BLOOD MARKERS OF THE QUALITY OF FAT IN THE DIET AND INCIDENCE OF ISCHAEMIC STROKE IN A MEDITERRANEAN POPULATION

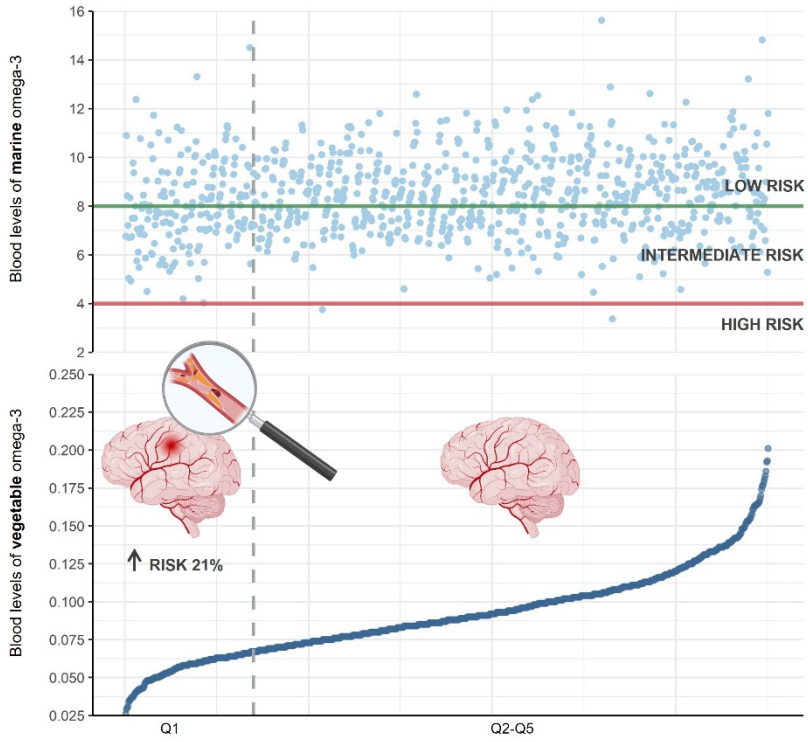
Dr Aleix Sala Vila

Barcelona Beta Brain Research Center

1. Summary of the project

One of the most studied dietary elements in relation to stroke is the long-chain omega-3 fatty acids, which are supplied by fatty fish (sardine, anchovy, salmon, tuna etc.). However, marine resources are limited; this, coupled to concerns related to marine pollutants, and the increasing relevance of vegetarianism and veganism have put the spotlight into the vegetable omega-3 fatty acids such as alpha-linolenic acid (ALA).Until recently it was commonly thought that this type of omega-3 fatty acid, found in walnuts and soy, provided a modest benefit on brain health, and always lower than that from marine omega-3 fatty acids. For this reason, brain benefits of ALA were only expected in populations with a low intake of fatty fish. Spain is a country where customarily fatty fish consumption is still high (especially in elderly population).Therefore, it is an ideal setting to explore whether marine and vegetable omega-3 can work synergistically reducing the risk of ischemic stroke. We have used data and samples from EPIC-SPAIN cohort, which includes more than 40,000 healthy individuals with a follow-up of several years (in some cases even decades).Among these 40,000 participants we selected 438 individuals (named cases) who had an ischemic stroke during follow-up period. We identified a matched control for each case. The matching criteria were sex, age at enrolment, centre and date of blood sampling. Thus, we had 438 control-case pairs. One novelty of our study relays on the fact that we did not ask for dietary habits, as other studies still do, because this can generate imprecise and inaccurate data. We decided to determine the levels of omega-3 (marine and vegetable) in blood samples (erythrocyte membranes) from the participants at the time of enrolment. This gave us an objective and unbiased idea of the regular intake of these omega-3 fatty acids by the participants. The two main findings are shown in the *Graphical abstract*. First, we proved that the studied population consumed large amounts of fatty fish and, therefore, it was highly protected by marine omega-3 fatty acids. It is well established that marine omega-3 fatty acids confer a maximum level of protection when their erythrocyte levels are above 8% (low risk, green line). On the contrary, when their levels are below 4%, there is a state of vulnerability (high risk, red line). By carefully looking at marine omega-3 blood levels (sky-blue dots, upper part of the graph) we observed that almost all participants were located in the intermediate- and low-risk zone, with only a few below the 4% line. The second, and more important, finding relates to vegetable omega-3 fatty acids (blue dots, lower part of the graph). Although participants with lowest levels of ALA (Q1, to the left of the

grey dashed line) had remarkable levels of marine omega-3 fatty acids (in many cases greater than 8%), they also had a 21% increased risk of suffering an ischemic stroke than participants who consumed more ALA (Q2-Q5, to the right of the grey line).



2. Results

The project has been translated in a scientific article. Next, we present the findings that constitute the backbone of this article.

First, we looked at the distribution of omega-3 fatty acid levels in the population (n=876, distributed between 438 cases and 438 matched controls). As we have previously mentioned, the population studied has a high intake of marine omega-3 fatty acids (translated into a large accumulation of these molecules in the erythrocyte membranes). It should be noted that more than half of the population in our study (59%) had an omega-3 index (defined as the sum of the percentages of the two most abundant marine fatty acids) greater than 8% (green stripe, Figure 1), which is a threshold considered to be "protective against cardiovascular risk". In contrast, only two participants (0.2% of the total) have an omega-3 index below 4% (red band),

considered "high risk". The rest of the population (41%) is located between 4 and 8%, meaning "intermediate risk". Therefore, we reinforce the idea that if there is a beneficial association of vegetable omega-3 fatty acids, this will be despite the protection (already very high) conferred by marine omega-3.

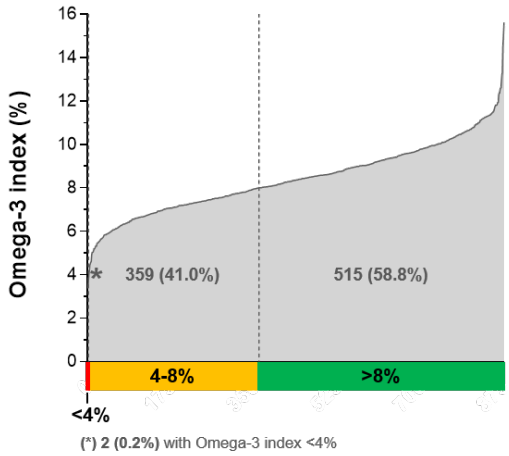


Figure 1 – Distribution of omega-3 index (sum of the percentages in erythrocyte membranes of the two most abundant marine fatty acids) in the studied population.

Next, we focus on exploring the distribution of ALA levels in erythrocytes in the population of our study. In the following figure we can see the percentages of ALA with respect to the total of fatty acids quantified in the membranes of the erythrocytes of our population. The red horizontal lines indicate the cut-off points that determine the division of our population into quintiles (Q1=0-20%; Q2=20-40%; Q3=40-60%; Q4=60-80%; Q5=80-100%).

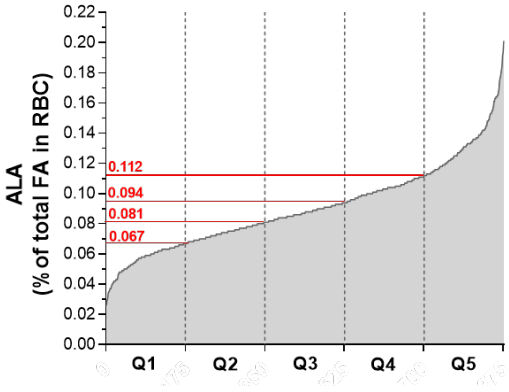


Figure 2 – Distribution of ALA proportion (alpha-linolenic acid – the main vegetable omega-3 fatty acid) in erythrocyte membranes in the studied population.

Table 1 – Distribution of the demographic, clinical and lifestyle variables of the study population, divided according to the quintiles of the proportion of linolenic acid (ALA) in the erythrocyte membrane.

Variable	Quintiles of ALA					P
	Q1 (<0.067%; median, 0.059%) n = 175	**Q2 (0.067 to 0.081%; median, 0.074%) n = 175**	**Q3 (0.081 to 0.094%; median, 0.088%) n = 176**	**Q4 (0.094 to 0.112%; median, 0.103%) n = 175**	**Q5 (>0.112 %; median, 0.103%) n = 175**	
Age, years	53.8 ± 7.4	54.8 ± 7.4	55.7 ± 6.9	56.0 ± 6.9	55.8 ± 7.1	0.026
Female, N (%)	81 (46.3)	71 (40.6)	84 (47.7)	85 (48.6)	89 (50.9)	0.382
Center						<0.001
Granada, N (%)	20 (11.4)	30 (17.1)	17 (9.7)	27 (13.7)	23 (13.1)	-
Murcia, N (%)	39 (22.3)	43 (24.6)	65 (36.9)	64 (36.6)	79 (45.1)	-
Navarra, N (%)	116 (66.3)	102 (58.3)	94 (53.4)	87 (49.7)	73 (41.7)	-
Body mass index, kg/m^2	29.6 ± 4.4	29.5 ± 3.6	29.2 ± 3.9	29.1 ± 3.9	29.2 ± 4.1	0.628
Diabetes, N (%)	14 (8.0)	15 (8.6)	15 (8.5)	24 (13.7)	21 (12.0)	0.291
Hypertension, N (%)	64 (36.6)	51 (29.1)	58 (33.0)	72 (41.1)	73 (41.7)	0.068
Smoking status						0.011
Never, N (%)	88 (50.3)	91 (52.0)	96 (54.5)	107 (61.1)	108 (61.7)	-
Current, N (%)	22 (12.6)	34 (19.4)	35 (19.9)	26 (14.9)	34 (19.4)	-
Former, N (%)	65 (37.1)	50 (28.6)	45 (25.6)	42 (24.0)	33 (18.9)	-
Dietary data - consumption						
Fruit and vegetables, g/d	597.4 ± 283.1	597.5 ± 339.0	612.8 ± 270.5	631.0 ± 280.4	639.5 ± 306.2	0.276
Red meat, g/d	50.6 ± 41.64	49.9 ± 39.8	38.7 ± 32.7	38.1 ± 38.2	33.5 ± 33.0	<0.001
Dairy products, g/d	256.1 ± 166.8	249.8 ± 149.6	227.9 ± 161.8	281.3 ± 178.8	273.1 ± 167.7	0.039
Fiber, g/d	25.6 ± 8.0	25.6 ± 9.2	25.7 ± 8.5	26.2 ± 8.6	26.4 ± 9.5	0.868
Erythrocyte marine omega-3s						
EPA, %	0.80 ± 0.35	0.87 ± 0.31	0.93 ± 0.36	0.99 ± 0.42	1.05 ± 0.41	<0.001
DHA, %	7.32 ± 1.46	7.54 ± 1.19	7.45 ± 1.23	7.59 ± 1.41	7.53 ± 1.37	0.360
Omega-3 index, % *	8.12 ± 1.74	8.41 ± 1.42	8.38 ± 1.49	8.59 ± 1.73	8.58 ± 1.69	0.044

P valued obtained by the chi-square test (categorical variables) or by 1-way ANOVA (continuous variables; those skewed were rank-transformed prior to ANOVA).

EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

* Sum of the proportions of EPA and DHA

Next, we investigated the associated risk of ischemic stroke for each of the ALA quintiles, establishing the first quintile as a reference. To do this, we developed Cox regression models, where the dependent variable was ischemic stroke (stroke/control), and the independent variable was the erythrocyte ALA quintile. Different models were created: Model 1 does not include adjustments for other variables. Model 2 adjusts for case-control matching variables (i.e., recruitment centre [Navarra, Murcia, Granada], sex [male, female], and age at enrolment [years]). In Model 3, variables that confer cardiovascular risk are also included, such as body mass index (kg/m^2), baseline diagnosis of diabetes (yes, no), baseline diagnosis of hypertension (yes, no), smoking status at baseline (never smoker, current smoker, former smoker). In Model 4, dietary variables are also included (fruit and vegetables, red meat, dairy products and derivatives, fibre), the consumption of which can increase or reduce the risk of ischemic stroke. Finally, Model 5 includes the omega-3 index. The data is represented in the following table.

Table 2 – Risk of incident ischemic stroke (*Hazard Ratio,* 95% confidence Interval) for the quintiles of alpha-linolenic acid (ALA) proportion in erythrocyte membranes.

	Quintiles of ALA					
	Q1 n = 175	**Q2** n = 175	**Q3** n = 176	**Q4** n = 175	**Q5** n = 175	**P**
Cases of stroke	104	80	85	77	92	
Model 1	1.00 (Ref)	0.74 (0.55; 0.98)*	0.76 (0.57; 1.02)	0.73 (0.55; 0.98)*	0.95 (0.72; 1.26)	0.081
Model 2	1.00 (Ref)	0.69 (0.51; 0.92)*	0.73 (0.54; 0.97)*	0.67 (0.50; 0.90)*	0.85 (0.64; 1.13)	0.036
Model 3	1.00 (Ref)	0.76 (0.57; 1.02)	0.78 (0.58; 1.04)	0.70 (0.52; 0.95)*	0.92 (0.69; 1.24)	0.106
Model 4	1.00 (Ref)	0.75 (0.56; 1.01)	0.78 (0.58; 1.04)	0.72 (0.53; 0.98)*	0.95 (0.71; 1.28)	0.104
Model 5	1.00 (Ref)	0.75 (0.56; 1.01)	0.78 (0.58; 1.04)	0.72 (0.53; 0.98)*	0.95 (0.71; 1.28)	0.106

From this table, we can extract a couple of comments. First, that compared to the first quintile (Q1-reference), all other quintiles have hazard ratios lower than 1 (and therefore there is a reduction in risk, although only statistically significant in those marked with "*"). The subsequent addition of adjustment variables does not change the observed associations in terms of their meaning. There is a slight attenuation of the strength of the associations as the adjustment variables are incorporated. Second, the behaviour is not homogeneous between quintiles.

This apparent dissonance is due to the fact that, at least for the risk of ischemic stroke in relation to ALA levels in erythrocytes, a linear relationship is not observed. This fact led us to explore other types of non-linear associations. In the following figure we can

see what is known as a "spline", which consists of a graphical representation of how the association behaves in a nonlinear model, including the adjustment variables described for Model 5 in the previous table. The red curve represents the risk of ischemic stroke, while the dashed curves are the 95% confidence intervals. Dashed vertical lines mark the five quintiles. The most interesting finding here is that a cut-off point is observed that separates participants at higher risk from participants at lower risk. This cut-off point, which coincides with the point that determines the bottom quintile (Q1), is represented by a green line.

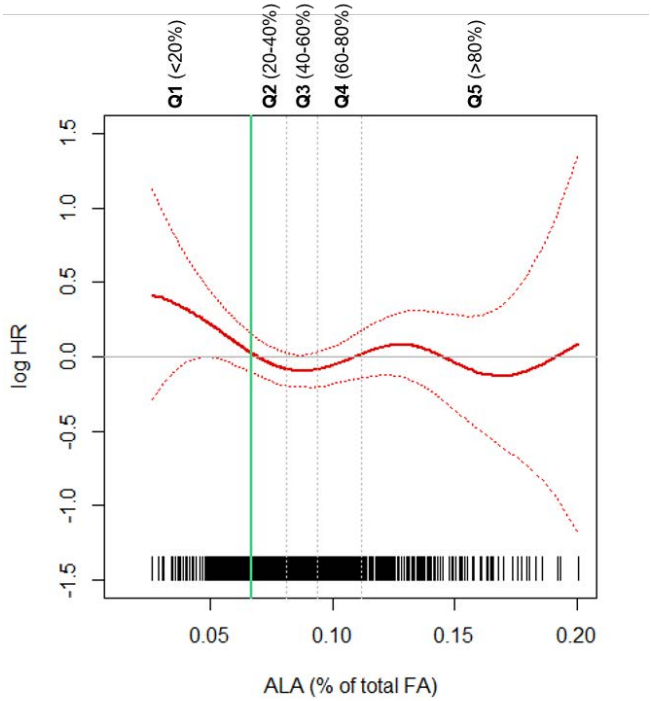


Figure 3 – "Spline" of the distribution of the proportion of ALA (alpha-linolenic acid) in erythrocytes and the prevalence of ischemic stroke in the population studied.

Therefore, this graph indicates that the presence of ischemic stroke is higher in those who are in the lower quintile of ALA in erythrocytes (that is, below 0.067% of the total fatty acids identified) than in the rest of the quintiles (those with a higher erythrocyte ALA). This led us to repeat the Cox regression models, not comparing all five quintiles but instead comparing the bottom quintile (below 0.067% ALA, to the left of the green line) with the other top four quintiles together (starting at 0.067% ALA, to the right of the green line). The results can be seen in the following table.

Table 3 – Risk of incident ischemic stroke (*Hazard Ratio*, 95% confidence interval) for Q2-Q5 quintiles of alpha-linolenic acid (ALA) proportion in erythrocytes.

	Q1 (<0.067%; median, 0.059%) n = 175	**Q2-Q5** (≥0.067%; median, 0.094%) n = 701	P
Cases of stroke	104	334	
Model 1	1.00 (Ref)	0.79 (0.63; 0.99)	0.037
Model 2	1.00 (Ref)	0.73 (0.58; 0.91)	0.036
Model 3	1.00 (Ref)	0.79 (0.63; 0.98)	0.036
Model 4	1.00 (Ref)	0.79 (0.63; 0.99)	0.045
Model 5	1.00 (Ref)	0.79 (0.63; 1.00)	0.046

We can conclude the following: compared to being in the bottom quintile (ALA<0.067%), having higher erythrocyte ALA levels is associated with a 21% reduced risk of ischemic stroke incidence (hazard ratio, 0.79), which is statistically significant in all models.

3. Relevance to potential future implications

We consider that the relevance of the findings is based on two aspects:

· We have identified a cut-off point for the concentration of ALA in blood that discriminates a higher risk of suffering ischemic stroke. This fosters two notions. First, to assess the clinical use that can be made of this cut-off. In other words, should we treat the people who are below it differently at a clinical level? Second, and linked to the concept of "personalized nutrition", there is increasing evidence of populations that can receive more benefits from interventions based on lifestyle. So, in regard to the interventions focused on supplementing with ALA (inclusion of ALA-rich foods in the diet, for instant walnuts or flaxseed oil), does it make sense to keep including any participant, or might it be worth while to identify and include in these studies only the 20% of the population with the lowest blood ALA levels?

· At the mechanistic level, we observed that there may be beneficial associations for ALA (vegetable omega-3 fatty acid) even in a background of abundant marine omega-3, to date the most studied omega-3 in relation to ischemic stroke. Until now, it has

been suggested that high levels of ALA could only be associated with a lower risk of ischemic stroke in those populations that consume little fish and, therefore, have low levels of marine omega-3 (these, being more powerful, would mask any protection that ALA can provide). We refute this belief, showing that vegetable and marine omega-3 are not competitors or antagonists.

4. Scientific bibliography

The project has been translated in a scientific article, which is already written and about to be distributed among the co-authors. It is expected to be sent in late spring 2022, to a specific journal in the field (*"Stroke"*), in the first decile in its category.