



## Original article

# Zinc supplementation among zinc-deficient vegetarians and vegans restores antiviral interferon- $\alpha$ response by upregulating interferon regulatory factor 3



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## SUMMARY

**Background & aims:** Sufficient zinc status is crucial for undisturbed immune function. Further, dietary zinc requirements are mainly covered by animal products. Consequently, plant-based diets have been repeatedly linked to zinc deficiency. In the light of increasing popularity of plant-based diets, elucidating the impact of zinc deficiency on the innate immune system of vegetarians and vegans is of great interest. Although it has been previously shown that interferon (IFN) - $\alpha$  response towards viral stimuli can be enhanced by administering zinc *in vitro*, the underlying molecular process remains unknown. In this project, we investigate the effect of short-term, oral zinc supplementation *in vivo* on antiviral immune response and describe the potential molecular mechanism behind zinc-dependent interferon- $\alpha$  production.

**Methods:** Zinc status of omnivore, vegetarian and vegan subjects was determined by serum zinc measurement and a food frequency questionnaire (FFQ). Antiviral IFN- $\alpha$  response was measured in whole-blood and peripheral blood mononuclear cells upon viral stimulus. Furthermore, expression of interferon regulatory factor (IRF) 3, a positive regulator of IFN $\alpha$ , was quantified in each cohort. If zinc deficiency was detected, participants received oral zinc supplementation and tests were repeated afterwards.

**Results:** We observed a high prevalence of imminent or manifest zinc deficiency among vegetarians and vegans. Compared to omnivores, IFN $\alpha$  response and IRF3 levels were significantly reduced. Moreover, we were able to enhance zinc status, increase IRF3 expression and reconstitute IFN $\alpha$  response by oral zinc supplementation.

**Conclusion:** We identified zinc-dependent IRF3 expression as an essential cellular mechanism behind impaired IFN $\alpha$  response in zinc-deficient subjects. This may contribute to disturbed antiviral immunity and cause increased susceptibility to virus infections *in vivo*. Oral zinc supplementation effectively restored IRF3 and IFN $\alpha$  levels. Hence, nutritional interventions may become increasingly important in order to prevent health implications from micronutrient deficiencies among vegetarians and vegans.

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## 1. Introduction

The prevalence of different plant-based diets has been continuously increasing in Germany throughout the past decade [1,2]. A similar trend was observed in several other European countries and the United States of America [3–5].

The main motivations for individuals adhering to this certain nutritional approach range from ethical beliefs (e.g. animal-

welfare) and environmental considerations to health concerns [6]. In the wake of rapid global population growth, plant-based diets are also a promising opportunity to restructure the food system in order to reduce the negative impact of food production on ecosystems and climate [7]. Furthermore, there is increasing evidence concerning the protective effect of a vegetarian or vegan diet on various disease entities such as cardiovascular diseases, hypertension, diabetes and different types of cancer [8–11].

On the other hand, recent studies have shown a surging prevalence of several micronutrient deficiencies among individuals following a plant-based diet. If adequate nutrient sources and

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certain supplements are not sufficiently included, this would ultimately undermine the positive intrinsic effects of this dietary approach [12]. Zinc status has been at the center of attention in many research projects, strongly indicating increased risk for zinc deficiency due to insufficient dietary zinc intake [13–15]. Zinc deficiency is partly caused by the reduced total amount of zinc in plant-based foods, but is mainly the result of impaired zinc bioavailability due to high phytate levels in plant-based diets [16]. Phytic acid is a zinc-binding ligand that disrupts intestinal zinc resorption through chelation [17–19]. Therefore, in the light of increasing popularity of plant-based diets, there is urgent need for elucidating potential harmful effects of zinc deficiency in vegetarians and vegans.

Zinc, as an essential trace element, plays a crucial role in over 300 enzymes and transcription factors, impacting a wide range of cell types of the human body [20,21]. Consequently, adequate zinc status is essential for a large number of physiological processes in various organ systems, including the immune system, intestinal function and many more [22–24]. Signaling pathways in both the adaptive and innate immune system have shown zinc-dependent properties, ultimately affecting proliferation, differentiation and cytokine-mediated communication of immune cells [24,25].

In the case of RNA-virus infection, innate immune response via the interferon family, especially interferon (IFN)  $\alpha$ , is initiated upon detection of viral RNA by different pattern recognition receptors (PRRs). Mainly, signaling pathways are activated through the endosomal Toll-like receptors (TLRs) TLR3, TLR7, TLR8 and TLR9, the cytosolic DNA sensor cyclic GMP-AMP synthase (cGAS) and the retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) [26]. Finally, synthesis of IFN $\alpha$  is initiated by interferon regulatory transcription factors (IRFs), especially IRF3 [27]. In ongoing viral infections, plasmacytoid dendritic cells (pDCs) are usually the main source of type I interferons, but, depending on virus, route of infection (systemic vs. local) and timepoint, monocytes, tissue-resident macrophages and conventional dendritic cells (cDCs) also play an important role in interferon response [28]. IFN $\alpha$  induces a broad variety of antiviral mechanisms including increased expression of pro-apoptotic proteins and MHC-I antigen presenting molecules in infected cells or by activating antiviral enzyme complexes (e.g. RNA-dependent protein kinase (PKR), 2'5'oligoadenylate synthetase (OAS) and RNAses), T cells and natural killer cells [29].

The causal interrelation between impaired production of certain cytokines and zinc status has long been subject of scientific research. Recently, decreased interleukin (IL)  $-2$  levels among zinc-deficient elderly subjects were successfully reconstituted by oral zinc supplementation [30]. Furthermore, it has already been shown *in vitro* that IFN $\alpha$  and IFN $\gamma$  response towards viral stimuli is diminished in zinc deficiency and increased after administering zinc *in vitro* [31–33]. In addition, zinc is known to enhance IFN $\alpha$ -mediated protection of host cells against virus infection [34].

Therefore, the aim of this study was to determine, whether vegetarian and vegan individuals, another cohort prone to zinc deficiency, show reduced IFN $\alpha$  production. If so, the question arises if oral zinc supplementation restores IFN $\alpha$  output. Moreover, the goal was to elucidate the underlying molecular mechanism. In order to do so, we focused on potentially zinc-dependent components of the signaling pathway initiating IFN $\alpha$  secretion, such as interferon regulatory factor (IRF) 3 [35].

Current literature on the regulation of IRF3 gene expression itself is scarce, since most projects focus on its posttranslational modification upon viral stimuli. IRF3 is constitutively expressed and the zinc-finger transcription factors Sp1 and Sp3 predominantly regulate its transcription [36]. The principal mechanism of IRF3 function is not an upregulation of its expression, but its activation through phosphorylation. IRF3 steady-state mRNA

levels remain unchanged upon viral infection [37,38]. To the best of our knowledge, this the first project to investigate a potential relationship between zinc status and IRF3 levels. Many viruses subvert host cell antiviral responses by increasing proteasomal degradation or inhibiting dimerization of IRF3 [39,40]. Accordingly, if IRF3 levels are reduced in knock-down mice models, transcription of  $\alpha/\beta$  interferon genes is ultimately disrupted, IFN type I secretion decreases and viral replication increases [41]. Further, it has been shown that the structure of the DNA-binding domain of IRF3 is zinc-dependent [42], thereby identifying it as a promising molecular target for detecting effects of zinc deficiency on antiviral immunity [31].

Results from this project are valuable to enhance identification of individuals at risk for compromised antiviral immune response and shift attention to the promising role of oral zinc supplementation in mitigating deficiencies of certain plant-based diets. In addition, we provide a deepened understanding of zinc as a player in IFN $\alpha$  signaling.

## 2. Material and methods

### 2.1. Subject recruitment and study design

The project was carried out from February to September 2024. The study design underwent review and received approval by the institutional ethics committee of the RWTH Aachen Medical Faculty (EK 23–234). Throughout the course of this research project, we complied with the principles of the Declaration of Helsinki. Participants provided written informed consent upon registration. The study included young participants aged 18–35 years, who were recruited via call from the Medical Faculty of RWTH Aachen University. Participants with omnivorous, vegetarian or vegan diets were all eligible for inclusion if they had adhered to their respective diet for at least 3 months in self-report.

Subjects were classified as vegetarians if they had followed at least a lacto-ovo-vegetarian diet, allowing the consumption of dairy and eggs but omitting meat and fish. As per definition, vegans completely abstained from any animal products and additives.

Exclusion criteria applied to all subjects included: 1) autoimmune diseases, 2) any infection (e.g. flu-like symptoms, fever  $>37.5/37.7$  C° in sublingual/axillary measurement) and 3) any antimicrobial/antiviral therapy two weeks prior to participation.

The omnivore control group contained 65 healthy young adults, compared to 45 vegetarians and 18 vegans. Consistent with similar studies, zinc deficiency (ZD) was identified by a serum zinc concentration below 70  $\mu\text{g}/\text{dL}$ . Participants with serum zinc levels above 70  $\mu\text{g}/\text{dL}$  and an adjusted zinc diet score below 113 points in the food frequency questionnaire (FFQ) were classified as “at risk of zinc deficiency” (rZD) [43–45]. Vegetarian and vegan subjects with diagnosed zinc deficiency received daily supplementation of 50 mg zinc-bis-(dl-hydrogen aspartate) equivalent to 10 mg zinc for 14 days (Unizink®, Köhler Pharma, Albach-Hähnlein, Germany). The testing procedure described in the following was conducted before and after zinc supplementation of each participant.

### 2.2. Blood collection, whole-blood assay and isolation of peripheral blood mononuclear cells (PBMC)

15 ml of blood was drawn via venipuncture into 50 ml tubes (Sarstedt, Nümbrecht, Germany) together with 150  $\mu\text{l}$  of heparine (5000 U/ml, Braun, Melsungen, Germany). Participants were not fastened. For whole-blood assay 100  $\mu\text{l}$  of heparinized blood were diluted 1:10 in culture medium per tube. The culture medium consisted of RPMI 1640 containing 10 % fetal calf serum (FCS,

Capricorn, Ebsdorfergrund, Germany), 2 mM L-glutamine, 100 U/mL potassium penicillin and 100 U/mL streptomycin sulfate (all Sigma–Aldrich, Steinheim, Germany). Remaining heparinized blood was used for PBMC isolation. PBMC were separated by centrifugation without brake over Biocoll Separating Solution (1.077 g/mL, Capricorn, Ebsdorfergrund, Germany) for 20 min at 600×g and then cleaned from remaining serum by centrifugation with phosphate-buffered saline (PBS, Sigma–Aldrich, Steinheim, Germany) for 10 min at 300×g. The PBMC pellet was then washed three times with PBS at 300×g for 5 min each. Centrifugation was carried out at 20 °C and PBMC were adjusted to a final concentration of  $1 \times 10^6$  cells/mL in culture medium.

Whole-blood assays and PBMC samples (950 µl each) were stimulated with 50 µl of culture medium (negative control; C), Sendai virus (SDV) with a multiplicity of infection (MOI) of  $6.92 \times 10^{-5}$  or Newcastle disease virus (NDV) with a MOI of  $8.65 \times 10^{-6}$  in a humidified 5 % CO<sub>2</sub> atmosphere at 37 °C for 24 h. The infectious virus titer was determined by means of the Spearman–Kärber method and corresponds to  $2.21 \times 10^4$  PFU/ml for each virus. In preliminary titration experiments with SDV and NDV virus stock, this MOI showed optimal IFN $\alpha$  response.

### 2.3. Assessment and calculation of study participants' baseline characteristics

At the time of enrollment, gender, age, and diet of study participants were recorded. The dietary zinc intake was assessed by an established 18-item FFQ (<https://www.zink-app.de>) focused on zinc [45]. Based on the instructions from the questionnaire, a phytate-corrected zinc diet score was calculated. Food frequency, serving types, and quantities were recorded. Based on the FFQ responses, individuals with an adjusted zinc diet score of less than 113 points and an serum zinc concentration above 70 µg/dL were classified as “at risk of zinc deficiency” (rZD) [45].

### 2.4. Serum zinc measurement

Serum samples were obtained using 9 ml serum monovettes designed for zinc measurement (Sarstedt, Nümbrecht, Germany) and allowed to clot for a minimum of 30 min at room temperature. Following this, the samples were centrifuged for 10 min at 1841×g. Prior to measuring serum zinc, the serum was diluted 1:2 in deionized water and stored in sterile Eppendorf tubes at –20 °C. The final serum zinc concentration was measured using flame atomic absorption spectrometry (AAS) with an AAnalyst 800 (Perkin–Elmer, Waltham, United States), as previously described [46]. Consistent with similar studies, serum zinc concentrations below 70 µg/dL were deemed zinc-deficient (ZD).

### 2.5. IFN $\alpha$ quantification

After 24 h of incubation, supernatants for IFN $\alpha$  determination were taken from the whole-blood assays and the PBMC samples. The supernatants were then stored at –80 °C. After dilution, IFN $\alpha$  protein concentrations in the supernatants were determined using the panIFN $\alpha$  ELISA (Antibodies, Stockholm, Sweden) according to the manufacturer's instructions, with a detection limit of 6.25 pg/mL. Each sample was analyzed in duplicate, and the absorption was measured with a Spark 10M wellplate reader (Tecan, Crailsheim, Germany).

### 2.6. Western blotting

Post PBMC isolation,  $2 \times 10^6$  cells per sample were prepared for quantification via Western blot, as previously detailed [47].

Samples were resuspended in 100 µl of sampling buffer (65 mM Tris–HCl (pH 6.8), 2 % (w/v) SDS, 1 mM sodium orthovanadate, 26 % (v/v) glycerol, 1 % (v/v)  $\beta$ -mercaptoethanol, 2 % proteinase inhibitor and 0.01 % (w/v) bromphenol blue).

3 µl of a colored, prestained standard (New England BioLabs, Frankfurt a.M., Germany) were used in a pocket of each 10 % polyacrylamide gel for protein mass estimation. Sample size per pocket was set to 20 µg by protein detection (Pierce 660 nm Protein Assay Reagent, Thermo Fisher, Rockford, USA) beforehand. Primary antibodies against IRF3 (#4302, Cell Signaling Technology, Leiden, Netherlands) and the housekeeping gene  $\beta$ -actin (#4967, Cell Signaling Technology, Leiden, Netherlands) were diluted 1:1000 in tris buffered saline (TBS: 20 mM Tris–HCl, 150 mM NaCl; AppliChem, Darmstadt, Germany), supplemented with 0.4 % Tween 20 (Sigma–Aldrich) and containing 5 % bovine serum albumin (BSA, AppliChem). Membranes were incubated with the primary antibodies overnight at 4 °C. The following day, membranes were washed three times with TBS-T (20 mM Tris–HCl (pH 7.6), 150 mM NaCl, 0.1 % (v/v) Tween 20) before the secondary antibody incubation. The secondary antibody, an HRP-coupled anti-rabbit IgG (Cell Signaling Technology, Frankfurt a.M., Germany), was diluted 1:2000 in TBS-T with 5 % powdered milk and incubated for 3 h at room temperature. Afterwards, membranes were washed three times with TBS-T. Westar Antares reagent (Cyanagen, Bologna, Italy) was employed for visualizing the specific protein bands at 55 kDa (IRF3) and 42 kDa ( $\beta$ -actin). Luminescence was analyzed using an LAS 3000 (Fujifilm Lifescience, Düsseldorf, Germany) and relative protein expression was quantified with ImageJ (NIH, USA) by calculating the ratio of IRF3 and  $\beta$ -actin expression for each sample. When samples across different blots were compared, the relative protein expression of each sample was normalized to a corresponding loading control present in each gel.

### 2.7. Statistics

Statistical analysis was performed using GraphPad Prism (Version 10.2.1). All data were cleaned from outliers (ROUT,  $Q = 1$  %) and tested for normality by Shapiro–Wilk test. The methods implemented to test for statistical significance are listed in the respective figure legends. Whether data were considered as paired or unpaired parameters, is stated in the respective figure legend as well. Correlations were tested by calculating the Spearman correlation coefficient  $r$ . P-values <0.05 were deemed statistically significant.

### 2.8. Power analysis

In order to determine the required sample size ( $n$ ) for our study, we conducted a power analysis based on a previous investigation from our institute examining the effects of zinc supplementation on CREM $\alpha$ -mediated IL-2 suppression in elderly individuals. In that study, 10 elderly participants (from an initial cohort of 31) received zinc supplementation [30]. By utilizing paired t-test data from this respective project concerning serum zinc concentrations before and after supplementation, we calculated a standardized effect size of 1.23 (derived from a pooled standard deviation of 10.77 and a mean difference of 13.25). The power analysis (effect size: 1.23, significance level: 0.05, target power: 0.8) indicated a minimum required sample size of 8 participants per cohort. Our study included 65 omnivores, 46 vegetarians, and 18 vegans, thereby exceeding this minimum threshold to enable preliminary comparisons between the three dietary groups (omnivores, vegetarians, and vegans;  $n \geq 8$  per group) prior to zinc supplementation. We further accounted for potential ineligibility for zinc

supplementation and anticipated dropouts to ensure adequate sample sizes for zinc supplementation experiments. Ultimately, 32 vegetarians and 16 vegans completed the supplementation.

### 3. Results

#### 3.1. Participant demographics

The study, conducted at RWTH Aachen University Hospital from February to September 2024, included a total of 128 participants. Sample population was subdivided into three dietary groups, each consisting of individuals with either an omnivore (n = 65), vegetarian (n = 45) or vegan (n = 18) nutritional approach. Table 1 contains information on baseline characteristics such as age, gender and prevalence of zinc deficiency. The average age of the omnivore participants (23.9 ± 3.2 years) was consistent with the respective means of the vegetarian (22.6 ± 2.6 years) and vegan (23 ± 3.2 years) cohort. Female subjects accounted for the majority of volunteers in all three dietary groups, with matching gender distribution in the vegetarian and vegan study collectives (84 % vs. 83 %). In the omnivore cohort, female gender led in quantity with 63 %.

#### 3.2. Zinc status is affected by plant-based diet

Upon recruiting participants Fig. 1 for the study, zinc status was evaluated by using two different assessment methods, which enabled us to verify previous reports on increased prevalence of zinc deficiency among individuals who abstain from animal products [13]. Serum zinc measurement is the most common and validated biomarker for assessing severe zinc deficiency, but lacks reliability in detecting mild to moderate deficiency [48,49]. Unfortunately, even marginal disparities in one's zinc status can lead to impaired function of the immune system [50]. Thus, we also monitored dietary zinc intake by using a FFQ [45]. The FFQ assesses the intake of bioavailable zinc by monitoring the phytate level in each diet and then calculating the concomitant reduction in zinc resorption [45]. Therefore, the questionnaire is a valuable tool for detecting individuals at risk for deficiency or with moderate severity. Ultimately, this data also builds the foundation for hypothesizing a potential causal relationship between zinc status and IFN $\alpha$  production.

Data acquired through the FFQ revealed a significant difference in prevalence of insufficient dietary zinc intake between omnivores and the two subgroups following a plant-based diet. While

only 2 % of the omnivore cohort showed inadequate zinc intake, 71 % of vegetarian and 94 % of vegan participants (Table 1) fell below cut-off. The mean adjusted (adj.; phytate-corrected) zinc diet scores (Fig. 1 A) differed significantly (OM vs. VT: p < 0.0001; OM vs. VG: p < 0.0001), thereby further underlining the discrepancies in bioavailable zinc intake due to a plant-based diet.

Moreover, serum zinc levels were measured using atomic absorption spectrometry (AAS), thereby, assessing the number of individuals not only at risk but with more severe zinc deficiency (cut-off <70  $\mu$ g/dL). Accordingly, we observed a similar trend, especially evident among vegan subjects. The mean serum zinc levels of all three subgroups exhibited significant disparities (OM vs. VT: p = 0.031; OM vs. VG: p < 0.0001; VT vs. VG: p = 0.0003). Omnivores presented an average serum zinc of 83.0 ± 11.1  $\mu$ g/dL, vegetarians 78.2 ± 13.0  $\mu$ g/dL and, remarkably, vegans reached 64.9 ± 12.1  $\mu$ g/dL, remaining below the cut-off value for zinc deficiency (Fig. 1 B). Therefore, 67 % of the vegan subgroup showed manifest zinc deficiency, whereas the frequency within cohorts that included animal products in their diet, appeared to be significantly lower (Table 1, Fisher's exact test).

Furthermore, we subdivided each cohort into male and female participants and tested for sex-dependent differences concerning serum zinc concentrations (Fig. 1 C). In the omnivorous and vegetarian subgroup, male subjects displayed significantly higher serum zinc levels than their female counterparts (m OM vs. f OM: p = 0.0431; m VT vs. f VT: p = 0.0126). While a comparable trend was evident in the vegan cohort, the result was not statistically significant.

To further differentiate the findings, we performed sex-specific analyses by comparing only male or only female participants of each cohort across the respective subgroups. While comparing only female participants in each subgroup, we observed a statistical trend similar to Fig. 1 B, with all three female-only cohorts differing significantly in serum zinc (t-test: f OM vs. f VT: p = 0.0341; f OM vs. f VG: p < 0.0001; f VT vs. f VG: p = 0.0004). At the same time, only the male vegans differed significantly from the male omnivores, concerning serum zinc (t-test: m OM vs. m VG: p = 0.0109). No further differences of statistical significance between male-only subgroups were observed (t-test: m OM vs. m VT: p = 0.7861; m VT vs. m VG: p = 0.2064).

#### 3.3. Reduced IFN $\alpha$ production among vegetarians and vegans

It has been shown that zinc has ubiquitous immunomodulatory effects on human antiviral immune response [51]. Further, data

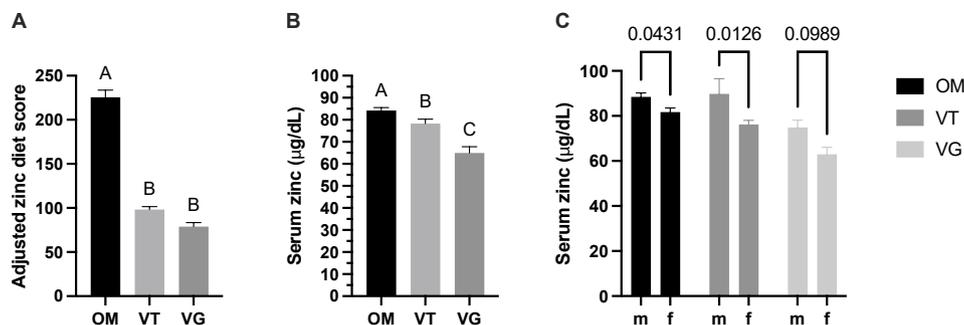
**Table 1**  
Characteristics of study participants.

Characteristics		Omnivores (OM) n = 65	Vegetarians (VT) n = 45	Vegans (VG) n = 18
Gender <sup>a</sup>	Female, n (%)	41 (63) <sup>a</sup>	38 (84) <sup>b,c</sup>	15 (83) <sup>a,c</sup>
	male, n (%)	24 (37)	7 (16)	3 (17)
Age <sup>b</sup> , years	Mean ± SD	23.9 ± 3.2 <sup>a</sup>	22.6 ± 2.6 <sup>a</sup>	23 ± 3.2 <sup>a</sup>
	Median	23	22	22.5
	Min, Max	19, 35	19, 30	20, 30
Zinc status <sup>a</sup>	ZA in FFQ, n (%)	64 (98) <sup>a</sup>	13 (29) <sup>b</sup>	1 (6) <sup>b</sup>
	ZD in FFQ, n (%)	1 (2)	32 (71)	17 (94)
	ZA in AAS, n (%)	58 (89) <sup>a</sup>	34 (76) <sup>a</sup>	6 (33) <sup>b</sup>
	ZD in AAS, n (%)	7 (11)	11 (24)	12 (67)
	rZD, n (%)	0 (0)	23 (51)	5 (28)

**Abbreviations:** zinc-adequate (ZA), zinc-deficient (ZD), at risk for zinc deficiency (rZD); subjects are ZD in FFQ and ZA in AAS), food frequency questionnaire (FFQ), atomic absorption spectrometry (AAS).

<sup>a</sup> **Fisher's exact test.** Means not sharing the same letter indicate statistical difference (p < 0.05). **Gender:** OM vs. VT: p = 0.0177; OM vs. VG: p = 0.1555; VT vs. VG: p > 0.9999, **Zinc status in FFQ:** OM vs. VT: p < 0.0001; OM vs. VG: p < 0.0001; VT vs. VG: p = 0.0509, **Zinc status in AAS:** OM vs. VT: p = 0.0694; OM vs. VG: p < 0.0001; VT vs. VG: p = 0.0032.

<sup>b</sup> **One-way ANOVA and Tukey's post-hoc test.** Means not sharing the same letter indicate statistical difference (p < 0.05). **Age:** OM vs. VT: p = 0.0528; OM vs. VG: p = 0.4824; VT vs. VG: p = 0.8563.



**Fig. 1. Baseline zinc status is reduced in vegetarians and vegans.** (A) Dietary zinc intake of omnivores (OM:  $n = 65$ ), vegetarians (VT:  $n = 44$ ) and vegans (VG:  $n = 17$ ) was assessed using a short 18 item food frequency questionnaire (OM vs. VT:  $p < 0.0001$ ; OM vs. VG:  $p < 0.0001$ ; VT vs. VG:  $p = 0.372$ ). Cut-off value for increased risk of zinc deficiency was set to an adjusted zinc diet score of 113. (B) Serum zinc concentrations of OM ( $n = 65$ ), VT ( $n = 45$ ) and VG ( $n = 18$ ) were measured with AAS (OM vs. VT:  $p = 0.031$ ; OM vs. VG:  $p < 0.0001$ ; VT vs. VG:  $p = 0.0003$ ). (C) Serum zinc concentrations of each cohort subdivided into male (m) and female (f) participants (m OM:  $n = 24$ ; f OM:  $n = 41$ ; m VT:  $n = 7$ ; f VT:  $n = 38$ ; m VG:  $n = 3$ ; f VG:  $n = 15$ ). All data are considered unpaired parameters and were cleaned from outliers (A: VT:  $n = 1$ ; VG:  $n = 1$ ; B and C: no outliers; ROUT, 1 %). Results are presented as the mean + SEM and are normally distributed. Statistical significance was calculated by ordinary one-way ANOVA followed by Tukey's post-hoc test (A + B) and by two-way ANOVA followed by Holm-Sidak's multiple comparisons test (C). Means not sharing any letter indicate significantly different results ( $p < 0.05$ ). Exact  $p$ -values are reported above.

has been published that indicates an interaction of zinc with IFN $\alpha$  release [31]. Thus, we wanted to elucidate if decreased zinc uptake due to plant-based diets would compromise immune function through reduced IFN $\alpha$  response upon virus stimulation.

In order to simulate a virus infection, blood samples were drawn from each participant and a whole-blood assay was conducted to test cellular responsiveness. Furthermore, PBMC were isolated and used as an additional approach to reduce confounding interindividual disparities. Samples were stimulated for 24 h *in vitro* with either NDV or SDV, two commonly used viruses to test IFN $\alpha$  production in research settings. The results align with the pattern previously observed during the evaluation of zinc status in each respective cohort.

The subgroups with higher prevalence of impaired zinc status showed the tendency to have reduced IFN $\alpha$  responses. Interestingly, the trend was consistent throughout all four different experimental approaches except for SDV stimulation in whole-blood assay (Fig. 2 A). While the omnivore cohort showed significantly higher IFN $\alpha$  levels than the vegetarian cohort ( $p = 0.0065$ ), production of vegans did not differ significantly from either group. In response to NDV application in whole-blood assay (Fig. 2 B), omnivores ( $896.6 \pm 404.7$  pg/ml) showed thrice as high cytokine output compared to vegetarians ( $278.0 \pm 171.4$  pg/ml; OM vs VT:  $p < 0.0001$ ) and vegans ( $261.2 \pm 208.3$  pg/ml; OM vs VG:  $p < 0.0001$ ). A matching trend was observed in PBMC stimulation with SDV (Fig. 2 C) and NDV (Fig. 2D).

In addition, we analyzed IFN $\alpha$  response by grouping participants by their zinc status, indifferent of their respective dietary approach (Fig. 2 E). Both, individuals with adequate serum zinc levels but reduced adjusted zinc diet score (rZD) and participants with impaired serum zinc levels (ZD), showed significantly decreased IFN $\alpha$  production compared to the zinc-adequate cohort.

### 3.4. IRF3 levels show same trend as IFN $\alpha$ response among omnivores, vegetarians and vegans

The transcription factor IRF3 regulates the expression of different interferon subtypes in case of infection [52]. After pathogen recognition, IRF3 is activated by phosphorylation and it is translocated into the nucleus, where transcription of IFN type I genes is induced [53]. Correlation between baseline levels of IRF3 and zinc status in each subgroup would strongly indicate a causal relationship. To investigate the molecular mechanism behind the

zinc-dependent IFN $\alpha$  response, we, therefore, measured IRF3 levels in unstimulated samples throughout all three cohorts.

Paralleling the trends detected in dietary zinc uptake (Fig. 1 A), serum zinc levels (Fig. 1 B) and IFN $\alpha$  production (Fig. 2), baseline value of transcription factor IRF3 more than halved among vegetarians (OM vs. VT:  $p = 0.0043$ ) and vegans (OM vs. VG:  $p = 0.0097$ ) in contrast to omnivores (Fig. 3).

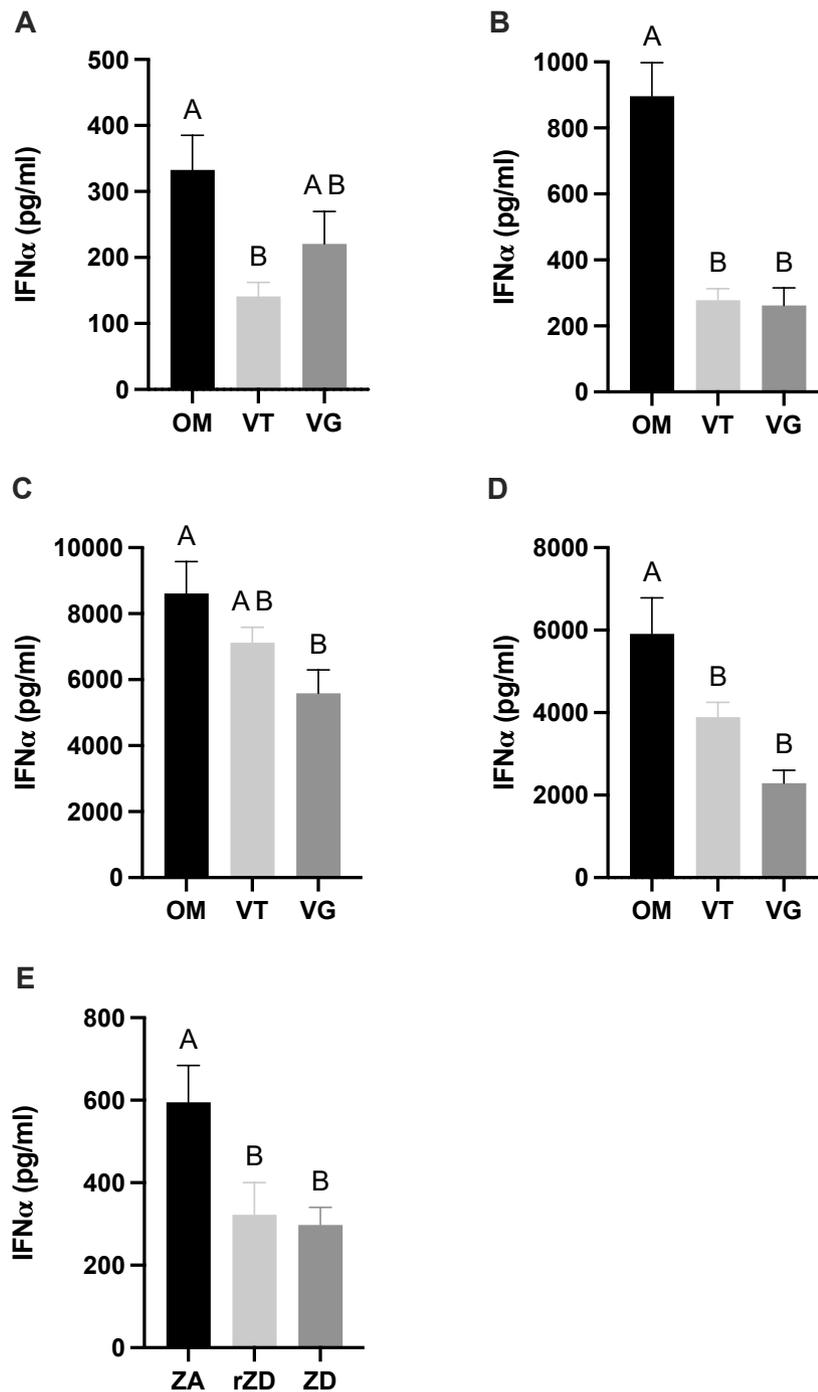
### 3.5. Zinc supplementation of vegetarians and vegans with zinc deficiency improves zinc status and IFN $\alpha$ production by increasing IRF3 expression

We further examined if the antiviral immune response of zinc-deficient vegetarians and vegans (serum zinc  $< 70$  µg/dL) can be improved by oral zinc supplementation. Omnivores that showed zinc deficiency in serum ( $n = 6$ ), were not supplemented and are not included in the following experiments. 10 mg of zinc was administered as zinc-aspartate for 14 days. To verify the effect of the therapy, serum zinc levels were measured before and after zinc supplementation (Fig. 4 A). Vegetarians and vegans with zinc deficiency in serum were pooled and showed mean serum zinc levels of  $58.9 \pm 5.9$  µg/dL before treatment. After supplementation, serum zinc increased significantly ( $p < 0.0001$ ), reaching an average of  $74.0 \pm 7.1$  µg/dL, which is deemed zinc sufficient [54].

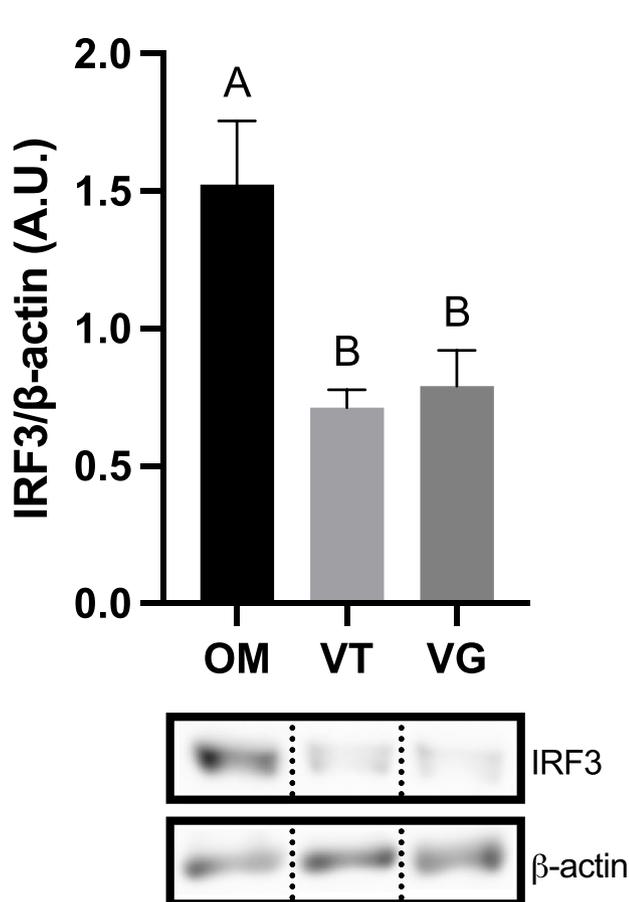
Furthermore, we conducted Western blot analysis for IRF3 before and after the 14-day cycle of zinc supplementation (Fig. 4 B). Evidently, expression of IRF3 increased significantly after administering zinc. In line with previous projects, IRF3 expression levels were not increased upon viral stimuli, since the effect of IRF3 signaling predominantly relies on posttranslational phosphorylation [37,38].

### 3.6. IRF3 expression correlates significantly with serum zinc status

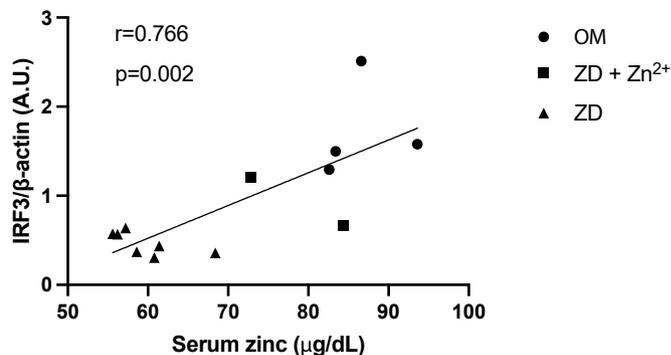
We already observed elevated IRF3 protein levels after zinc supplementation, but if zinc status correlates with the respective extent of IRF3 expression had to be further analyzed. Therefore, we conducted a correlation analysis with the previously introduced Western blot data from the OM, ZD + Zn $^{2+}$  and ZD cohort (Fig. 5). Thereby, we were able to substantiate a significant statistical relationship, which further argues a causal correspondence between serum zinc and IRF3 levels.



**Fig. 2. IFN $\alpha$  production is reduced in vegetarians and vegans.** Graphs A–D depict decreasing IFN $\alpha$  production of vegetarians (VT) and vegans (VG) compared to omnivores (OM). Graph E shows reduced IFN $\alpha$  response among subjects classified as “at risk of zinc deficiency” (rZD) and “zinc-deficient” (ZD) compared to “zinc-adequate” (ZA) subjects, indifferent of their individual dietary approach. (A) IFN $\alpha$  production in whole-blood assay after SDV stimulation (OM: n = 16, VT: n = 33, VG: n = 16; OM vs. VT: p = 0.009; OM vs. VG: p = 0.3553; VT vs. VG: p = 0.7425) or (B) NDV stimulation in whole-blood (OM: n = 16, VT: n = 24, VG: n = 15; OM vs. VT: p < 0.0001; OM vs. VG: p < 0.0001; VT vs. VG: p = 0.9803). (C) IFN $\alpha$  production in PBMC after SDV stimulation (OM: n = 16, VT: n = 33, VG: n = 18; OM vs. VT: p = 0.2569; OM vs. VG: p = 0.0155; VT vs. VG: p = 0.2124) or (D) NDV stimulation in PBMC (OM: n = 16, VT: n = 26, VG: n = 14; OM vs. VT: p = 0.0225; OM vs. VG: p = 0.0002; VT vs. VG: p = 0.1048). (E) NDV stimulation in whole-blood (ZA: n = 23, rZD: n = 15, ZD: n = 16; ZA vs. rZD: p = 0.0453; ZA vs. ZD: p = 0.0234; rZD vs. ZD: p = 0.9778). All data were cleaned from outliers (A: VT: n = 2; VG: n = 2; B: VT: n = 4; VG: n = 2; C: no outliers; D: VT: n = 1; VG: n = 3; E: rZD: n = 1; ZD: n = 5; ROUT, 1 %) and are considered unpaired parameters. Data is presented with mean + SEM. Statistical significance was calculated by Kruskal–Wallis test followed by Dunn’s multiple comparison test in case of non-normal distribution (A) or by ordinary one-way ANOVA followed by Tukey’s post-test for gaussian normal distribution (B – E). Means not sharing any letter indicate statistical significance (p < 0.05). Exact p-values are reported above.



**Fig. 3. IRF3 levels are reduced in vegetarians and vegans.** IRF3 protein expression was examined in omnivores (OM), vegetarians (VT) and vegans (VG) by Western blot from PBMC. Samples had been incubated in medium for 24 h but were not stimulated with virus. IRF3 expression was normalized to β-actin. A representative Western blot is shown. Statistically significant differences between OM (n = 8), VT (n = 8) and VG (n = 8) were calculated by ordinary one-way ANOVA followed by Tukey's post-hoc test (OM vs. VT: p = 0.0043; OM vs. VG: p = 0.0097; VT vs. VG: p = 0.9354). All data are considered unpaired parameters. No outliers (ROUT, 1 %). Results are presented as the mean + SEM and are normally distributed. Means not sharing any letter indicate statistical significance (p < 0.05). Exact p-values are reported above.



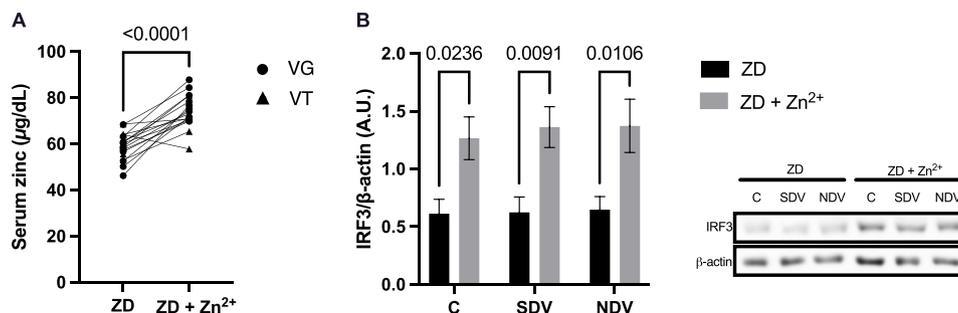
**Fig. 5. Serum zinc levels correlate with IRF3 expression.** Serum zinc and Western blot data from zinc-deficient vegetarians and vegans (ZD; n = 7), participants after zinc supplementation (ZD + Zn<sup>2+</sup>; n = 2) and omnivores (OM; n = 4) was analyzed. Correlation was determined by using Spearman rank correlation coefficient (p = 0.002). To achieve comparability of separate blots, results were normalized to a positive control run on every gel. No outliers (ROUT, 1 %).

Consequently, we tested if there was an imminent effect of IRF3 upregulation on IFNα production by performing ELISA analysis before and after two weeks of zinc supplementation (Fig. 6). Results of the supplemented group (ZD + Zn<sup>2+</sup>) revealed a consistent increase in IFNα production in whole-blood assay (Fig. 6 A,B) and PBMC (Fig. 6 C,D). Moreover, application of either SDV (Fig. 6 A,C) or NDV (Fig. 6 B,D) led to matching trends concerning elevated IFNα levels after zinc treatment.

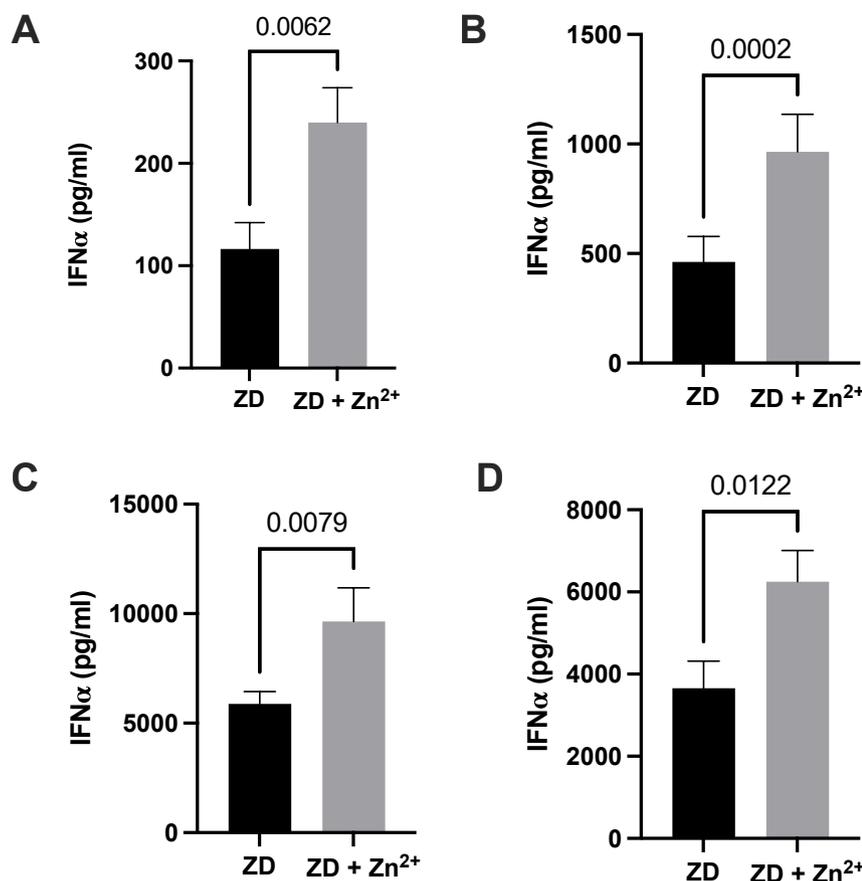
### 3.7. Zinc supplementation of vegetarians and vegans with risk for zinc deficiency improves IFNα production

The data presented so far primarily concerned vegetarians and vegans that had clinically manifest zinc deficiency confirmed by serum zinc measurement in AAS (ZD cohort). The question was raised, if vegetarians and vegans, who only showed an increased “risk for zinc deficiency” (rZD cohort) in the FFQ, would also benefit from zinc supplementation, even if serum zinc levels were not yet reduced. Therefore, participants with an adjusted zinc diet score below 113 and serum zinc levels above 70 μg/dL were summarized as the “rZD” cohort, with the aim to detect possible changes to serum zinc status, IRF3 expression and IFNα response.

Unlike the ZD cohort (Fig. 4A), oral zinc supplementation in the rZD group did not lead to changes in serum zinc status (Fig. 7A).



**Fig. 4. Serum zinc levels and IRF3 expression increase in the ZD cohort after zinc supplementation.** Data was collected from vegetarians and vegans zinc-deficient in serum (ZD). (A) Serum zinc of all participants was measured with AAS. Zinc levels prior to supplementation (ZD; n = 18) differed significantly from results observed after treatment (ZD + Zn<sup>2+</sup>; n = 18). Data were considered paired parameters, since the exact same subjects were once analyzed before and once after supplementation. Statistical significance was calculated by paired t-test. No outliers (ROUT, 1 %) (B) Samples for Western blot analysis were drawn from PBMC that were unstimulated (control, C) or had been stimulated with either SDV or NDV for 24 h. IRF3 expression was normalized to β-actin. A representative Western blot is shown. In all three experimental approaches (C, SDV, and NDV) IRF3 was significantly upregulated in the ZD + Zn<sup>2+</sup> treatment group (n = 8) compared to the ZD cohort (n = 8) prior to supplementation. Statistical significance was calculated by ordinary two-way ANOVA followed by Sidák's multiple comparisons test. No outliers (ROUT, 1 %). Results are presented as the mean + SEM and are normally distributed. Exact p-values are reported in each graph.



**Fig. 6.** IFN $\alpha$  production increases in the ZD cohort after zinc supplementation. Data includes vegetarians and vegans who initially showed zinc deficiency in serum (ZD) and completed supplementation. IFN $\alpha$  levels were measured by ELISA before and after zinc supplementation (ZD + Zn<sup>2+</sup>). (A) IFN $\alpha$  production in whole-blood assay after SDV stimulation (ZD: n = 12; ZD + Zn<sup>2+</sup>: n = 12) or (B) NDV stimulation (ZD: n = 14; ZD + Zn<sup>2+</sup>: n = 14). (C) IFN $\alpha$  production in PBMC after SDV stimulation (ZD: n = 17; ZD + Zn<sup>2+</sup>: n = 17) or (D) NDV stimulation (ZD: n = 12; ZD + Zn<sup>2+</sup>: n = 12). All data were cleaned from data pairs identified as outliers (A: n = 5; B: no outliers; C: no outliers; D: n = 1; ROUT, 1 %) and are considered paired parameters, since the exact same subjects were once analyzed before and once after supplementation. Data is presented with mean + SEM. Statistical significance was calculated by paired t-test in case of normal distribution (A) or by Wilcoxon-test for non-normal distribution (B – D). Exact p-values are reported in each graph.

In addition, the effect of zinc supplementation on IRF3 expression was less extensive than in the ZD cohort (Fig. 4B). Despite a trend towards slightly increased levels of IRF3 in the zinc-supplemented rZD cohort (rZD + Zn<sup>2+</sup>), it did not reach statistical significance (Fig. 7B).

All in all, we observed unaltered serum zinc levels and no significant increase in IRF3 expression after zinc supplementation in the rZD cohort. Nevertheless, IFN $\alpha$  response upon viral stimuli was elevated following 14-days of zinc treatment (Fig. 8). Significant enhancement of cytokine production after zinc supplementation was observed in PBMC stimulated with either SDV or NDV (Fig. 8 C+D) and whole-blood assay incubated with NDV (Fig. 8 B). Whole-blood assays with SDV stimulus followed the same trend but did not show significant IFN $\alpha$  increase (Fig. 8A).

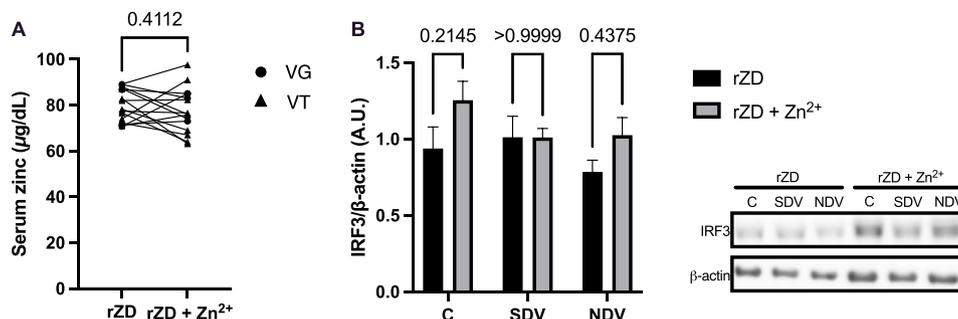
#### 4. Discussion

The essential role of zinc in a multitude of immunological properties underscores the importance of sufficient dietary zinc intake [24]. Common plant-based diets contain reduced amounts of zinc and are further characterized by reduced bioavailability [13]. Especially in the light of the continuously increasing prevalence of plant-based diets, investigating the impact of zinc

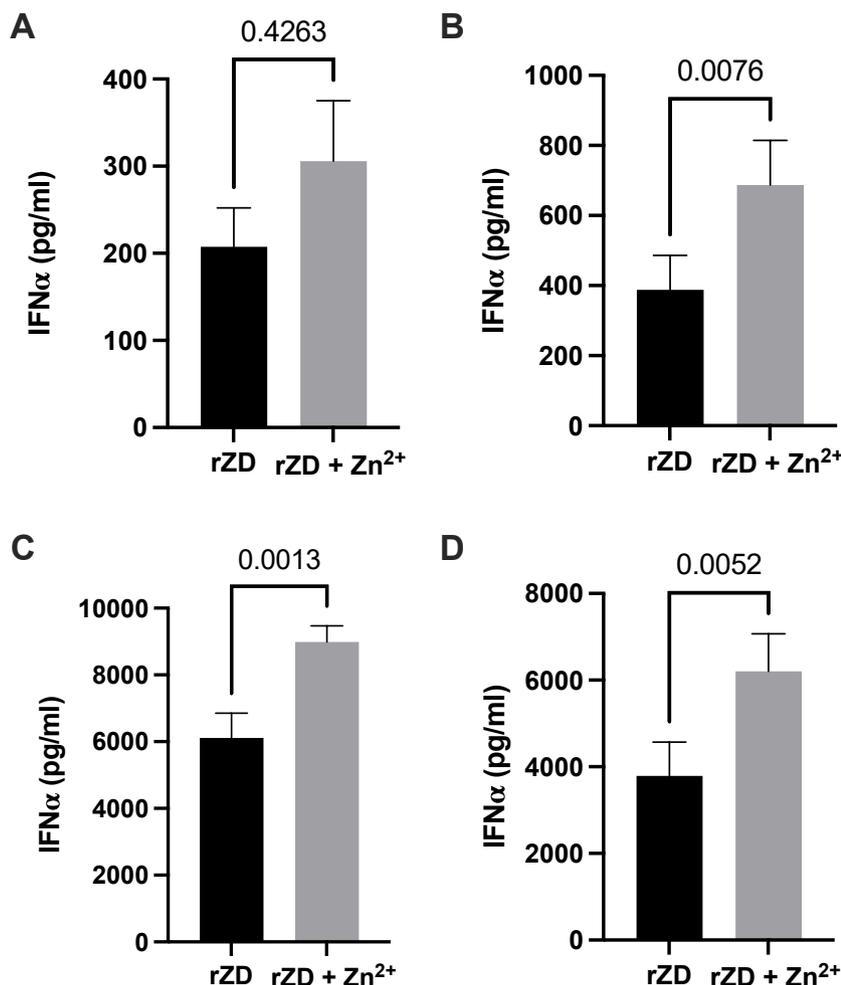
deficiency among vegetarians and vegans on their immune system is of great interest. Findings on this matter might be valuable to optimize dietary patterns and prevent health implications.

In this study, we observed an impaired IFN $\alpha$  response towards viral stimuli as a result of zinc deficiency-induced IRF3 down-regulation in vegetarian and vegan individuals. Furthermore, we were able to reconstitute IFN $\alpha$  production by oral zinc supplementation *in vivo*. Precedingly, Cakman et al. had shown reduced production of IFN $\alpha$  in PBMC of elderly stimulated *in vitro* and could improve cytokine production by zinc supplementation *in vitro* [31]. However, they did not assess the individual zinc status nor did they elucidate the underlying molecular mechanism. Therefore, based on our current knowledge, this is the first project to describe impaired antiviral immune response among vegetarians and vegans in terms of reduced IFN $\alpha$  production mediated by reduced IRF3 expression. In addition, this is the first time oral zinc supplementation was successfully implemented to restore IRF3 expression and, thereby, enhance antiviral immunity by optimizing IFN $\alpha$  output.

Before carrying out any analyses concerning zinc status, IFN $\alpha$  production and IRF3 expression, we had to define the minimum time span of diet adherence for individuals eligible for inclusion. In a prospective twin study by Landry et al., first metabolic



**Fig. 7. Serum zinc levels and IRF3 expression do not increase in the rZD cohort after supplementation.** Data was collected from vegetarians and vegans at risk for zinc deficiency in the FFQ (rZD). (A) Serum zinc of participants was measured with AAS. Zinc levels prior to supplementation (rZD; n = 15) did not differ from results after treatment (rZD + Zn<sup>2+</sup>; n = 15). Data were considered paired parameters, since the exact same subjects were once analyzed before and once after supplementation. Statistical significance was calculated by paired t-test. No outliers (ROUT, 1 %). (B) Samples for Western blot analysis were drawn from PBMC that were unstimulated (control, C) or had been stimulated with either SDV or NDV for 24 h. IRF3 expression was normalized to β-actin. A representative Western blot is shown. In all three experimental approaches (C, SDV, and NDV) IRF3 was not significantly upregulated in the rZD + Zn<sup>2+</sup> treatment group (n = 8) compared to the rZD cohort (n = 8) prior to supplementation. Statistical significance was calculated by ordinary two-way ANOVA followed by Šidák's multiple comparisons test. No outliers (ROUT, 1 %). Results are presented as the mean + SEM and are normally distributed. Exact p-values are reported in each graph.



**Fig. 8. IFN $\alpha$  production increases in the rZD cohort after zinc supplementation.** Data includes vegetarians and vegans who showed increased risk for zinc deficiency (rZD) in the FFQ and completed supplementation. IFN $\alpha$  levels were measured by ELISA before and after zinc supplementation (rZD + Zn<sup>2+</sup>). (A) IFN $\alpha$  production in whole-blood assay after SDV stimulation (rZD: n = 14; rZD + Zn<sup>2+</sup>: n = 14) or (B) NDV stimulation (rZD: n = 11; rZD + Zn<sup>2+</sup>: n = 11). (C) IFN $\alpha$  production in PBMC after SDV stimulation (rZD: n = 15; rZD + Zn<sup>2+</sup>: n = 15) or (D) NDV stimulation (rZD: n = 12; rZD + Zn<sup>2+</sup>: n = 12). All data were cleaned from data pairs identified as outliers (A: n = 1; B: n = 1; C: no outliers; D: no outliers; ROUT, 1 %) and are considered paired parameters, since the exact same subjects were once analyzed before and once after supplementation. Data is presented with mean + SEM. Statistical significance was calculated by Wilcoxon-test in case of non-normal distribution (A) or by paired t-test for normal distribution (B – D). Exact p-values are reported in each graph.

differences were shown two months after converting from an omnivore to a vegan diet [55]. Due to possible errors in each individual's self-report, we further extended the minimum duration of diet adherence to three months.

The first objective was to accurately assess individual zinc status. Despite significant efforts in the past decades, there is no well-established zinc biomarker that reliably evaluates zinc status [56]. In line with Klein et al. who recently investigated zinc status in vegetarians and vegans, we also decided to determine zinc status by measuring serum zinc and by using the FFQ (<https://www.zink-app.de>) of Trame et al. [13,45].

The results of the FFQ showed that zinc and phytate intake in vegetarians and vegans differed significantly from the omnivore control group, indicating increased risk for zinc deficiency for 71 % (VT) and 94 % (VG) of the respective subgroup in contrast to only 2 % of the omnivores. The data concerning the FFQ were consistent with Klein et al. [13].

Our analysis of serum zinc levels revealed that vegetarians had significantly lower mean serum zinc levels compared to omnivores, with vegans showing even lower mean levels than vegetarians. Regarding serum zinc, Klein et al. could not show significant differences among omnivores, vegetarians and vegans [13]. Interestingly, even the omnivore cohort in Klein et al. had a 22.5 % prevalence of zinc deficiency in serum zinc (11 % in our study). Furthermore, mean serum zinc levels of each cohort in Klein et al. (omnivores, vegetarians and vegans) were lower compared to the measured means in our project. At this point, it should be noted that different technical methods were implemented for measuring serum zinc.

In addition, a subgroup analysis by Klein et al. only considering female vegans revealed a significant deviation from the omnivore subgroup, implying that varying gender ratios in the respective study need to be considered as well. All three subgroups in our study differed significantly in serum zinc levels, compatible with results from Schupbach et al., who also used atom absorption spectrometry for detection. Anyhow, compared to Schupbach et al., a larger proportion of vegetarians (24 % vs 18.9 %) and vegans (67 % vs 47.2 %) from our project showed zinc deficiency in serum. This, again, might be attributed to a greater share of female participants in each subgroup [15]. In line with previous work on sex-dependent serum zinc levels by Barman et al. [57], our subgroup analysis (Fig. 1 C) observed significantly lower serum zinc concentrations among females compared to their male counterparts in the omnivorous and vegetarian cohort. The same trend was seen in the vegan subgroup.

Furthermore, we carried out sex-dependent analysis of serum zinc across the three cohorts. Female-only omnivores, vegetarians and vegans all differed significantly in serum zinc, similar to the trend observed in Fig. 1 B, which took subjects of both sexes into account. In the male-only sub-analysis, solely the male vegans showed significantly lower serum zinc levels than the male omnivores. No other differences of statistical significance were detected whilst comparing the male-only cohorts. We assume, that this might be due to the small number of male participants in the vegan ( $n = 3$ ) and vegetarian ( $n = 7$ ) subgroup, which falls below the threshold of our power analysis.

Upon reviewing the results of zinc status assessment, it becomes apparent that a greater proportion of both vegetarians (FFQ: 71 % ZD vs serum zinc: 24 % ZD) and vegans (FFQ: 94 % ZD vs serum zinc: 67 % ZD) fell below cut-off in the FFQ compared to serum zinc measurement.

Especially FFQ and serum zinc results in the vegetarian cohort differ drastically, indicating that a notable number of individuals with moderately impaired zinc status would not have been detected if we had only relied on the biomarker serum zinc. These

results were anticipated, considering the role of serum zinc as a biomarker in more severe zinc deficiency and its reduced reliability in assessing mild to moderate zinc deficiency. Serum zinc levels may fluctuate due to circadian variations, recent food intake, hydration levels and present inflammation [24]. Moreover, even serum zinc levels above cut-off (70  $\mu\text{g/dL}$ ) do not rule out zinc deficiency, since moderate deficits might be hidden despite already causing health implications [16].

As discussed beforehand, particularly vegetarians and vegans consume vast amounts of phytate. Hence, additionally monitoring dietary zinc and phytate intake by FFQ is a helpful tool to detect individuals with hidden zinc shortage or increased risk of developing deficiency. Therefore, we propose that reduced bioavailability of zinc due to chelation by phytate should be taken into account by using the FFQ alongside serum zinc measurements while assessing zinc status in vegetarians and vegans [13,17]. Concluding, we have shown that plant-based diets greatly increase the risk of mild to moderate zinc deficiency, and a heightened prevalence of more severe zinc deficiency is seen in vegans.

In the next step, we investigated how the current diet impacts  $\text{IFN}\alpha$  response upon viral stimuli. SDV and NDV, two RNA viruses of the family Paramyxoviridae, were used for stimulation [58,59]. Furthermore, we used samples with normalized concentrations of PBMC in addition to whole-blood assays in order to detect potential confounders like interindividual differences in cell count and plasma composition. Especially in our experimental approach using PBMC, we found a gradually declining  $\text{IFN}\alpha$  response from omnivores over vegetarians to vegans which correlated with the mean serum zinc status of each cohort. In whole blood, vegetarians and vegans showed a similarly reduced  $\text{IFN}\alpha$  output despite differences in zinc status. This data aligns with reports from Cakman et al. on lowered  $\text{IFN}\alpha$  response towards NDV in whole blood from elderly subjects, another study population with high prevalence of zinc deficiency [31].

Surprisingly, only our results for SDV stimulation in whole-blood in the vegan cohort showed a different trend, presenting with higher cytokine production than in vegetarians and not differing significantly from the omnivore subgroup. A possible explanation is that due to the smaller sample size in the vegan cohort, host-dependent differences in susceptibility towards SDV-induced hemolysis may cause elevated  $\text{IFN}\alpha$  response [60]. Hemolysis is known to induce type I interferons and, therefore, the vegan cohort might have more individuals with increased hemolysis [61]. Once again, it is important to address the lower number of female participants in the omnivore cohort, as we already did while discussing differences in zinc status. Previous studies have shown significantly higher levels of  $\text{IFN}\alpha$  in antiviral immune response of females compared to male subjects [62–65]. Still, we observed significantly higher  $\text{IFN}\alpha$  production in the omnivore subgroup, despite a greater proportion of female subjects in the vegetarian and vegan cohort. Therefore, the difference in sex distribution between the cohorts more likely underscores the observed effect, rather than being a limitation.

Lastly, we were able to show that individuals with adequate serum zinc levels and mild to moderate zinc deficiency detected in the FFQ (rZD), exhibited a significant decrease in  $\text{IFN}\alpha$  response, comparable to the  $\text{IFN}\alpha$  production of the cohort deficient in serum (ZD) (Fig. 2 E). This further underscores the potential of implementing the FFQ in detecting individuals with impaired zinc status, who are not sufficiently identified in serum zinc measurements.

With the aim of further elucidating the underlying molecular mechanism of zinc-dependent  $\text{IFN}\alpha$  response, we aimed to investigate if IRF3, one of the components of the signaling pathway behind viral  $\text{IFN}\alpha$  induction [41], shows zinc-dependent protein

levels. Sp1 and Sp3, two zinc-finger transcription factors, predominantly induce constitutive expression of IRF3 [36] and the structure of the IRF3 DNA-binding domain appears to be zinc-dependent [42], suggesting its potential as a promising molecular target.

Therefore, we subsequently measured IRF3 levels in all three cohorts and demonstrated a significant decrease in IRF3 expression in vegetarian and vegans, correlating with their diminished IFN $\alpha$  production. Considering the central role of IRF3 in initiating type I interferon response towards RNA-viruses, these findings strongly indicate a causal relationship between impaired zinc status, lowered IRF3 levels and reduced IFN $\alpha$  response among individuals adhering to a plant-based diet. These results comply with results from Sato et al. who reported a decrease in type I interferons on mRNA and protein level in IRF3-knockdown mice infected with NDV [41]. Further research is needed to elucidate to what extent expression of Sp1 and Sp3, the main IRF3 regulators, is impacted by zinc status.

With regards to the highly homologous functional sites of IRF3 and IRF7 [26,66], further research is required on the potential involvement of IRF7 in zinc-dependent reduction of IFN $\alpha$  response. Interestingly, Molony et al. recently published on decreased IFN $\alpha$  production in monocytes from elderly subjects stimulated with influenza virus *in vitro* but did not detect any changes in baseline expression of IRF3 [35]. They attributed the impaired IFN $\alpha$  response to reduced IRF3 phosphorylation due to increased tumor necrosis factor receptor associated factor 3 (TRAF3) degradation. TRAF3 is essential for TANK-binding kinase 1 (TBK-1) activity and, ultimately impacts IRF3 activation, hence phosphorylation [67]. Therefore, IRF3 phosphorylation and signaling compounds further upstream in the RIG-I signaling pathway (e.g. TBK-1 and TRAF3) should also be focus of additional research on zinc-dependent interferon production. Next to the RIG-I pathway, components of the cGAS-STING pathway are promising candidates for future research as well. This is due to relevant cross-talk between these pathways, synergistically inducing type I interferon response [68].

Furthermore, we investigated the impact of oral zinc supplementation on the IFN $\alpha$  response in zinc-deficient participants. The dose of 10 mg daily zinc used in the treatment group was chosen in accordance with a similar *in vivo* short-term zinc supplementation of elderly by Baarz et al. and with the Population Reference Intake (PRI) for dietary zinc issued by the European Food Safety Authority (EFSA) [30,69]. The PRIs range from 7.5 to 12.7 mg/day for women and 9.4–16.3 mg/day for men [69]. Since individuals with manifest zinc deficiency or increased risk for impaired zinc status almost certainly fall below the population reference intake, 10 mg is sufficient to meet physiological requirements without imminent risk of exceeding the tolerable upper intake level of 25 mg/day [70].

Serum zinc levels of initially deficient subjects revealed sufficient increase upon supplementation. It already has been shown that oral zinc supplementation enhances serum zinc status dose-dependently in elderly subjects, therefore, our project demonstrates a concordant effect in zinc-deficient vegetarians and vegans [71].

Moreover, the relative IRF3 levels more than doubled after zinc supplementation, further indicating zinc-dependent IRF3 expression. Zinc-dependent upregulation of IRF3 was comparable in unstimulated PBMCs and PBMCs incubated with SDV or NDV. These results are in line with previous projects, which showed that IRF3 mRNA levels are independent of viral stimulation and follow a steady-state expression pattern [37,38]. The proposed causal nature of this interrelation between IRF3 and zinc was additionally substantiated by correlation analysis. We showed a significantly increased IFN $\alpha$  response after zinc supplementation of zinc-

deficient participants, consistent in both SDV and NDV stimulation of whole-blood assay and PBMC. To date, this is the first project in which zinc supplementation of zinc-deficient vegetarians and vegans has been successfully used to reconstitute IFN $\alpha$ -dependent antiviral immune response via upregulation of IRF3 expression.

Lastly, the rZD cohort was also analyzed for serum zinc levels, IRF3 expression and IFN $\alpha$  response after completing zinc supplementation. Individuals assigned to this subgroup initially showed sufficient serum zinc levels but decreased dietary intake in the FFQ. Still, this could lead to hidden zinc deficiency and functional impairments despite physiological serum zinc concentrations [16]. In line with results from previous studies, serum zinc levels of participants in the rZD cohort did not further increase to supplementation [56]. Furthermore, IRF3 expression showed a slight increase in the zinc-supplemented group but failed to reach significance.

Yet, impaired IFN $\alpha$  response of individuals at risk of zinc deficiency was significantly improved after receiving zinc supplementation. This further indicates that the absolute level of IRF3 expression is not the only zinc-dependent mechanism responsible for reduced IFN $\alpha$  production in individuals at risk for zinc deficiency. As mentioned beforehand, additional research on the relation of zinc and posttranslational activation of IRF3 via phosphorylation is needed.

In addition, these results underscore the persisting demand for a more precise zinc biomarker. As seen in the rZD cohort, serum zinc does not reliably unmask moderate zinc deficiency which already may disturb proper immune function [24]. In our case, individuals at risk of zinc deficiency, as identified by the FFQ, showed functional impairment in terms of reduced IFN $\alpha$  output.

To date, the ameliorating effect of zinc on progression of infectious diseases has been subject of several clinical trials [72]. Previous randomized, controlled and double-blinded studies showed beneficial impact of zinc supplementation on duration and severity of the common cold [73–76]. Similar results have been shown for COVID-19 patients, who showed poor outcomes, if zinc deficient [77]. These observations from a clinical perspective go in line with our findings on zinc-dependent antiviral IFN $\alpha$  response and may be linked in a causal manner.

Further clinical research is required to investigate whether this enhancement of antiviral response by zinc supplementation also reduces susceptibility to other virus infections and whether it decreases the incidence of virus infections among supplemented vegetarian and vegan patients *in vivo*.

Since there is no storage system for zinc [78], vegetarians and vegans who continue their respective diet should consider long-term zinc supplementation. Zinc deficiency does not only impact antiviral immune response, but a broad variety of immunological processes and it has been linked to autoimmune diseases, allergies and some types of cancer [24]. Ultimately, a well-balanced diet is crucial for physiological zinc status and, therefore, overall health. Supplementation is strongly advised if sufficient zinc intake is not met.

For long-term zinc supplementation, the individual dietary zinc intake must be taken into consideration in order not to exceed tolerable upper intake levels. Weipenborn et al. have calculated that a maximum dosage of 6.5 mg zinc per day in addition to adequate zinc intake causes no detrimental health implications [79]. This upper intake level is also recommended by the German Federal Institute for Risk Assessment (BfR) and, therefore, this might be a promising approach for future studies on long-term zinc supplementation. Again, the FFQ may be implemented as a cost- and time-efficient tool to assess the individual need for zinc supplementation.

In conclusion, we showed that the high prevalence of imminent or manifest zinc deficiency among vegetarians and vegans is directly linked to reduced IFN $\alpha$  response due to significantly lower IRF3 levels, a positive regulator of IFN $\alpha$  production. This may contribute to impaired antiviral immune response in vegetarians and vegans *in vivo*. Moreover, we were able to enhance zinc status, increase IRF3 expression and reconstitute IFN $\alpha$  response by oral zinc supplementation. In the light of increasing popularity of plant-based diets, these nutritional interventions become increasingly important in order to prevent health implications from micronutrient deficiencies. With regards to zinc deficiency in vegetarians and vegans, we strongly advocate further clinical research on differences in susceptibility to viral infections *in vivo* in order to make validated recommendations for supplementation.

### Author contribution

Conceptualization: J.J. and L.R.; Data curation: F.V.; Formal analysis: F.V.; Investigation (All of the experiments except for atomic absorption spectrometry and virus concentration determination): F.V.; Investigation – atomic absorption spectrometry: J. J.; Investigation – virus concentration determination: H.S.; Project administration: F.V., J.J. and L.R.; Resources: L.R.; Supervision: J.J. and L.R.; Validation: F.V. and J.J.; Visualization: F.V.; Writing – original draft: F.V.; Writing – review & editing: J.J. and L.R.

### Declaration of Generative AI and AI-assisted technologies in the writing process

The authors acknowledge the use of large language models for improving the written English of the manuscript. These tools were not employed in the research or data interpretation of this work.

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### Conflict of interest

The authors declare that they have no competing interests.

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