

# Vitamin D and $\omega$ -3 Supplementations in Mediterranean Diet During the 1<sup>st</sup> Year of Overt Type 1 Diabetes: A Cohort Study

Current title: Mediterranean diet, Vitamin D<sub>3</sub> and  $\omega$ -3 in 1<sup>st</sup> Year of T1D child

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**Abstract:** Vitamin D and  $\omega$ -3 fatty acids ( $\omega$ -3) co-supplementation potentially improve type 1 diabetes (T1D) by attenuating autoimmunity and counteracting inflammation. This cohort study preliminary to a randomized control trial (RCT) is aimed to evaluate, in a series of T1D children assuming Mediterranean diet and taking cholecalciferol 1000U/day from T1D onset, if  $\omega$ -3 co-supplementation preserves the residual endogen insulin secretion (REIS). Therefore, 22 new onsets of 2017 received  $\omega$ -3 [eicosapentenoic acid (EPA) plus docosahexaenoic acid (DHA), 60mg/kg/day], and were compared retrospectively vs. 37 previous onsets without  $\omega$ -3 supplementation. HbA1c%, daily insulin demand (IU/Kg/day) and IDAA1c, a composite index (calculated as IU/Kg/day $\times$ 4 + HbA1c%) as surrogate of REIS, were evaluated at recruitment (T0) and 12 months later (T12). In the  $\omega$ -3 supplemented group, dietary intakes were evaluated at T0 and T12. As outcome, decreased insulin demand ( $p < 0.01$ ), particularly pre-meal boluses ( $p < 0.01$ ), and IDAA1c ( $p < 0.05$ ), were found in the  $\omega$ -3 group while HbA1c% were not different in the two groups. Diet analysis, at T12 vs. T0, showed that the intake of arachidonic acid (AA) was decreased ( $p < 0.01$ ) in the  $\omega$ -3 supplemented group while other nutrients were unchanged; at T0, the AA intake was inversely correlated with HbA1c% ( $p < 0.05$ ;  $r: 0.411$ ). In conclusion, the results suggest that vitamin D plus  $\omega$ -3 co-supplementation and AA reduction in Mediterranean diet display benefits for T1D children and deserve further investigation.

**Keywords:** cholecalciferol; omega3; EPA; DHA; Arachidonic Acid; AA/EPA ratio; type 1 diabetes; remission period; honeymoon period

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## Background

Nutraceutical is a neologism from "nutrition" and "pharmaceutical" coined in 1989 by Stephen de Felice, to represent the science oriented to nutrient principles contained in foods with beneficial

effects on health. This discipline has its roots in the old past, but it has taken on scientific values in the last fifty years. However, it faces inherent difficulties in the assessment of clinical outcomes for a specific nutrient outside of the whole nutrition[1], which would be relevant for management of specific diseases. Particular nutrients, such as vitamin D and omega-3 polyunsaturated fatty acids ( $\omega$ -3), are of concern in type 1 diabetes (T1D), an autoimmune chronic disease due to progressive selective destruction of pancreatic  $\beta$ -cells producing insulin, beginning in infancy with the appearance of specific autoantibodies, and with clinical outbreak in 90% of cases during the pediatric age. Hyperglycemia is the characteristic sign of the disease and requires a precise administration of insulin throughout life to restore the metabolic control, but it does not abolish disabling complications over time. T1D entails a great individual, family and social commitment. The raise in incidence of T1D in most developed countries and its shift in younger ages of life implies that a genetic predisposition must be associated with environmental factors, including nutrients or other potentially reversible causative agents.

In 2003, Lars C. Stene found a low incidence of T1D in the coastal countries of Norway with greater availability for fish, compared to the countries of the hinterland[2]. This finding suggested a role of  $\omega$ -3 and vitamin D as possible environmental determinants of the disease. A prevention capability of 2000 IU of vitamin D assumed daily in the first year of live to prevent T1D was reported by Elina Hyppönen et al in 2001[3]. Surprisingly, little of these strong statements has become of clinical utility in the prevention or treatment of T1D, except for a consensus on correcting vitamin D deficiency[4]. Recently in TEDDY, a large network cohort study, vitamin D or  $\omega$ -3 supplementation during pregnancy did not confer any effect in the development of islet autoantibodies[5]. Conversely, in the first years of open disease, vitamin D and  $\omega$ -3 supplementation have been found to play a clinical role, as shown by SEARCH, two large prospective nutritional studies, reporting that the amount of  $\omega$ -3 in the diet correlates inversely with glycosylated hemoglobin and positively with persistent fasting C-peptide (FCP)[6,7].

A possible therapeutic role of vitamin D in T1D might be mediated by its regulatory activity on the immune system and the autoimmune response through vitamin D receptors expressed on both immune cells and pancreatic  $\beta$ -cells. The first clinical evidence in T1D was given by Gerlies Treiber et al, with the administration of high doses of cholecalciferol (70 IU/kg/day) in a randomized controlled trial on children with a recent onset of the disease, that showed a capacity to restore immunosuppressive Treg cells and potentially counteract the elective autoimmune damage to pancreatic  $\beta$ -cells[8]. In this study, supplementation with vitamin D did not display significant effect on stimulated C-peptide (SCP). Recently in Persian children at onset of overt T1D, Narges Habibi et al provided evidence of vitamin D as an environmental factor decreasing the C-peptide falling in children with recent T1D[9]

The ability of  $\omega$ -3 to counteract the pathogenic pathways of T1D has been highlighted in two translational researches showing that its nutritional intake may reduce blood glucose levels[10] and limit  $\beta$ -cells apoptosis mediated by glucolipotoxicity[11]. Specifically, diet supplementation with  $\omega$ -3 was found to lead to reduction of postprandial glycemia and improvement of glycemic variability, mediated by inhibition of neoglucogenesis[10], and to counteract  $\beta$ -cell apoptosis through activation of the *Eovl2*/DHA enzyme axis [11]. These reports suggest both an immunologic and metabolic role of  $\omega$ -3, limiting the post-prandial blood glucose increase, and protecting  $\beta$ -cell apoptosis induced by glucolipotoxicity. The role of  $\omega$ -3 and omega 6 in the regulation of autoimmunity in NOD mice is detailed in study of Xinum Bi et al[12]. In this work, EPA and DHA reduced the imbalance of Th1/Th2 cells and the proportion of Th17 cells, and increased Treg. In contrast, arachidonic acid (AA) intake increased the number of Th17, without significant difference in counting of Treg cells. In the same direction,  $\omega$ -3 supplementation decreased levels of IFN- $\gamma$ , IL-17, IL-6, and TNF- $\alpha$ , supporting its anti-inflammatory action.

The ideal time point of patient's investigation to test a possible role of vitamin D and  $\omega$ -3 should be at clinical onset of T1D when, after the starting of insulin therapy and achievement of a stable metabolic compensation, about 80% of children and adolescents experience a partial remission reducing insulin demands to keep the euglycemia. Afterwards, a resumption of the autoimmune

process determines a progressive growth of insulin requirements and concludes the so called "honeymoon phase" of T1D[13]. To sustain the recovery of endogen insulin secretion (REIS) should be the ultimate goal of T1D therapy to reduce severe hypoglycemia and avoid its long-term complications[14].

Given that insulin therapy is the first factor conditioning the restoration of the critical mass of  $\beta$ -cells during the honeymoon phase through stabilization of blood glucose, to date there is limited experience of the effect of combined supplementations with vitamin D and  $\omega$ -3 in the first year of overt disease in children. Since anecdotal cases suggested that this co-supplementation may prolong REIS [15–17], we considered to introduce in a series of T1D children, already assuming Mediterranean diet and supplementation of 1000 IU of cholecalciferol from onset, an additional  $\omega$ -3 supplement starting from the first semester of overt disease and evaluate outcomes as a pilot study before a possible randomized controlled intervention trial (RCT).

## PATIENTS AND METHODS

**Intervention group:** From January 2017 we studied prospectively 26 consecutives new T1D onsets referred to the pediatric diabetes service of University Hospital at Novara. Everyone received training to Mediterranean diet, cholecalciferol supplementation 1000 IU/day, and within the first semester of over disease, also  $\omega$ -3 supplement of fish oil ultra refined, equivalent to 60 mg/kg/day of eicosapentenoic acid (EPA) and docosahexaenoic acid (DHA).

**Retrospective controls:** All patients with T1D onset in 2014-2016 were admitted to the study as controls, after written consent of parents. One patient (n.38) was excluded since he was the anecdotic case (n.38) who received  $\omega$ -3 supplementation before this study. Data were structured in a database. They had been introduced to Mediterranean diet (as reported in the Diet section) and 1000 UI/day cholecalciferol supplementation since the first month of clinical disease, without  $\omega$ -3 supplement.

**Inclusion and exclusion criteria** The patients carrying renal cysts, or affected of sarcoidosis, histoplasmosis, hyperparathyroidism, lymphoma and tuberculosis were excluded. T1D patients with thyroiditis and celiac disease at gluten free diet and tissue transglutaminase autoantibody (tTGA) negative were not excluded. Patients treated with drugs that could affect immunity or glucose metabolism, including corticosteroids, ciclosporin and tacrolimus, were excluded.

The study was performed at the Division of Pediatrics, AOU Novara (Novara, Piedmont, Italy), with recruitment from January 1<sup>st</sup> 2017 to December 31<sup>st</sup>, 2018. The intervention lasted one year for each case. This study was approved by the Ethics Committee of AOU Novara, and all patient's parents signed the informed consent form. This trial was registered at ClinicalTrials.gov website (identifier: NCT03911843).

Diagnosis of T1D was performed according to the American Diabetes Association criteria[18]. Micro or macro vascular complications were defined according to the ISPAD criteria[19]. Children were evaluated using the Italian growth charts[20]. At the onset of the disease, presence of diabetic ketoacidosis, HbA1c%, insulin requirement, 25(OH)D levels, thyroid function, antibody title of GADA, IAA, IA-2, antibody pattern of celiac disease, lipid profile were collected. Evidence of ketoacidosis was assessed according to the ISPAD criteria[19], and severe DKA was considered if pH  $\leq$ 7.1. The vitamin D status, as 25(OH)D levels, sufficiency, deficiency or insufficiency, were defined according to the Endocrine Society criteria, graded as sufficiency 30-100 ng/ml (75-250 nmol/l), insufficiency 21-29 ng/ml (52.5-72.5 nmol/l), deficiency  $\leq$ 20 ng/ml (<50 nmol/l)[21,22]. The annual screening (blood count, lipid profile, urine test, thyroid function and aPTT coagulation tests) was performed and evaluated at T0 and T12. Blood glucose meter (Conturnext USB® Ascensia Diabetes Care) had been supplied to patients enrolled to standardize blood glucose measurements and their data were downloaded with dedicated software (Glucofacts® Ascensia Diabetes Care, FreeStyleLibreLink®, Medtronic CareLink Pro®, Accu-ChekChekSmartPix®, Diasend® or Dexcom Clarity®). Clinical data, insulin demand, laboratory analysis results, were entered in a structured database.

## Supplementations

Supplementation of  $\omega$ -3 was considered the intervention (T0). The preparation administered was a fish oil highly purified to avoid pollutants, containing a mixture of PUFA standardized for contents of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), in capsules or in liquid preparation (Ener Zone Omega 3 RX® Equipe Enervit). A liquid preparation was used in the case of difficulties of swallowing capsules or concomitant celiac disease because certified without gluten; the preparations contained antioxidants to preserve PUFA, tocopherol (1 mg in 1 g of  $\omega$ -3), palmitate and rosemary extract. EPA and DHA at 60 mg/kg/day, were administered for 12 months. The supplementation goal was AA/EPA ratio between 1.5–3. The investigation of AA/EPA ratio was performed in cases at enrolment (T0), and repeated after 3 (T3), 6 (T6) and 12 months (T12).

Cholecalciferol supplementation was fixed 1000 IU/day (25 mcg/day) both in cases and controls[21]. Vitamin D was determined as 25(OH)D level in cases and controls at clinical onset of T1D and at enrolment (T0); and in cases, 3 (T3), and 12 (T12) months after.

## Diet

At T1D clinical onset, a dietician performed a training to Mediterranean diet[23]. The educational item was standardized[24] (see attached file). A dietitian counseling was provided to each patient and accompanying parent every 3 months to sustain Mediterranean diet over time. At T0, a written information was provided on the foods main source of PUFA, to sustain the intake of food rich in  $\omega$ -3 (blue fish, nuts, walnuts, poultry, eggs, olive oil, flax seeds and leafy vegetable) and limit food rich in  $\omega$ -6 (beef, pork and offal).

At T0 and T12, dietary intakes were assessed based on a food diary of the last week, and further interview to determine the macro and micronutrients intakes as precisely as possible. The amounts of the nutrients have been evaluated with the support of the Food Composition Database for Epidemiological Studies (BDA version 1-2015)[23], assessing protein (g/day), energy (Kcal/day), cholesterol (mg/day), simple carbohydrates (g/day), dietary fiber (g/day), vitamin D (g/day), PUFA (g/day), arachidonic acid (AA g/day), EPA (g/day) and DHA (g/day).

## Assays

Plasma glucose levels (mg/dl; 1 mg/dl:0.05551 mmol/liter) were measured by the gluco-oxidase colorimetric method (GLUCOFIX, by Menarini Diagnostics, Florence, Italy). HbA1c% levels were measured through the high-performance liquid chromatography (HPLC), using a Variant machine (Biorad, Hercules, CA); intra- and inter-assay coefficients of variation are respectively lower than 0.6% and 1.6%. Linearity is excellent from 3.2% (11 mmol/mol) to 18.3% (177 mmol/mol). The presence and titration of antibodies GAD65, IA2 and IAA, expressed in IU/ml, was carried out by IRMA (Immunoradiometric Assay) with analytical coefficient of analysis of 13%, 8.4% and 13%, respectively. The semi-quantitative determination of TGA, AGAD-G and AGAD-A, expressed in IU/ml, was carried out on serum by QUANTA Flash, a rapid response immunochemiluminescence test (CIA) performed on the BIO-FLASH instrument; the coefficient of analytical variability was, respectively, 5.5%, 6.7% and 4.3%. The quantitative determination of the anti-TG and anti-TPO autoantibodies, expressed in IU/ml, of the TSH, expressed in  $\mu$ UI/ml, and of the fT4, expressed in ng/dl, was performed by a competitive immunoassay using immunochemiluminescence technique direct, using the Siemens ADVIA centaur XPT system.

The circulating Vitamin D level, were measured by serum 25(OH)D assay, using an immunochemiluminescence (CLIA) method (DiaSorin Liaison® Test, Stillwater MN-USA); the coefficient of inter-individual variability of the method (CVa%) was 10%.

Fatty acids (AA, EPA, DHA percentages and respective AA/EPA ratio) were determined by high-resolution capillary gas chromatography in whole blood using dried blood spots testing[25].

The levels of circulating C-peptide, expressed in ng/ml, were measured, on citrate or heparinized plasma, both by chemiluminescent "sandwich" immunoassay (DiaSorin Liaison) and by

immunochemiluminescence with automatic analyzer Immulite 2000, Medical Systems with coefficient of variability of 7.40%.

To assess a possible link between  $\omega$ -3 administration and persistent REIS, we used a composite index of insulin demand and metabolic control IDAA1c [calculated as HbA1c (%) + 4 insulin dose (UI/kg/24 h)]. A score <9 meet definition of partial remission and REIS, according with the previous studies in TrialNet[26].

Tests of glycosylated hemoglobin were performed every three months, and data on glucometer and if assigned CGM were discharged.

### Statistical analysis

Data were expressed as mean  $\pm$  SD of absolute values. The differences between groups were evaluated for the continuous variables through U Mann-Whitney test. Chi-square test was used for comparison of nominal variables between groups. In supplemented subjects, the evaluation of variation between T0 and T12 for all metabolic parameters was performed with T test for repeated measures. The association between the variables was evaluated according to Pearson test, after proper logarithmic transformation of the parameters, when necessary. Trend evaluation across 25(OH)D levels was performed at onset through multinomial regression analyses. Moreover, in supplemented cases, a trend evaluation of metabolic parameters across each time point and timing of supplementation (T0  $\rightarrow$  T12) was performed through multinomial regression analyses. Significant p values were less than 0.05. All statistical analyses were performed using SPSS 22.0 (SPSS Inc., Chicago, IL, USA).

## PATIENTS

Starting from 64 patients (26 cases and 38 controls), the analysis was carried out on 59 patients whose observations were available, from recruitment (T0), to 3, 6 and 12 (T12) months after, as HbA1c%, average daily insulin needs (IU/Kg/day) and IDAA1c. Patients whose  $\omega$ -3 supplementation started beyond 6 months from T1D onset, or without complete follow-up at 3, 6 and 12 months, were not considered either as cases or as controls. Of those entered, 2 cases were dropped out because not adhering to therapy, 2 cases because stopped  $\omega$ -3 for side effects, and 1 control because he changed residence. Finally, 59 patients were evaluated (22 cases - 37 controls)(**Tab 1**). Cases and controls received the same diet training and supplementations, except for  $\omega$ -3 supplementation that was given to the case group only.

**Table 1**

### Characteristics of participants at baseline.

Data presented as mean + SD or percentages as appropriate.

	CASES	CONTROLS	p value
n.	26	37	
Gender (female/male)	14/12	20/17	
Age (years)	8.7 $\pm$ 4.6	8.8 $\pm$ 3.6	NS
Body weight (Kg)	30.7 $\pm$ 17.5	32 $\pm$ 14	NS
BMI (Kg/m <sup>2</sup> )	-0.92 $\pm$ 1.1	-1.1 $\pm$ 2	NS
HbA1c%	11.3 $\pm$ 2.2	11.6 $\pm$ 2.6	NS

Insulin dose (UI/Kg/day)	0.61±0.22	0.69±0.28	NS
CSII/MDI device	2/24	11/27	

## RESULTS

**At onset of T1D.** Between the whole series (64) of T1D children we found in 82.5% vitamin D insufficiency [ $\leq 21$  ng/ml ( $\leq 52.5$  nmol/l)] in T1D children at time of clinical onset of T1D. A severe deficiency [ $\leq 10$  ng/ml (25 nmol/l)] was present in 12.7%, and that displayed a significantly lower FCP level ( $p < 0.02$ ) and pH ( $p < 0.02$ ) than the others. Variability in pH ( $p < 0.01$ ) and FC-P ( $p < 0.02$ ) across 25(OH)D levels was observed.

**Follow up of vitamin D and  $\omega$ -3 co-supplementation.** After 12 months of co-supplementation of  $\omega$ -3 and cholecalciferol, cases at T12 (n.22) showed a decrease of AA/EPA (50.8±38 vs. 8.8±7 ng/dl;  $p < 0.01$ ) and an increase of 25(OH)D (22.5±13 vs. 35.5±12 ng/dl;  $p < 0.01$ ) compared to cases at T0 (n.26). Thus, the target level of AA/EPA (1.5-3) was not achieved. The insulin demand increased ( $p < 0.0001$ ) and FCP decreased ( $p < 0.0001$ ) (Table 2).

**Patients supplemented vs. not supplemented.** At 12 months of  $\omega$ -3 supplementation (T12), the cases (n.22) showed significantly lower insulin needs than the controls (n.37). In particular a lower daily insulin needs (0.49±0.24 vs. 0.63±0.19 IU/Kg/day;  $p < 0.01$ ) and pre-meal bolus (0.22±0.16 vs. 0.34±0.14 IU/Kg/day;  $p < 0.01$ ) were found, without differences in HbA1c% ( $p$  NS). Analysis of IDAA1c index at T12 showed IDAA1c $< 9$ , consistent with a partial remission, in 12/22 (54.5%) cases vs. 7/37 controls (18%;  $p < 0.01$ ) (Fig.1).

**The impact of the diet.** Evaluation of the composition of the diet at T12 showed that macronutrients intake was slightly lower in terms of carbohydrates ( $p < 0.09$ ), fibers ( $p < 0.05$ ), and proteins ( $p < 0.05$ ) in cases vs. controls, whereas non-substantial differences were found in terms of lipids and vitamin D (NS). Moreover, at T12, the diet intake of AA was significantly lower (0.25±0.1 vs. 0.20±0.1 g/die;  $p < 0.01$ ) in the cases, whereas non significant differences were found in PUFA, EPA and DHA intakes (NS) (Table 3).

**Correlations** At disease onset, 25OHD levels were correlated with pH ( $r: 0.359$ ,  $p < 0.01$ ); HbA1c% values were correlated with insulin daily requirement ( $r: 0.568$ ,  $p < 0.0001$ ) and weakly inversely correlated with pH ( $r: -0.245$ ,  $p = 0.05$ ). The AA intake at starting point of  $\omega$ -3 supplementation (T0) was inversely correlated with HbA1c% ( $p < 0.05$ ,  $r: -0.411$ ). After 12 months of  $\omega$ -3 supplementation, vitamin D was inversely correlated with HbA1c ( $p < 0.05$ ,  $r: -0.462$ ).

**Limitations:** There are several limitations to conclude on efficacy of  $\omega$ -3 on T1D children: 1) the study was not randomized; it is therefore a preliminary study finalized to subsequent RCTs. 2) the IDAA1c index is a surrogate of REIS and not a direct evaluation of insulin secretion. 3) Controls are retrospective, so the comparability of series (Tab 1) are only for some data, such as HbA1c%, insulin needs and IDAA1c, and not for FCP, nor AA/EPA. 4) The target of AA/EPA level wasn't reached with the doses assigned (Tab 2).

**Side effects:** One female child reported diarrhea; she stopped the fish oil supplement with a quick return to normality. A female teenager with preexisting thyroiditis presented a transient suppression of the TSH, which returned to normal values after  $\omega$ -3 suspension. One child at T12 showed a lengthening of clotting time (aPTT); the parameters returned gradually to the norm after suspension of  $\omega$ -3 supplement. No other side effects have been reported, no hemorrhagic clinical symptoms or manifestations that could suspect coagulation involvement.

## DISCUSSION

In the management of T1D, there is a general agreement that the Mediterranean diet improves the metabolic control[27], and administration of vitamin D avoids its deficiency[4]; a recent systematic review on vitamin D supplementation at the onset of T1D concludes that alphacalcidole and cholecalciferol supplementations has beneficial effects on daily insulin doses, HbA1c%, FCP, and SCP; and indicates that further randomized controlled trials based on biomarkers are needed to define the optimal contribution[28]. Instead, there are no directions to date on supplementation of  $\omega$ -3 in young T1D subjects, despite some recent anecdotal data showed persistent partial remissions resulting up on vitamin D and  $\omega$ -3 supplementation introduced early after the disease onset[15,17]. Beyond these anecdotal cases, there is little scientific production of clinical outcomes on co-supplementation of vitamin D and  $\omega$ -3 in recent T1D. In this study involving a series of T1D children already assuming Mediterranean diet and cholecalciferol, the additional supplement of  $\omega$ -3 in recent onsets seems to have beneficial effects, which suggest the need to design further RCT.

Our previous findings in this context showed a widespread insufficiency of vitamin D at clinical onset of T1D and a relationship between its severe deficiency with reduced FCP and pH at clinical onset in line with reports of other authors [29–31]. This highlights the importance of vitamin D status in childhood to determine the severity of T1D at clinical onset. Ultimately, is mandatory the correction of vitamin D deficiency from the onset of T1D.

In our study, the daily supplementation of  $\omega$ -3 starting since the 1<sup>st</sup> semester from clinical onset entails, one year later, less insulin demand without affecting the glycemic control since had similar HbA1c%. Considering IDAA1c (Insulin Dose Adjusted for HbA1c%) as a surrogate index of residual  $\beta$ -cell function, and IDAA1c  $\leq 9$  indicative of partial remission of T1D[25], our data show that  $\omega$ -3 may preserve  $\beta$ -cell secretion. This finding, if confirmed in further randomized investigations, might be considered in setting the assistance for children since the onset of the disease. The clinical outcome of a reduced insulin demand mainly at meals is compatible with the hypothesis of the inhibition of on postprandial protein neoglucogenesis[10]. Interestingly, the effects of co-supplementation of vitamin D and  $\omega$ -3 are also reported in randomized clinical trials outside of T1D, on gestational diabetes and on multiple sclerosis[32,33]. Those studies reported a reduction of blood glucose and fasting plasma insulin, and an increase of insulin sensitivity. Particularly, the trial on gestational diabetes highlighted a synergism of vitamin D and  $\omega$ -3[33]. While clinical randomized trials on co-supplementation of vitamin D and  $\omega$ -3 in T1D are ongoing, our data suggest a benefit, and point to finalize further randomized investigations to the topic.

Unexpectedly, we found a decreasing trend of fasting C-peptide (FCP) from the start of  $\omega$ -3 supplement (T0) to one year later (T12). A plausible explanation of a similar metabolic control with less insulin administration, despite decreased FCP, is probably related to the counteraction of  $\omega$ -3 on neoglucogenesis, that limits the postprandial glucose increase and reduces insulin need for meals. The lowering of the blood glucose excursions could in turn affect the process of apoptosis of  $\beta$ -cells reducing glucolipototoxicity, and so could preserve REIS, according to findings in translational models[11]. This assumption might be investigated through analysis of stimulated C-peptide (SCP) after standardized mixed-meal tolerance test, that represent a direct measurement of insulin secretion, in future randomized trials. A possible immune regulatory role of  $\omega$ -3 supplementation could concur, together with vitamin D, to improve T1D, and should be investigated by assessing biomarkers of autoimmunity (e.g. lymphocyte subpopulations Treg or Th-17). Anyway, this study cannot determine if the decreased insulin demand is sustained only by reduced needs, or also by preserved REIS over time, because IDAA1c is a composite index of insulin needs and metabolic control, but not a direct measurement of REIS.

Another limit of this study is the failure to achieve the target levels of AA/EPA ratio, probably due to reduced compliance of pediatric patients in assumption of fish oil, or insufficient dosage. Reaching an AA/EPA range 1.5-3 will be therefore a goal in future trials.

From the diet analysis, in the  $\omega$ -3 supplemented group, no changes were found from T0 to T12 in terms of caloric intake and nutrients (carbohydrates, proteins, lipids, fibers and PUFA), except for decrease of AA. From a regression analysis, the AA intake was a strong determinant of the HbA1c% at T0. Since it was significantly decreased at T12, the AA

reduction concurred with  $\omega$ -3 supplementation indetermining AA/EPA and outcomes. This is likely related to a competition between  $\omega$ -3 and  $\omega$ -6 for common metabolic PUFA pathways and indicates that better outcome might result if supplementation of  $\omega$ -3 will be contextual to the reduction of AA in the diet.

## Conclusion

Given that this study is preliminary to future randomized controlled ones, our findings suggest that a  $\omega$ -3 supplement, in the context of Mediterranean diet and of a vitamin D administration, gives benefits to children with T1D, with a reduction of insulin requirements after 12 months of supplementation, despite a decrease in FCP. Vitamin D and the dietary intakes of  $\omega$ -3 and AA play a role in the result of a better/worse metabolic control and perhaps favorable/unfavorable autoimmunity. Particularly, further investigations should consider direct biomarkers of insulin secretion (as SCP) and of T-cell immune-regulatory subgroups (as Tregs, Th17), to define if AA/ $\omega$ -3 assign metabolic or immune effect, or both.

Attached file:

### A structured dietician training to a Mediterranean-style diet

The recommended composition of the dietary regimen was suggested as 50-55% of carbohydrates; 10-15% of proteins or 0.80-1.34 g/Kg/d according to age and gender; 30-35% of total fats of which saturated less than 10%; cholesterol consumption 100 mg every 1000 Kcal. Two or more servings of fish per week and two servings of fruit per day (400 g), three of vegetables (300 g), per day were recommended. Walnuts and olive oil consumption were suggested. A fiber intake of 12-14 g every 1000 Kcal, and intake of whole foods was encouraged choosing a variety of fiber containing food (whole grains, fruits, vegetables, vegetable soups). The nutritional counseling was scheduled into 3 structured and managed steps.

The first step was straight to detect the proportion of daily caloric intakes (carbohydrates, proteins and lipids) and to promote the intake of monounsaturated fatty acids. Rates of lipids were according to age. The second step was more structured and detected the intakes of fibers, fruits, and vegetables in the daily diet; their consume was emphasized and promoted. A progressive increasing consumption of fibers was suggested to reduce a refusal due to palatability or gastrointestinal side effects. The third step aimed to better explain the concept of portion size and life-style flexibility, in order to manage exceptions and fit in events that not allow the usual meal consumption. Pictorial copies of meals and nutrients and booklets were used in this step. One hour per step was reserved at every subjects and/or parents[24].

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## Bibliography

1. McClements, D.J.; Li, F.; Xiao, H. The Nutraceutical Bioavailability Classification Scheme: Classifying Nutraceuticals According to Factors Limiting their Oral Bioavailability. *Annual Review of Food Science and Technology***2015**, *6*, 299–327.
2. Stene, L.C.; Joner, G.; Norwegian Childhood Diabetes Study Group Use of cod liver oil during the first year of life is associated with lower risk of childhood-onset type 1 diabetes: a large, population-based, case-control study. *The American Journal of Clinical Nutrition***2003**, *78*, 1128–1134.
3. Hyppönen, E.; Läärä, E.; Reunanen, A.; Järvelin, M.-R.; Virtanen, S.M. Intake of vitamin D and risk of type 1 diabetes: a birth-cohort study. *The Lancet***2001**, *358*, 1500–1503.
4. Mathieu, C. Vitamin D and diabetes: Where do we stand? *Diabetes Research and Clinical Practice***2015**, *108*, 201–209.
5. Silvis, K.; Aronsson, C.A.; Liu, X.; Uusitalo, U.; Yang, J.; Tamura, R.; Lernmark, Å.; Rewers, M.; Hagopian, W.; She, J.; et al. Maternal dietary supplement use and development of islet autoimmunity in the offspring: TEDDY study. *Pediatric Diabetes***2018**.
6. Lamichhane, A.P.; Crandell, J.L.; Jaacks, L.M.; Couch, S.C.; Lawrence, J.M.; Mayer-Davis, E.J. Longitudinal associations of nutritional factors with glycated hemoglobin in youth with type 1 diabetes: the SEARCH Nutrition Ancillary Study. *The American Journal of Clinical Nutrition***2015**, *101*, 1278–1285.
7. Mayer-Davis, E.J.; Dabelea, D.; Crandell, J.L.; Crume, T.; D'Agostino, R.B.; Dolan, L.; King, I.B.; Lawrence, J.M.; Norris, J.M.; Pihoker, C.; et al. Nutritional Factors and Preservation of C-Peptide in Youth With Recently Diagnosed Type 1 Diabetes: SEARCH Nutrition Ancillary Study. *Diabetes Care***2013**, *36*, 1842–1850.
8. Treiber, G.; Prietl, B.; Fröhlich-Reiterer, E.; Lechner, E.; Ribitsch, A.; Fritsch, M.; Rami-Merhar, B.; Steigleder-Schweiger, C.; Graninger, W.; Borkenstein, M.; et al. Cholecalciferol supplementation improves suppressive capacity of regulatory T-cells in young patients with new-onset type 1 diabetes mellitus — A randomized clinical trial. *Clinical Immunology***2015**, *161*, 217–224.
9. Habibian, N.; Amoli, M.M.; Abbasi, F.; Rabbani, A.; Alipour, A.; Sayarifard, F.; Rostami, P.; Dizaji, S.P.; Saadati, B.; Setoodeh, A. Role of vitamin D and vitamin D receptor gene polymorphisms on residual beta cell function in children with type 1 diabetes mellitus. *Pharmacological Reports***2019**, *71*, 282–288.
10. Wang, B.; Smyl, C.; Chen, C.-Y.; Li, X.-Y.; Huang, W.; Zhang, H.-M.; Pai, V.; Kang, J. Suppression of Postprandial Blood Glucose Fluctuations by a Low-Carbohydrate, High-Protein, and High-Omega-3 Diet via Inhibition of Gluconeogenesis. *International Journal of Molecular Sciences***2018**, *19*, 1823.
11. Bellini, L.; Campana, M.; Rouch, C.; Chacinska, M.; Bugliani, M.; Meneyrol, K.; Hainault, I.; Lenoir, V.; Denom, J.; Véret, J.; et al. Protective role of the ELOVL2/docosahexaenoic acid axis in glucolipototoxicity-induced apoptosis in rodent beta cells and human islets. *Diabetologia***2018**, *61*, 1780–1793.
12. Bi, X.; Li, F.; Liu, S.; Jin, Y.; Zhang, X.; Yang, T.; Dai, Y.; Li, X.; Zhao, A.Z.  $\omega$ -3 polyunsaturated fatty acids ameliorate type 1 diabetes and autoimmunity. *Journal of Clinical Investigation***2017**, *127*, 1757–1771.
13. Regnell, S.E.; Lernmark, Å. Early prediction of autoimmune (type 1) diabetes. *Diabetologia***2017**, *60*, 1370–1381.
14. Steffes, M.W.; Sibley, S.; Jackson, M.; Thomas, W. -Cell Function and the Development of Diabetes-Related Complications in the Diabetes Control and Complications Trial. *Diabetes Care***2003**, *26*, 832–836.
15. Baidal, D.A.; Ricordi, C.; Garcia-Contreras, M.; Sonnino, A.; Fabbri, A. Combination high-dose Omega-3 fatty acids and high-dose cholecalciferol in new onset type 1 diabetes. 6.

16. Cadario, F.; Savastio, S.; Ricotti, R.; Rizzo, A.M.; Carrera, D.; Maiuri, L.; Ricordi, C. Administration of vitamin D and high dose of omega 3 to sustain remission of type 1 diabetes. 4.
17. Cadario, F.; Savastio, S.; Rizzo, A.M.; Carrera, D.; Bona, G.; Ricordi, C. Can Type 1 diabetes progression be halted? Possible role of high dose vitamin D and omega 3 fatty acids. 6.
18. American Diabetes Association 2. Classification and Diagnosis of Diabetes. *Diabetes Care* **2017**, *40*, S11–S24.
19. Donaghue, K.C.; Wadwa, R.P.; Dimeglio, L.A.; Wong, T.Y.; Chiarelli, F.; Marcovecchio, M.L.; Salem, M.; Raza, J.; Hofman, P.L.; Craig, M.E. Microvascular and macrovascular complications in children and adolescents: Microvascular and macrovascular complications. *Pediatric Diabetes* **2014**, *15*, 257–269.
20. 15.pdf.
21. Holick, M.F.; Binkley, N.C.; Bischoff-Ferrari, H.A.; Gordon, C.M.; Hanley, D.A.; Heaney, R.P.; Murad, M.H.; Weaver, C.M. Evaluation, Treatment, and Prevention of Vitamin D Deficiency: an Endocrine Society Clinical Practice Guideline. *The Journal of Clinical Endocrinology & Metabolism* **2011**, *96*, 1911–1930.
22. Holick, M.F.; Binkley, N.C.; Bischoff-Ferrari, H.A.; Gordon, C.M.; Hanley, D.A.; Heaney, R.P.; Murad, M.H.; Weaver, C.M. Guidelines for Preventing and Treating Vitamin D Deficiency and Insufficiency Revisited. *The Journal of Clinical Endocrinology & Metabolism* **2012**, *97*, 1153–1158.
23. The DIAMOND Project Group Incidence and trends of childhood Type 1 diabetes worldwide 1990–1999. *Diabetic Medicine* **2006**, *23*, 857–866.
24. Cadario, F.; Prodam, F.; Pasqualicchio, S.; Bellone, S.; Bonsignori, I.; Demarchi, I.; Monzani, A.; Bona, G. Lipid profile and nutritional intake in children and adolescents with Type 1 diabetes improve after a structured dietician training to a Mediterranean-style diet. *Journal of Endocrinological Investigation* **2012**.
25. Rizzo, A.M.; Montorfano, G.; Negroni, M.; Adorni, L.; Berselli, P.; Corsetto, P.; Wahle, K.; Berra, B. A rapid method for determining arachidonic:eicosapentaenoic acid ratios in whole blood lipids: correlation with erythrocyte membrane ratios and validation in a large Italian population of various ages and pathologies. *Lipids in Health and Disease* **2010**, *9*, 7.
26. Hao, W.; Gitelman, S.; DiMeglio, L.A.; Boulware, D.; Greenbaum, C.J. Fall in C-Peptide During First 4 Years From Diagnosis of Type 1 Diabetes: Variable Relation to Age, HbA<sub>1c</sub>, and Insulin Dose. *Diabetes Care* **2016**, *39*, 1664–1670.
27. Zhong, V.W.; Lamichhane, A.P.; Crandell, J.L.; Couch, S.C.; Liese, A.D.; The, N.S.; Tzeel, B.A.; Dabelea, D.; Lawrence, J.M.; Marcovina, S.M.; et al. Association of adherence to a Mediterranean diet with glycemic control and cardiovascular risk factors in youth with type I diabetes: the SEARCH Nutrition Ancillary Study. *European Journal of Clinical Nutrition* **2016**, *70*, 802–807.
28. Gregoriou, E.; Mamais, I.; Tzanetakou, I.; Lavranos, G.; Chrysostomou, S. The Effects of Vitamin D Supplementation in Newly Diagnosed Type 1 Diabetes Patients: Systematic Review of Randomized Controlled Trials. *The Review of Diabetic Studies* **2017**, *14*, 260–268.
29. Bener, A.; Alsaied, A.; Al-Ali, M.; Al-Kubaisi, A.; Basha, B.; Abraham, A.; Guiter, G.; Mian, M. High prevalence of vitamin D deficiency in type 1 diabetes mellitus and healthy children. *Acta Diabetologica* **2009**, *46*, 183–189.
30. Greer, R.M.; Portelli, S.L.; Hung, B.S.-M.; Cleghorn, G.J.; McMahon, S.K.; Batch, J.A.; Conwell, L.S. Serum vitamin D levels are lower in Australian children and adolescents with type 1 diabetes than in children without diabetes: Vitamin D in Australian children with diabetes. *Pediatric Diabetes* **2013**, *14*, 31–41.
31. Franchi, B.; Piazza, M.; Sandri, M.; Mazzei, F.; Maffei, C.; Boner, A.L. Vitamin D at the onset of type 1 diabetes in Italian children. *European Journal of Pediatrics* **2014**, *173*, 477–482.

32. Jamilian, M.; Samimi, M.; Ebrahimi, F.A.; Hashemi, T.; Taghizadeh, M.; Razavi, M.; Sanami, M.; Asemi, Z. The effects of vitamin D and omega-3 fatty acid co-supplementation on glycemic control and lipid concentrations in patients with gestational diabetes. *Journal of Clinical Lipidology***2017**, *11*, 459–468.
33. Kouchaki, E.; Afarini, M.; Abolhassani, J.; Mirhosseini, N.; Bahmani, F.; Masoud, S.A.; Asemi, Z. High-dose  $\omega$ -3 Fatty Acid Plus Vitamin D3 Supplementation Affects Clinical Symptoms and Metabolic Status of Patients with Multiple Sclerosis: A Randomized Controlled Clinical Trial. *The Journal of Nutrition***2018**, *148*, 1380–1386.