variability we observed increasing negative skewness of %ALA (Figure 9, upper panel, left), and increasing positive skewness of %EPA and %AA. (Figure 9, upper panel, right; lower panel).

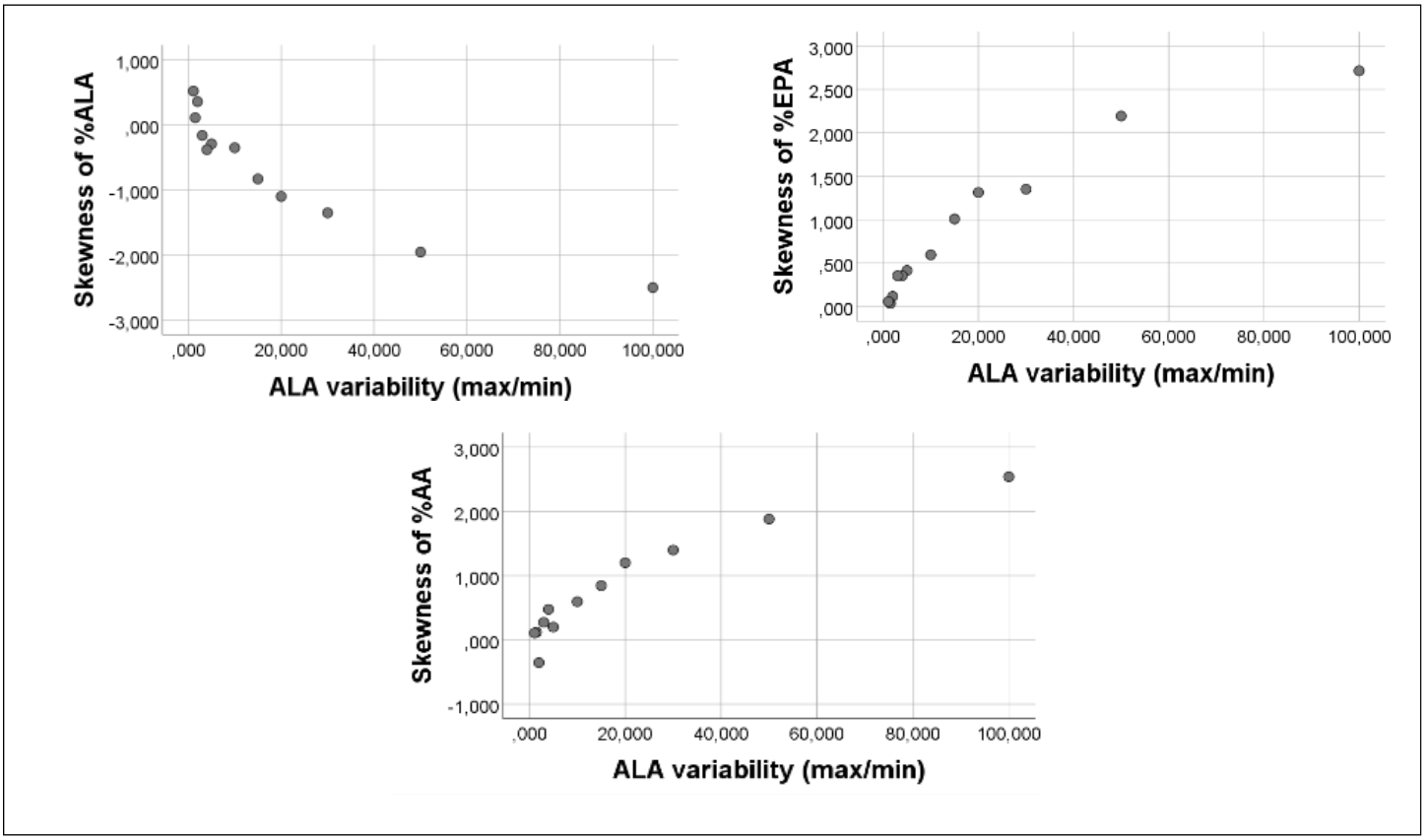

Figure 9. Scatterplots showing the association between ALA variability and skewness of the %ALA distribution (upper panel, left), %EPA distribution (upper panel, right) and %AA distribution (lower panel); the figure relates to the eq. % EPA + %AA + %ALA =100, or %AA = - %EPA + (100 - %ALA), see text. For each of the 12 points on the figure, we generated 163 RANDOM numbers with uniform distribution, sampled within the true (measured) concentration range, i.e. for EPA 0.13 – 0.24 g/kg, and for AA 0.25 – 0.42 g/kg. For ALA we used the following 12 *hypothetical* ranges: 0.1 – 10.0; 0.1 – 5.0; 0.1 – 3; 0.1 – 2.0; 0.1 – 1.5; 0.1 – 1.0, 0.1 – 0.5; 0.1 – 0.4; 0.1 – 0. 3, 0.1 – 0. 2; 0.1- 0.155; and 0.1 – 0.11.

Skewness of the %ALA distribution and correlation between %AA and %EPA

We previously reported that *skewness of distributions* might be involved in some correlations between percentages of 3 scale variables in general. [21]. We point out again that skewness of relative amounts of variables is encountered with variables having different ranges, and skewness increases with increasing differences between ranges of the actual variables [19, 21]. Computer experiments with random numbers seem to substantiate the hypothesis. We computed skewness of % ALA in response to the 12 ALA ranges shown above, however keeping the true ranges for EPA and AA. The following skewness values for % EPA / % AA / % ALA were observed: 2.714/2.540/ -2.500; 2.191/1.882/ -1.955; 1.351/1.399/1.352;1.131/1.201/1.101;1.008/0.845/0.833;0.595/0.453/0.353;0.415/0.199/-0.294; 0.355/0.475/-0.383; 0.355/0.273/-0.165; 0.117/-0.320/0.354; 0.035/0.121/0.137; 0.058/0.107/0.516. The association between skewness of the % ALA distribution and Spearman's rho for the correlation between percentages of AA and EPA is shown in Figure 10; as observed previously [21], the relationship seemed like a mirror image of a sigmoidal scatter of points. With increasing *negative skewness* of %ALA we observed a progressive improvement towards *positive correlations* between percentages of AA and EPA. With *decreasing* negative skewness of %ALA, eventually reaching the positive side, the positive correlation between %EPA and %AA was increasingly attenuated and the correlation moved towards the negative side (Figure 10).

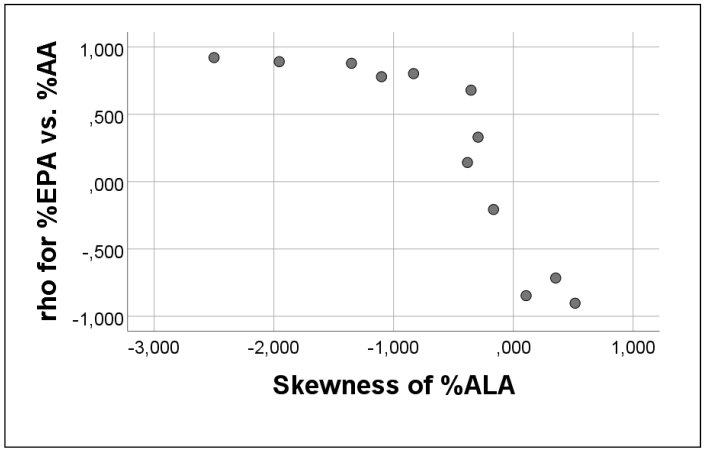

Figure 10. Scatterplot showing the association between skewness of the %ALA distribution and Spearman's rho for %AA vs. %EPA; the figure relates to the eq. % EPA + %AA + %ALA =100, or %AA = - %EPA + (100 - %ALA), see text. For each of the 12 points on the figure, we generated 163 RANDOM numbers with uniform distribution, sampled within the true (measured) concentration range, i.e. for EPA 0.13 – 0.24 g/kg, and for AA 0.25 – 0.42 g/kg. For ALA we used the following 12 hypothetical ranges: 0.1 – 10.0; 0.1 – 5.0; 0.1 – 3; 0.1 – 2.0; 0.1 – 1.5; 0.1 – 1.0, 0.1 – 0.5; 0.1 – 0.4; 0.1 – 0. 3, 0.1 – 0. 2; 0.1- 0.155; and 0.1 – 0.11. p<0.001 for all correlation coefficients, except for those close to the Turning Point, i.e. where rho turns from being positive to becoming negative.

Turning Point

With 3 positive scale variables (A, B, C) , we previously suggested that there should be a *Turning Point* where a positive (negative) correlation between percentages of A and B turns to become negative (positive), in response to varying the range of C [19, 21, 23]. Furthermore, the Turning Point was observed when skewness of %C approached zero. In accordance with this previous observation, also in the current experiment we observed a Turning Point when skewness of %ALA was approaching zero (i.e. symmetrical %ALA distribution). Thus, high negative (positive) skewness values of %ALA are associated with high positive (negative) rho values for %AA vs. %EPA.

It would appear, accordingly, that when skewness of the %ALA distribution approaches zero (symmetrical histogram), then rho **(%AA vs. %EPA)** varies greatly in response to minor changes in skewness of %C. Thus, close to a symmetrical distribution of the histogram of %ALA, the correlation between percentages of AA and EPA is very sensitive to changes in skewness of %ALA. On the other hand, with very high (positive or negative) skewness of the %C distribution, only small changes in the size of Spearman's rho for the %AA vs. %EPA correlation is allowed. Thus, skewness of the %ALA distribution may seem to partly explain the correlation between percentages of AA and EPA. However, when the %ALA histogram is close to become symmetrical there is appreciable alterations in rho for the %AA vs. %EPA correlation, in response to even minor changes in %ALA skewness. This finding would make skewness of %ALA a poor predictor of the strength of correlation between percentages of AA and EPA. Nevertheless, these and our previous experiments [12, 23] seem to suggest that skewness of the %ALA distribution, as well as the equation %AA = -%EPA + (100 - %ALA), might be used when trying to predict whether correlations between percentages of AA and EPA will be positive or negative, and also whether we might expect associations to be strong or weak.

It is not surprising that percentages of fatty acids may be correlated, since they are all computed from the same sum. Indeed, in 1897 Karl Pearson [24] reported that there will be a spurious correlation between two indexes with the same denominator, even if the variables used to produce the indexes are selected at random with no correlation between them. Our results show that significant correlations (positive and negative) between percentages of the same sum can indeed be obtained, but not always, and add that *range* of the variables is essential for the outcome. In our opinion, such correlations may serve as a novel regulatory mechanism in biology, rather than being "spurious correlations".

We previously observed a positive association between percentages of EPA and AA [16-18], and an inverse relationship between percentages of AA and OA [13-15]. The present finding of a positive correlation between %EPA and %AA both when using the real values of the fatty acids and with random numbers (sampled within the true concentration ranges) strongly suggest that *range* might be the real target for biological regulation. This conclusion seems to apply for the modifying influence of ALA as well. Thus, evolution may have "chosen" specific concentration ranges for each of the many fatty acids to ensure that the relative amounts of some of them must be positively correlated whereas others are negatively associated. Biochemical mechanisms behind the particular concentration ranges could, in general, involve regulation of the synthesis of enzymes catalyzing metabolism of fatty acids, allosteric regulation of enzyme activities, and interconversion between phosphorylated and dephosphorylated forms of key enzymes. Whatever the mechanisms might be, the examples in this work suggest that correlation between percentages of EPA and AA, and the modifying influence of ALA, might be predicted using the equation %AA= - %EPA + (100 – %ALA), and by considering skewness of the %ALA distribution.

Limitations of the Study

Since this work was confined to studying the association between percentages AA, and EPA, as modified by ALA, we do not know to what extent the suggested phenomenon of Distribution dependent correlations/-regulation is valid for other fatty acids as well. Furthermore, the analyses was based upon the fatty acid pattern in breast muscle lipids of chickens and we do not know the generalizability of our results, as related to different organs, tissues or compartments, and to various species, including man. Thus, the ALA influence on the association between %AA and %EPA may be completely different in man and various species. Future work in this field should include studies to explore whether the fatty acid distribution might also govern the association between percentages of other fatty acids. To investigate whether our findings have a more general validity, comparable studies should be done in other animals and in humans as well.

Conclusion

The present analyses show that the concentration range (distribution, variability) per se of ALA, EPA, and AA, including where on the scale they are placed, will determine (possibly via skewness of the %ALA distribution) whether percentages of the fatty acids will be correlated. High tissue ALA (likely to be diet related) might improve the positive %AA vs. %EPA association. We suggest that Distribution Dependent Correlations/ -Regulation may be an evolutionary regulatory principle, being a mathematical consequence of type-specific distributions of variables (like fatty acids). The present analyses suggest that high ALA variability may improve the positive association between relative amounts of AA and EPA. We do not know whether a disturbance in this type of regulation could be linked to the risk of AA related conditions and diseases.

Acknowledgements

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