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Prenatal vitamin D deficiency induces an early and more severe experimental autoimmune encephalomyelitis in the F2 generation

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Abstract: To be written shortly (200 words)

GLOSSARY

DVD: Developmental Vitamin D Deficiency**; EAE:** Experimental Autoimmune Encephalomyelitis; **MOG**: Myelin Oligodendrocyte Glycoprotein; **MS**: Multiple Sclerosis; **REFGENSEP**: Reseau d'Etudes Francais GENetique sur la Sclerose En Plaques

Keywords: vitamin D3; experimental autoimmune encephalomyelitis; multiple sclerosis; deficiency; season of birth; transgenerational

(3-10 keywords separated by semi colons)

1. Introduction

A highly powered epidemiological study has reported that the risk of Multiple Sclerosis (MS) is greater for people born in May and lower for those born in November (Willer et al, 2005). Although disputed (Sadovnick et al, 1994; Givon et al, 2010), these findings were recurrently confirmed in various cohorts of MS patients (Sotgiu et al, 2006; Lewy et al, 2008; Fernandes de Abreu et al, 2009; Bayes et al, 2010; Salzer et al, 2010; Staples et al, 2010). This season of birth effect, combined with an observed latitude gradient (Simpson et al 2011), suggest that a sunlight-related factor, possibly vitamin -D, is involved in MS.

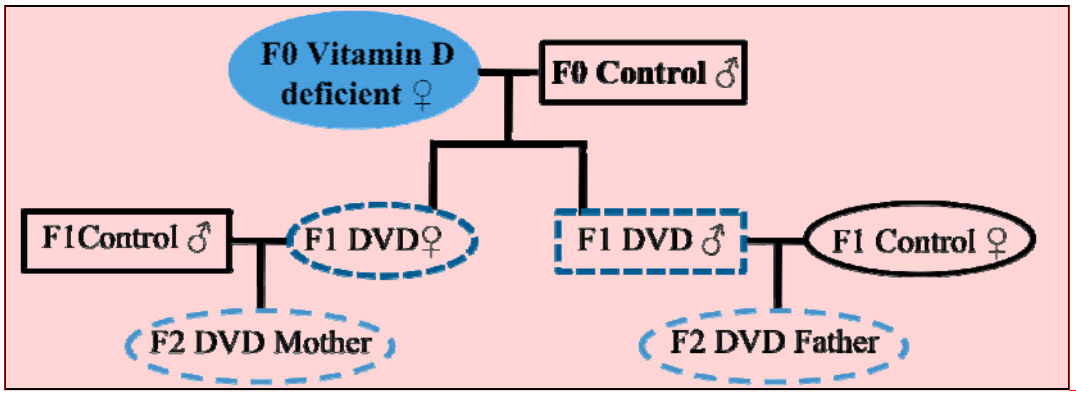
In order to test the hypothesis ~~according which~~that a low vitamin D status during gestation is a risk factor for MS, we devised a developmental vitamin D deficiency (DVD) rodent model based on a vitamin D-free diet delivered before (6 weeks) and during pregnancy (Eyles et al, 2003; Feron et al, 2005). Contrary to our prediction, we observed that C57BL/6 mouse adult offspring exposed to developmental vitamin D deficiency (DVD) developed a striking milder and delayed experimental autoimmune encephalomyelitis (EAE), when compared with~~to~~ control offspring (Fernandes de Abreu et al, 2010; Fernandes de Abreu et al, 2011). In parallel, another team similarly demonstrated that a vitamin D deficiency, maintained for two generations, diminished the severity and delayed the onset of EAE in B10PL mice (DeLuca and Plum, 2011).

Comment [A1]: I use a non-separating space for "Vitamin D" (shift+control+space), so that it doesn't get broken over two lines.

Though paradoxical, the behaviour of the first generation indicated that a transient maternal hypovitaminosis D imprints the developing offspring and induces an altered response of the immune system after immunization. It remained however to be seen whether this environment-related change could be inherited *via* the gametes, as brilliantly demonstrated in rats exposed to a fungicide (Anway et al, 2005). We surmised that the second generation (F_2) was also affected by the vitamin D status of the pregnant F_0 female mice. With the aim of (un)validating this hypothesis, we designed a new experimental model, schematized i~~o~~n Figure 1.

In this series of experiments, only the pregnant F_0 female mice and their growing fetuses were vitamin D deficient. F_0 female C57BL/6 mice were fed with a vitamin D-free diet before and during pregnancy. In order to highlight potential differences between male and female germlines, we set up two groups for the F_1 generation: adult DVD males were mated with control females, while DVD females ~~DVD~~ were mated with control males. A vitamin D-containing diet was provided to all

animals, before, during and after pregnancy. F2 offspring were weaned and adult females were subjected to EAE.



Comment [A2]: Needs a caption?

To our surprise, we observed that the vitamin D deficiency affecting the F_0 pregnant females induced an early onset and more severe EAE in the F_2 generation. This new and unexpected finding led us to refine our initial hypothesis and infer that an *in utero* low vitamin D status for the parents may be an additional risk factor for MS patients. Therefore, using the REFGENSEP database for MS trios (the patient and his/her parents), we collected the parents' dates of birth and assessed a potential season of birth effect that could potentially be indicative of the vitamin D status of the pregnant grandmothers.

2. Results and Discussion

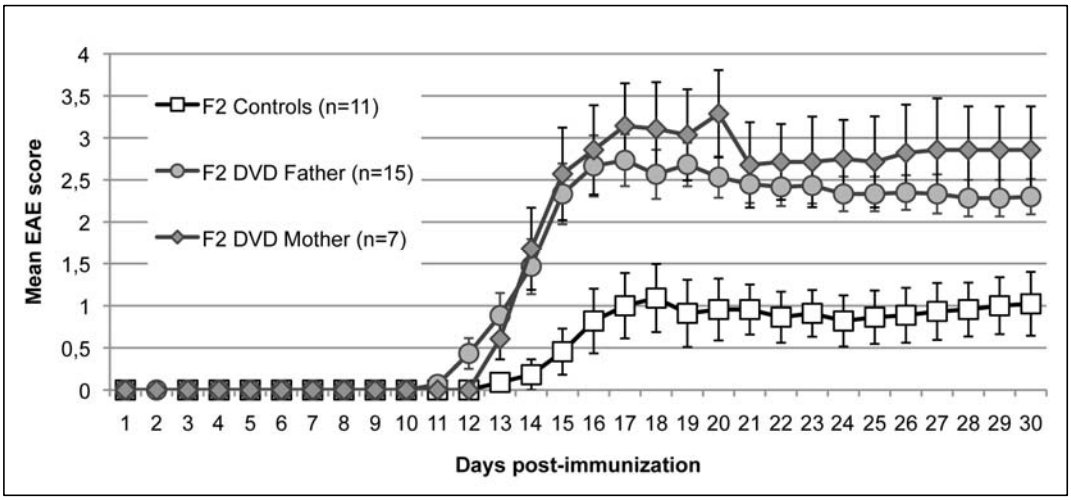
2.1. A prenatal vitamin D deficiency displays a transgenerational effect and induces a more severe EAE in F2 female adult mice

The three tested groups developed MOG35-55-induced EAE (Figure 2). However, developmental vitamin D deficiency in the F_0 generation significantly altered EAE course in the F_2 generation. Both DVD Mother and DVD Father developed an EAE with a significantly earlier onset (onset day x+/-y and v+/-w, respectively) when compared to controls (onset day t+/-u, F = a, p = 0,0…). Moreover, both DVD groups displayed an increased clinical score peak (x+/-y and v+/-w, respectively) when compared to controls (t+/-u, F = a, p = 0,0…). At Day_0 post-immunization, the mean weight of the three tested groups was similar (DVD Mother: x+/-y g; DVD Father: t+/-u g; controls: v+/-w g). DVD Mother and DVD Father exhibited similar clinical parameters.

Comment [A3]: Obviously waiting on numbers here.

In a previous experiment, we demonstrated that a prenatal vitamin D deficiency induces a milder and delayed EAE in the F_1 generation (Fernandes de Abreu et al, 2010). In line with other

studies describing a generational transmission of molecular disturbances (Daxinger and Whitelaw, 2012, for a recent review), we predicted that the F_2 generation would display a phenotype similar to the



F_1 generation. To our great surprise, we observed an inverted response to MOG immunization. This discordant behavior between the F_1 and the F_2 generations is unusual and, to the best of our knowledge, has never been reported. It is not unlikely that a positive effect of a potentially deleterious environmental factor remains unnoticed, especially in humans. Conversely, an impaired response is easily noticeable but sometimes can only be associated to the grandparents' and not the parents' lifestyle, as exemplified by a seminal epidemiological study on ancestral food supply (Pembrey et al, 2006). If confirmed, our findings may provide an additional evidence for future studies aiming to explain generation-skipping transmission of non genetic disturbances.

The molecular basis of this inheritance is unclear. The role of chromatin and DNA methylation in epigenetics have been extensively studied during the past three decades. However, recent evidence tend to support a bigger role for RNA in gametes, including piRNAs and miRNAs, that can travel between cells and silence transposable elements (Daxinger and Whitelaw, 2012). Among the current candidates, we can cite miR-22 that is induced by vitamin D and acts as an antiproliferative and antimigratory agent in cancer cells (Alvarez-Diaz et al, 2012) or miR-125b that regulates the expression of human vitamin D receptor and abolishes the anti-proliferative action of calcitriol (Mohri et al, 2009). Nonetheless, to date, not a single study has yet demonstrated a piRNA- or a miRNA-associated action of vitamin D on the immune or the nervous system.

2.2. Observation of a trend for a reduced number of births in Autumn for the parents of MS patients

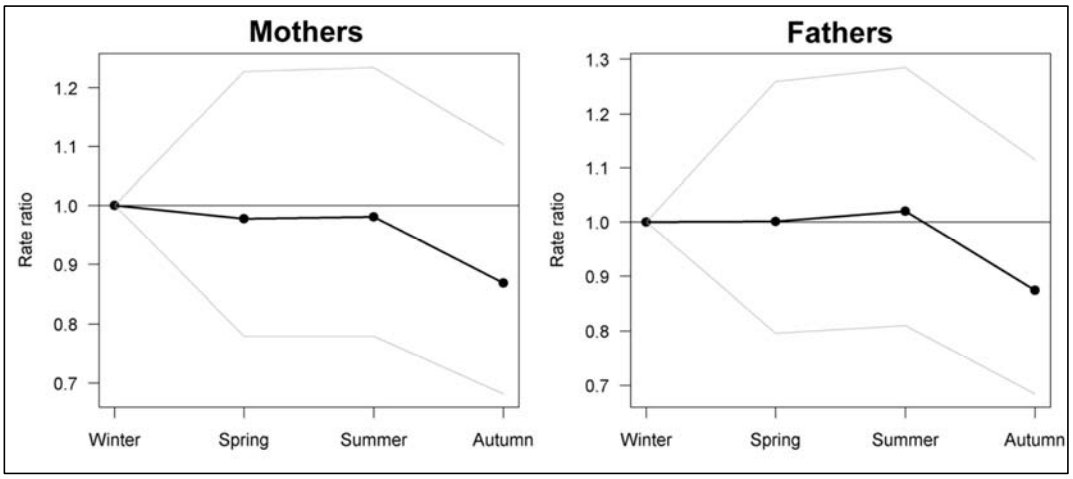
Parents' dates of births were clustered into seasons as follows: Winter (Dec, Jan, Feb), Spring (Mar, Apr, May), Summer (Jun, Jul, Aug) and Autumn (Sep, Oct, Nov). Figure 3 indicates that the lowest risk for both groups is consistently in Autumn, although this ~~trend~~ pattern did not reach statistical significance (p-values: 0.28 mothers, 0.25 fathers).

Comment [A4]: Give the p-values for Autumn in brackets for men and women?

Using the same database, we ~~then~~ performed a case–only analysis based on children, with the cases as children born in summer and autumn, and the controls born in winter and spring. The exposure variable was the circular distance from October 15 for the mother's and father's birthdays. The largest exposure value was π (half the circumference of a circle) ~~(pi)~~ for a birthday on April 15, exactly six months away from October 15. The aim of this analysis was to identify a difference in the pattern of the parents' birthdays for ~~diseased~~MS children born in the high risk time (winter and spring – cases), compared with the low risk time (summer and autumn – controls). Our prior belief was that children born in the low risk time ~~would~~could have had parents with higher risks, and that this might explain their MS instead of their in utero exposure~~a riskier~~. However, w~~W~~e found no evidence of any difference in the pattern of parents' birthdays for case and control children.

Comment [A5]: Is this right, maybe check with John.

Comment [A6]: Again, some p-values?



~~Once again, our prediction was not met.~~ The current study failed to find an ~~uprise~~ increased number of MS births in Spring, as suggested by our animal model. So once again, our prior beliefs were not met. Nonetheless, the reduced number of births in Autumn, in each arm of the trio (children, fathers, mothers), requires further attention. Although not statistically significant, this finding is consistent with previous studies demonstrating a nadir of MS patients born in November (Willer et al, 2005; Fernandes de Abreu et al, 2009). Being born in Autumn means that, during the third trimester of pregnancy, the foetus developed in a supposedly vitamin D rich environment. It is therefore likely that his/her immune and nervous systems benefited from this optimal condition. We previously

Comment [A7]: Data not shown?

demonstrated that a postnatal vitamin D supplementation reduced the severity of EAE and delayed the onset of symptoms (Fernandes de Abreu et al, 2011). It would be now of great interest to confirm that a similar phenotype is observed when the supplementation occurs during pregnancy. Additionally, we should perform a transgenerational study in order to assess whether the F_2 generation is positively affected by a high dose of vitamin D delivered to the F_0 generation.

Note: Unfortunately, so far, I have no answer from REFGENSEP about the scoring of the patients. Therefore, we cannot correlate the season of birth for the parents and disease severity for the children.

3. Experimental Section

Animal housing and feeding

All procedures were performed according to the French law on Animal Care Guidelines. Animal Care Committee of University Aix-Marseille II approved protocols. C57Bl/6 mice (Charles River, France) were maintained in a holding room at a constant temperature of 21±2 °C° and 60% relative humidity, on a 12 hour light–dark cycle. Food and water were provided ad libitum. Vitamin D deficiency was achieved by: i) feeding fertile adult F_0 female mice with a normo-phosphatic vitamin D3-free diet supplemented with lactose and calcium (INRA, France) and ii) using UV-free lighting. Control animals (males and females) were given a standard vitamin D3-containing (1,500 IU/kg) diet (INRA, France). Serum vitamin D depletion was assessed six weeks later using a commercial RIA (Diasorin, MN, USA) for 25-hydroxyvitamin D_3. Dams exposed to six weeks of vitamin D3 depletion exhibited a reduced production of 25-hydroxyvitamin D (mean of 3 ng/ml ± ~~+/-~~ 0.5) when compared ~~to~~ with control dams (40 ng/ml ± ~~+/-~~ 2.5). Vitamin D-deficient females were then mated with control males and kept under vitamin D3-free conditions throughout gestation. At birth, offspring and dams were placed on the vitamin D3-containing control diet. In parallel, control females were mated with control males in order to obtain control offspring (control mice). DVD F_1 offspring were weaned 28 days after birth and mated with control partners that were bred with the control diet during their whole life. DVD F_2 offspring were weaned and females, born to either DVD F1 males or DVD F1 females, were subjected to EAE at 12 weeks of age.

Active MOG35-55-induced EAE

A total of twenty--nine female offspring were immunized subcutaneously with 200 µg of 35–55 MOG peptide (sequence: MEVGWYRSPFSRVVHLYRNGK, Genepep, France), emulsified in complete Freund adjuvant (DIFCO, USA) and supplemented with 400 µg of H37Ra Mycobacterium tuberculosis (DIFCO, USA). 100 ng of pertussis toxin was injected ip, at Day_0 and Day_1 post-immunization. The dose for the MOG peptide was elected in order to induce a very mild EAE in

control animals. Immunized females were randomly placed in different cages. Weight and disease severity were blindly scored, once a day, according to the EAE clinical scale: 0 = no detectable sign of EAE; 1 = weakness of the tail; 2 = tail paralysis and hind limb weakness; 3 = partial paralysis of hind limbs; 4 = complete paralysis of hind limbs; 5 = complete paralysis of hind limbs with incontinence and partial or complete paralysis of forelimbs; 6 = dead~~, as previously described~~ (Fernandes de Abreu et al, 2010; Fernandes de Abreu et al, 2011).

Season of birth analysis

MS French family trios were prospectively recruited as part of a survey of MS patients identified throughout France by REFGENSEP, the French MS Genetics Group (Cournu-Rebeix, 2008). Trios were ascertained through one patient (child) per family and two parents available for typing. All patients included in the databank were examined by a neurologist, and fulfilled diagnosis criteria for definite MS (Poser, 1983). Informed consent was given by each individual participating to the study, in accordance with the Helsinki convention (1964) and French law relating to biomedical research. The whole set of 610 pairs of unrelated parents of MS patients were considered for the season of birth study. The numbers of births per month and year for the mothers and ~~the~~ fathers ~~groups~~ were compared with~~to~~ the ~~months of birth~~numbers for the general French population, during the same time period of ~~time, namely from~~ 1901 to 1960. ~~A ratio observed number of births/expected number of births was calculated for each month.~~

Comment [A8]: I'm not sure what you mean by this word here.

Statistical analysis

EAE data were analyzed using parametric (ANOVA) tests and GraphPad Prism software. In all analyses, $P<0.05$ was selected as the threshold for statistical significance.

Tests for the season of birth analysis??

The ratio of the observed to expected number of births for the parents was analysed using a general linear model assuming a Poisson distribution. The number of births was the dependent variable, with season as the independent variable and the log-transformed total number of births as an offset (in order to analyse the ratio of observed to expected). This analysis was made using the R software (www.r-project.org).

4. Conclusions

To be written shortly

Acknowledgments

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