



## RESEARCH ARTICLE

# Longitudinal association of inflammatory markers with markers of glycaemia and insulin resistance in European children

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## Abstract

**Purpose:** Subclinical systemic inflammation may lead to development of type 2 diabetes, but there has been no investigation into its relationship with early progression of glycaemic deterioration and insulin resistance, especially in younger population. In this study we assessed longitudinal associations of pro- and anti-inflammatory markers with markers that evaluate glycaemia and insulin resistance. **Methods:** This study includes 6537 initially nondiabetic children (mean age at baseline = 6.2 years) with repeated measurements from the IDEFICS/I.Family cohort study (mean follow-up = 5.3 years) from eight European countries. Markers of inflammation were used as independent variables and markers of glycaemia/insulin resistance as dependent variables. Associations were examined using two-level growth model. Models were adjusted for sex, age, major lifestyle, metabolic risk factors, early life markers, and other inflammatory markers in final model. **Results:** Children with 6 years of follow-up showed that a one-unit increase in z-score of leptin level was associated with 0.38 (95% CI = 0.32 to 0.44) unit

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increase in HOMA-IR z-scores. Leptin continued to be associated with HOMA-IR even when analysis was limited to children with no overall obesity, no abdominal obesity, and low to normal triglyceride levels. An inverse association was observed between IL-15 and HOMA-IR ( $\beta = -0.11$ , 95% CI =  $-0.15$  to  $-0.07$ ).

**Conclusions:** IL-15 should be evaluated further in the prevention or treatment of prediabetes whereas leptin may prove to be useful in early detection of prediabetes via their association with markers of insulin resistance in European children.

#### KEYWORDS

children cohort, HOMA-IR, inflammatory markers, interleukin-15, leptin

## 1 | INTRODUCTION

Inflammation plays a significant role in the pathogenesis of diabetes.<sup>1</sup> In the adult population, increased concentrations of proinflammatory and reduced anti-inflammatory markers were significantly associated with the incidence of type 2 diabetes.<sup>2-4</sup> However, these associations need to be confirmed for causality, as Mendelian randomisation studies yielded inconsistent results for some inflammatory markers.<sup>5-8</sup> Previously, there have been prospective studies that have investigated associations between inflammatory markers and glycaemia or insulin resistance, measured at one-time point.<sup>9,10</sup> However, few studies have addressed longitudinal associations between inflammatory markers and glycaemic traits<sup>11-13</sup> and even fewer studies have investigated on how inflammatory markers act in combination.<sup>14</sup> Particularly, longitudinal studies investigating the association between low-grade systemic inflammation and markers of glycaemic deterioration/insulin resistance in children are missing.<sup>7,8</sup>

Moreover, since higher HbA<sub>1c</sub> and HOMA-IR are important indicators of vascular complications in prediabetic conditions<sup>15</sup> and have also been closely related to higher risk of cardiovascular disease and all-cause mortality in nondiabetic people,<sup>16</sup> better biological markers are required to identify the subjects at high risk in very early phases, such as prediabetes which may open new directions for early prevention. As inflammatory markers may be used to refine diabetes risk prediction and thus better target individuals for lifestyle interventions, we aimed to investigate longitudinal associations between pro- and anti-inflammatory markers (individually and combined) and markers of glycaemia (fasting glucose [FG], HbA<sub>1c</sub>), and insulin resistance (HOMA-IR) in European children.

## 2 | RESEARCH DESIGN AND METHODS

### 2.1 | Study population

The study population was enrolled in the pan-European, multi-centre, prospective IDEFICS/I.Family cohort of 16,229 children aged between 2 and 9.9 years at  $T_0$ , from eight European countries (Belgium, Cyprus, Estonia, Germany, Hungary, Italy, Spain, and Sweden). The children were first examined in 2007 and 2008 with follow-up

examinations conducted after two ( $T_1$ ) and six ( $T_3$ , I.Family study) years; the design of this cohort study has been described in detail elsewhere.<sup>17,18</sup> In the IDEFICS/I.Family study, risk factors of lifestyle-related outcomes were investigated in young children and anthropometric and clinical examinations were conducted at each survey wave. Blood samples were considered fasting if the last meal or drink (other than water) was consumed >8 h before drawing blood. Before children entered the study, parents provided written informed consent. Additionally, children aged 12 years and older gave simplified written consent. Younger children gave verbal assent for examinations and sample collection. Ethics approval was obtained from the institutional review boards of all eight study centres.

### 2.2 | Markers of glycaemia/insulin resistance

At  $T_0$ , FG was assessed either with capillary blood from finger prick or with venous blood from venipuncture using a point-of-care analyser (Cholestech LDX, Cholestech Corp.) which reports plasma equivalent glucose concentrations/venous plasma glucose concentrations. In  $T_3$ , an enzymatic UV test (Cobas c701, Roche Diagnostics GmbH) was used for FG analysis from NaF plasma. At  $T_0$ , serum insulin concentrations were measured by luminescence immunoassay in a central laboratory. We used an AUTO-GA Immulite 2000, Siemens, Eschborn, Germany. At  $T_3$ , serum insulin was analysed (at the University of Bremen, Centre for Biomolecular Interactions Bremen) by multiplex analysis with electrochemiluminescence technology from Meso Scale discovery (MSD) using a MULTI-SPOT® Assay System; Human Leptin, Insulin Assay Kit. The HbA<sub>1c</sub> was analysed in K2-EDTA venous blood by high-performance liquid chromatography (AUTOGA variant, Biorad) in a central laboratory at both  $T_0$  and  $T_3$ . HOMA-IR was calculated as fasting insulin ( $\mu\text{IU ml}^{-1}$ )  $\times$  FG (mg/dl)/405.

### 2.3 | Inflammatory markers

Serum samples stored at  $-80^\circ\text{C}$  were used to detect levels of C-reactive protein (CRP), interleukin-1 receptor antagonist (IL-1Ra), IL-6, 8, 15, interferon gamma inducible protein (IP-10), TNF- $\alpha$ ,

adiponectin and leptin were measured at  $T_0$  and  $T_3$ , by ELISA using electrochemiluminescent multiplex assay (using either single or MULTI-SPOT® Assay Systems, Meso Scale Discovery). The choice of inflammatory markers were based on their role in endothelial function via either direct or indirect mechanisms such as reducing nitric oxide production and stimulating inflammation-oxidative stress pathways. IL-6, IL-8, TNF- $\alpha$ , IP-10, IL-15 and IL-1Ra were run together on a 6-plex assay, insulin and leptin run together on a 2-plex assay, whereas adiponectin, and CRP on single-plex assays each. The combination of markers for the assays were decided based on the feasibility of combinations with the help of MSD customer support.

## 2.4 | Covariables

Based on the validated and reproducibility tested FFQ data, a Healthy Diet Adherence Score (HDAS) was developed for all the study regions,<sup>19,20</sup> as a proxy-indicator of children's adherence to healthy dietary guidelines including a high consumption of fruits and vegetables, wholemeal, fish consumption of 2–3 times per week and a reduced intake of refined sugars and fat. The HDAS was used for the present analyses as a continuous variable and ranged from 0 to 50. A higher score represented a higher adherence to healthy dietary guidelines. The pubertal status was self-reported by children in  $T_3$ , and was defined as pre-pubertal or pubertal based on voice change in boys and age at menarche in girls. This definition of pubertal status has given similar results when compared to Tanner stage in this cohort previously.<sup>21</sup> We used the number of occasions reported for alcohol intake/cigarette smoking in lifetime to create binary indicator variables for alcohol intake and smoking of ever smokers/drinkers versus nonsmokers/non-drinkers. The alcohol and smoking questionnaire was completed at  $T_3$  by study participants 12 years of age or older at the time of examination. Sports club membership (yes/no) as an indicator of physical activity<sup>22</sup> and daily TV, DVD, video, computer or games-console use in hours which were summed to obtain the total screen time for the whole week as a proxy for sedentary behaviour were reported by parents in  $T_0$ . In  $T_3$ , these proxy measures were reported by parents if the child was younger than 12 years, or self-reported if the child was 12 years or older. Parents self-reported their history of diabetes which was categorised as positive (at least one parent with diabetes), negative (both parents without diabetes), or unknown (if diabetes status of mother and father were unknown). Parents reported medication use and medical history for their children by means of an interview based on the health and lifestyle questionnaire. Mothers were asked to retrospectively report starting and ending months of exclusive breast feeding and breast feeding combinations which were used to derive the total breast feeding duration.<sup>23</sup> Information on mother's height and weight assessed at cohort entry was used for calculating maternal BMI. A binary indicator for children delivered at term versus children born preterm ( $\leq 37$ th gestational week) and continuous variable for birthweight were derived from parental questionnaire data. As part of the standardised anthropometric examination

protocol, waist circumference (WC; cm) was measured in an upright position with relaxed abdomen and feet together, midway between the lowest rib margin and the iliac crest to the nearest 0.1 cm (inelastic tape: Seca 200; Seca). Height (cm) of the children was measured to the nearest 0.1 cm with a calibrated stadiometer (Seca 225 stadiometer), body weight (kg) was measured in fasting state in light clothing on a calibrated scale accurate to 0.1 kg (Tanita BC 420 SMA, Tanita Europe GmbH). BMI was calculated as weight (kg) divided by height (m) squared.

## 2.5 | Analysis dataset

The present analysis used only  $T_0$  and  $T_3$  measurements as inflammatory markers were not measured at  $T_1$ . Our analysis dataset included participants with measurements of at least one inflammatory marker from  $T_0$  or  $T_3$  ( $n = 7992$ ). Children diagnosed with type 1 or type 2 diabetes at cohort entry ( $n = 9$ ) or taking anti-diabetic drugs (ATC codes: A10), anti-inflammatory drugs (M01), or corticosteroids (H02) within the last 14 days of cohort entry or follow-up examination were excluded from the analysis ( $n = 560$ ). Children with acute infection defined as CRP level  $\geq 10$  mg/l at  $T_0$  or  $T_3$  were also excluded ( $n = 886$ ). Finally, for non-fasting blood samples the values of FG and HOMA-IR were set to missing, thus leading to a final study sample of 6537 children (Figure S1).

## 2.6 | Statistical analysis

Data were expressed as mean  $\pm$  SD or median with an inter-quartile range as appropriate. According to previously described methods,<sup>24–27</sup> age- and sex-specific z-scores were derived for waist-to-height ratio, WC, HbA<sub>1c</sub>, HOMA-IR, triglycerides, SBP, and FG in children and adolescents using the data collected in the IDEFICS/I. Family cohort. Since the laboratory methods to measure FG changed between  $T_0$  and  $T_3$ , age- and sex-specific reference percentiles were estimated for  $T_0$  and  $T_3$ , separately, and were used to calculate the respective z-scores for the analysis. We used stata module STNDZXAGE for calculating z-scores of inflammatory markers by standardising its raw values (irrespective of their distribution with respect to the detection limits) over age, sex, and survey.<sup>28</sup> Since the children were newly recruited in all surveys (i.e.  $T_0$ ,  $T_1$  and  $T_3$ ; Figure S1), we henceforth use the word 'baseline' for cohort entry and follow-up time for representing difference between age at follow-up and age at cohort entry. The follow-up time was used as a continuous variable, as it was different for different study participants.

To model the association between inflammatory markers and markers of glycaemia/insulin resistance, a two-level growth model was used, where one level accounts for differences between individuals and the other level for changes over time within individuals.<sup>29</sup> Markers of inflammation (continuous variable) were the exposure variables and markers of glycaemia/insulin resistance

(continuous variable) were the response variables. The between-subject effect estimate referred to the association between an inflammatory marker and HbA<sub>1c</sub>, HOMA-IR or FG, whereas the fixed-effect interaction between follow-up time and inflammatory markers represented the rate of change in the association between inflammation and glycaemic deterioration per 2-year increase in follow-up time.

The description of the crude model is as follows: let  $y_{ij}$  be  $j$ -th measurement of the  $i$ -th child (e.g., z-scores of HOMA-IR, HbA<sub>1c</sub>, FG),  $M_{ij}$  is an inflammatory marker (e.g., z-scores of CRP, IL-1Ra, IL-6, 8, 15, IP-10, TNF- $\alpha$ , adiponectin and leptin),  $time_{ij}$  is the follow-up time since cohort entry and  $\epsilon_{ij}$  is the error term for individual  $i$  at follow-up time  $j$ , then the crude model without adjustment was specified as follows:

$$y_{ij} = \gamma_{0i} + \gamma_{1i}time_{ij} + \epsilon_{ij} \text{ with}$$

$$\gamma_{0i} = \beta_{00} + \beta_{01}M_{ij} + u_{0i}$$

$$\gamma_{1i} = \beta_{10} + \beta_{11}M_{ij} + u_{1i}$$

where  $\beta_{00}$  is the overall mean intercept,  $\beta_{10}$  is the overall mean slope and  $u_{0i}$  and  $u_{1i}$  express how much the intercept and slope, respectively, of individual  $i$  deviates from the average intercept and slope with respect to the individuals's follow-up time. Crude models included age and sex in addition to one exposure variable and follow-up time. The adjusted models furthermore included the minimum sufficient adjustment set (MSAS) for estimating the association between each inflammatory marker and markers of glycaemia/insulin resistance. The MSAS was identified using directed acyclic graph (DAG)<sup>30</sup> built using DAGitty version 3.0 (Figure S2) and included age, sex, study region (proxy for ethnicity), waist-to-height ratio, lifetime smoking and alcohol status, family history of diabetes, membership in a sports club (proxy for physical activity), and screen time per week (proxy for sedentary behaviour), HDAS, pubertal status, birthweight and other inflammatory markers. The DAG was built from literature research and expert knowledge (Figure S2). The main assumptions of growth models were checked and confirmed. Post-hoc analyses were performed to evaluate the marginal effect of each inflammatory marker on FG, HbA<sub>1c</sub>, HOMA-IR at baseline and different follow-up times using effect estimates from the adjusted model. To investigate the differential effect of sex, we performed a sex-stratified analysis. The combined effect of all inflammatory markers was calculated by subtracting the sum of z-scores of the anti-inflammatory markers (IL-1Ra, IL-15, adiponectin) from the sum of z-scores of the proinflammatory markers (CRP, leptin, TNF- $\alpha$ , IP-10, IL-8 and IL-6). This sum was then dichotomised in high (=1; topmost quartile) and low (=0; lower three quartiles) using the 75th percentile as the cut-off, separately for each sex and survey ( $T_0$  and  $T_3$ ). Several sensitivity analysis were performed: (i) study participants with >90th percentile of HbA<sub>1c</sub>/HOMA-IR/FG at baseline were excluded to further eliminate bias due to undetected prevalent diabetes; (ii) to evaluate the robustness of our DAG MSAS, additional covariates were included in the model; (iii) the sample was limited to children

with no overall obesity (BMI category upto 0 by Cole & Lobstein), no abdominal obesity (waist circumference z-scores < 0.1) and low to normal triglyceride levels (z-scores < 0.1) on DAG suggested model to rule-out the confounding effects of overall and central obesity.

All covariates were treated as time-varying to account for changes in lifestyle and anthropometric factors over time. The results were reported as regression coefficients and their 95% confidence intervals. Bonferroni correction was used to account for multiple testing, that is the statistical significance level was set to  $\alpha = 0.05/10 = 0.005$  (nine independent inflammatory markers and one dichotomised sum score were tested for FG, HbA<sub>1c</sub>, HOMA-IR). All statistical tests were two-sided. Statistical analyses were performed using Stata 16 and R 4.0.3.

### 3 | RESULTS

#### 3.1 | Characteristics of the study population

Table 1 shows the characteristics of the study participants included in the analysis at  $T_0$  and  $T_3$ . The mean age of participants at  $T_0$  was 6.17 years (SD = 1.75) and 48% were girls (Table 1). The mean ( $\pm$ SD) duration of follow-up time was 5.3 years ( $\pm$ 1.1). The percentage of overweight or obese children were 15.9 and 25.2 at  $T_0$  and  $T_3$ , respectively. The prevalence of diabetes in parents was low at both  $T_0$  and  $T_3$ . There was an increase in the proportion of children with a sports club membership at  $T_3$  as well as an increase in screen time over this period. Moderate correlations were observed among most of the inflammatory markers (Figure S3).

#### 3.2 | Two-level growth models

Results of the two-level growth models for the association between inflammatory markers and HbA<sub>1c</sub> and HOMA-IR are depicted in Table 2. Figure 1 illustrates effect estimates at baseline and different follow-up time-points from adjusted model based on DAG. The interaction between inflammatory markers and follow-up time represents the rate of change in the association between z-scores of an inflammatory marker and z-scores of HbA<sub>1c</sub>, HOMA-IR or FG per 2-year increase in follow/up time. The increase in leptin levels was observed to be strongly associated with increased levels of HOMA-IR (Figure 1; Table 2). We also observed weak association between leptin and FG ( $\beta = 0.04$ , 95% CI = 0.004 to 0.09; Table S1). Further, a significant interaction was observed between leptin and follow-up time showing that the association between leptin and HOMA-IR increases by 0.05 units per 2-year increase in follow-up time (Table 2). In post-hoc analysis, children with 6 years of follow-up showed that a one-unit increase in z-score of leptin level showed an 0.38 (95% CI = 0.32 to 0.44) unit increase in HOMA-IR z-scores (Figure 1). Association between higher levels of CRP with increasing concentration of HOMA-IR were observed only in crude model (Table 2).

TABLE 1 Characteristics of the analysis group at  $T_0$  and  $T_3$ 

Parameters		IDEFICS/I.Family cohort <sup>a</sup>	
		$T_0$ (n = 5794)	$T_3$ (n = 4393)
Year of examination		2007–08	2013–14
Sex, female: n (%)		2781 (48.00)	2129 (48.46)
Age: years		6.17 ( $\pm 1.75$ )	11.64 ( $\pm 1.96$ )
Study region	Italy: n (%)	641 (11.06)	634 (14.43)
	Estonia: n (%)	915 (15.79)	731 (16.64)
	Cyprus: n (%)	824 (14.22)	1061 (24.15)
	Belgium: n (%)	777 (13.41)	235 (5.35)
	Sweden: n (%)	838 (14.46)	436 (9.92)
	Germany: n (%)	1000 (17.26)	541 (12.32)
	Hungary: n (%)	703 (12.13)	650 (14.80)
	Spain: n (%)	96 (1.66)	105 (2.39)
BMI category by Cole & Lobstein, 2012	Thinness grade 1–3: n (%)	636 (10.99)	369 (8.41)
	Normal weight: n (%)	4236 (73.11)	2918 (66.42)
	Overweight/obese: n (%)	922 (15.91)	1106 (25.18)
BMI z-score		0.19 ( $\pm 2.71$ )	0.89 ( $\pm 2.84$ )
WC z-score, n ( $T_0$ ) = 5705, n ( $T_3$ ) = 4308		0.13 ( $\pm 1.35$ )	0.43 ( $\pm 1.48$ )
Wasit-to-height ratio z-score, n ( $T_0$ ) = 5705, n ( $T_3$ ) = 4308		0.07 ( $\pm 1.32$ )	0.39 ( $\pm 1.45$ )
Antibiotic intake: n (%)		249 (4.30)	70 (1.59)
HDAS, n ( $T_0$ ) = 5397, n ( $T_3$ ) = 4031		21 (15–27)	22.0 (16–28)
SBP z-score, n ( $T_0$ ) = 5368, n ( $T_3$ ) = 4197		0.05 ( $\pm 1.01$ )	0.03 ( $\pm 1.02$ )
DBP z-score, n ( $T_0$ ) = 5369, n ( $T_3$ ) = 4197		−0.01 ( $\pm 1.01$ )	−0.02 (1.00)
TRG z-score, n ( $T_0$ ) = 4883, n ( $T_3$ ) = 3986		0.09 ( $\pm 0.85$ )	0.10 ( $\pm 1.04$ )
HDL z-score, n ( $T_0$ ) = 4887, n ( $T_3$ ) = 3976		0.02 ( $\pm 0.99$ )	−0.08 ( $\pm 1.02$ )
Pubertal status: n (%)		Not observed	1701 (38.72)
Ever smoking: n (%)		Not observed	203 (4.62)
Ever consumed alcohol: n (%)		Not observed	665 (15.14)
Parental history of diabetes: n (%)		87 (1.50)	117 (2.66)
Membership in sports club: n (%)		2617 (45.17)	2700 (61.46)
Screen time per week (hours), n ( $T_0$ ) = 5444, n ( $T_3$ ) = 4001		11.97 ( $\pm 0.10$ )	17.19 ( $\pm 0.17$ )
CRP (ng/ml), n ( $T_0$ ) = 3377, n ( $T_3$ ) = 3890		1006.17 (341.28–2720.94)	323.77 (109.85–1048.80)
Adiponectin ( $\mu$ g/ml), n ( $T_0$ ) = 2277, n ( $T_3$ ) = 3135		26.09 (19.76–34.84)	2.05 (14.41–29.56)
Leptin (pg/ml), n ( $T_0$ ) = 3154, n ( $T_3$ ) = 3969		1555.21 (923.75–2987.10)	5152.26 (2193–13,322.02)
IL-1Ra (pg/ml), n ( $T_0$ ) = 3195, n ( $T_3$ ) = 3561		313.92 (205.87–450.17)	272.94 (202.74–395.72)
IL-6 (pg/ml), n ( $T_0$ ) = 3124, n ( $T_3$ ) = 3605		0.27 (0.17–0.45)	0.41 (0.28–0.62)
IL-8 (pg/ml), n ( $T_0$ ) = 3194, n ( $T_3$ ) = 3633		3.25 (2.35–4.61)	6.18 (4.46–8.98)
IL-15 (pg/ml), n ( $T_0$ ) = 3170, n ( $T_3$ ) = 3633		1.79 (1.27–2.49)	2.26 (1.72–2.92)
IP-10 (pg/ml), n ( $T_0$ ) = 3195, n ( $T_3$ ) = 3636		175.28 (131.27–253.39)	213.09 (158.63–296.52)
TNF- $\alpha$ (pg/ml), n ( $T_0$ ) = 3195, n ( $T_3$ ) = 3633		2.18 (1.68–2.86)	2.57 (2.01–3.42)
HbA <sub>1c</sub> z-scores, n ( $T_0$ ) = 4225, n ( $T_3$ ) = 3889		−0.26 ( $\pm 0.94$ )	0.05 ( $\pm 0.99$ )

(Continues)

TABLE 1 (Continued)

Parameters	IDEFICS/I.Family cohort <sup>a</sup>	
	T <sub>0</sub> (n = 5794)	T <sub>3</sub> (n = 4393)
HOMA-IR z-scores, n (T <sub>0</sub> ) = 3515, n (T <sub>3</sub> ) = 3172	0.04 (±1.07)	0.18 (±1.18)
FG z-scores, n (T <sub>0</sub> ) = 4744, n (T <sub>3</sub> ) = 3220	−0.01 (±1.03)	−0.01 (±0.98)
HbA <sub>1c</sub> (%), n (T <sub>0</sub> ) = 4331, n (T <sub>3</sub> ) = 3926	4.7 (4.40–5.00)	5.0 (4.80–5.20)
HbA <sub>1c</sub> 5.7%–6.4%: n (%)	21 (0.36)	40 (0.91)
HOMA-IR, n (T <sub>0</sub> ) = 3596, n (T <sub>3</sub> ) = 3303	0.76 (0.45–1.17)	1.22 (0.77–1.86)
HOMA-IR > 2.5: n (%)	104 (1.79)	427 (9.72)
FG (mg/dl), n (T <sub>0</sub> ) = 4881, n (T <sub>3</sub> ) = 3354	84 (78.00–90.00)	93 (89.00–98.00)
FG 100 mg/dL to 125 mg/dL: n (%)	198 (3.42)	584 (13.29)

Note: n stated in case of missingness.

Abbreviations: CRP, C-reactive protein; DBP, diastolic blood pressure; FG, fasting glucose; HDAS, Healthy Diet Adherence Score; HDL, high density lipoprotein; IL-1Ra, interleukin-1 receptor antagonist; IP-10, interferon gamma inducible protein; NA, not applicable; SBP, systolic blood pressure; TRG, triglyceride; WC, waist circumference.

<sup>a</sup>Characteristics of the study participants are presented as number (percentages) for categorical variables and median (25<sup>th</sup> and 75<sup>th</sup> percentiles) or mean (±SD) for continuous variables.

An inverse association between IL-15 levels and HOMA-IR was observed with significant interaction between IL-15 and follow-up time (Figure 1; Table 2). An inverse association between IL-1Ra and HOMA-IR was observed in adjusted model, however the associations were no longer significant after Bonferroni correction for multiple comparisons (Table 2). TNF- $\alpha$  was associated with higher HbA<sub>1c</sub> levels only at baseline (Figure 1). The association between most inflammatory markers of glycaemia/insulin resistance did not differ between boys and girls (Table S2). We also observed high sum score of the combined effect variable to be positively associated with HOMA-IR (Table 3).

### 3.3 | Sensitivity analyses

The sensitivity analysis, in which we additionally excluded study participants with HbA<sub>1c</sub>, HOMA-IR, or FG levels >90th percentile at baseline, showed mostly very similar results to the main analyses concerning the direction of effect and the effect sizes (Figure S4). The association of leptin and IL-15 persisted with HOMA-IR after including triglyceride levels, systolic blood pressure, antibiotic intake and additional covariates of early markers such as duration of breastfeeding, preterm birth, maternal obesity (Table S3).

## 4 | DISCUSSION

This study primarily focussed to evaluate the relationship between systemic inflammation and its association with markers of glycaemia and insulin resistance, to better understand the cause-effect relationship of metabolic dysregulation in the development of type 2 diabetes. In initially nondiabetic children with a mean follow-up duration of 5.3 years, we found significant longitudinal associations

in markers of systemic inflammation with glycaemic deterioration and HOMA-IR. After adjustment for changes in other risk factors of type 2 diabetes including adiposity (measured using waist-to height ratio), our data showed that increased levels of leptin and decreased levels of IL-15 were associated with increase in HOMA-IR levels. Also, an increase in TNF- $\alpha$  was associated with glycaemic deterioration initially, however, later a decreasing trend in their association was observed.

We observed that the leptin levels were associated with marker of insulin resistance which was also reported in previous cross-sectional and longitudinal studies in children<sup>31,32</sup> and well-established risk factor studies on type 2 diabetes in the adult population showing that the association is mediated by insulin resistance.<sup>33,34</sup> The leptin levels continued to be associated with HOMA-IR even when the analysis was limited to children with no overall obesity (BMI category up to 0 by Cole & Lobstein), no abdominal obesity (waist circumference z-scores < 0.1) and low to normal triglyceride levels (z-scores < 0.1; Table S4) suggesting that the association is independent of overall and abdominal obesity. Similarly, a recent study in adults reported an association between type 2 diabetes risk and increased levels of leptin in abdominally non-obese participants.<sup>34</sup>

An observation not reported previously in large epidemiological studies is our finding on a beneficial role of IL-15 concerning HOMA-IR and HbA<sub>1c</sub> levels, which may be attributed to its involvement in the regulation of energy expenditure as observed in animal models and human studies.<sup>35,36</sup> Further, though we observed a positive association between TNF- $\alpha$  and HbA<sub>1c</sub> at baseline, a possible explanation for the inverse association between TNF- $\alpha$  with FG and HbA<sub>1c</sub> at follow-up could be due to the inhibitory effects of IL-15 production (induced by a simple event as physical activity) which may aid in decreasing the negative effects of TNF- $\alpha$ ,<sup>37</sup> however this needs to be further confirmed in future studies.

The previous cohort studies such as Whitehall II study, Health 2000, National FINRISK study, suggested that an upregulation of

TABLE 2 Association between markers of inflammation and HbA<sub>1c</sub>/HOMA-IR

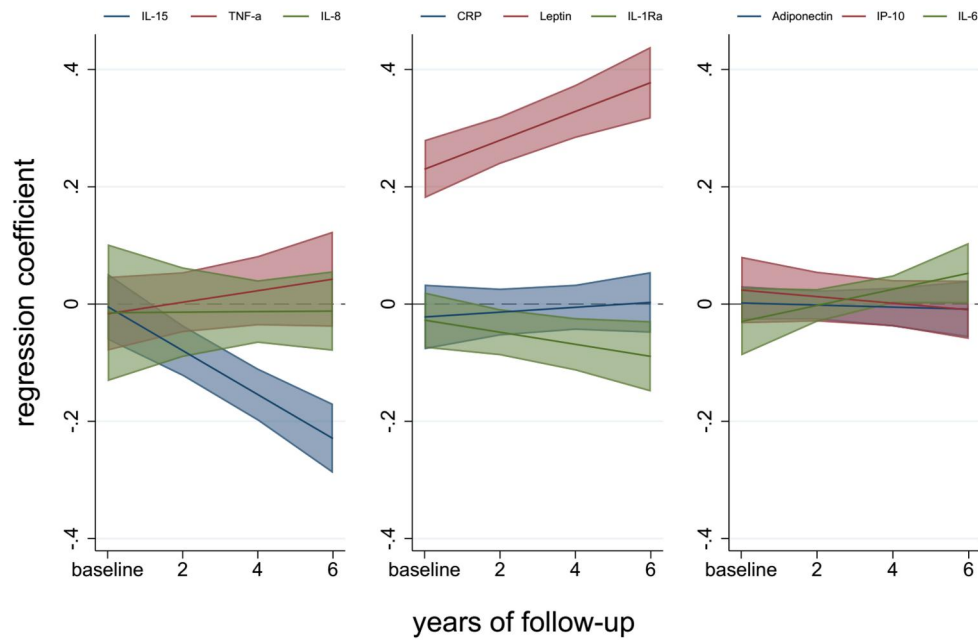
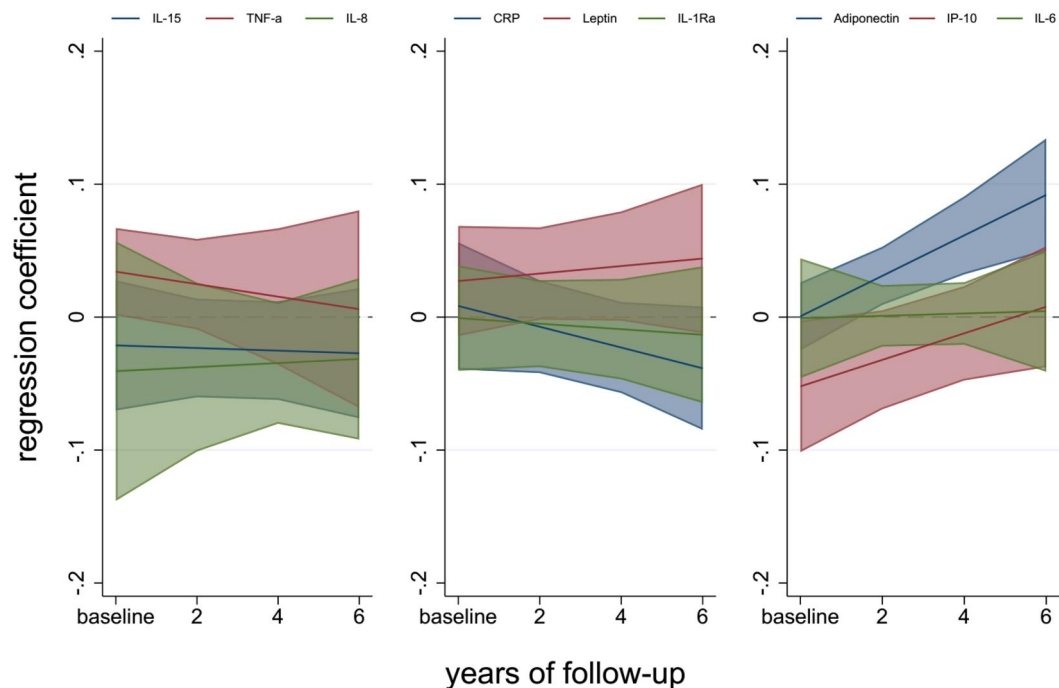
HOMA-IR	Crude <sup>a</sup> $\beta$ (95% CI)	Adjusted <sup>b</sup> $\beta$ (95% CI)
CRP	<b>0.06 (0.03 to 0.09)</b>	−0.01 (−0.05 to 0.03)
CRP × follow-up time	<b>0.04 (0.02 to 0.06)</b>	0.01 (−0.02 to 0.03)
Adiponectin	0.00 (−0.02 to 0.02)	0.00 (−0.03 to 0.02)
Adiponectin × follow-up time	−0.01 (−0.03 to 0.01)	0.00 (−0.02 to 0.02)
Leptin	<b>0.39 (0.36 to 0.41)</b>	<b>0.29 (0.25 to 0.33)</b>
Leptin × follow-up time	<b>0.04 (0.02 to 0.06)</b>	<b>0.05 (0.02 to 0.07)</b>
IL-1Ra	0.01 (−0.01 to 0.04)	<b>−0.05 (−0.09 to −0.01)</b>
IL-1Ra × follow-up time	<b>0.03 (0.02 to 0.05)</b>	−0.02 (−0.04 to 0.00)
IL-6	−0.01 (−0.03 to 0.01)	0.01 (−0.01 to 0.03)
IL-6 × follow-up time	0.00 (−0.02 to 0.03)	0.03 (0.00 to 0.06)
IL-8	<b>−0.03 (−0.06 to −0.01)</b>	−0.01 (−0.07 to 0.04)
IL-8 × follow-up time	0.02 (0.00 to 0.04)	0.00 (−0.05 to 0.05)
IL-15	<b>−0.06 (−0.09 to −0.03)</b>	<b>−0.11 (−0.15 to −0.07)</b>
IL-15 × follow-up time	<b>−0.06 (−0.08 to −0.04)</b>	<b>−0.07 (−0.10 to −0.05)</b>
IP-10	0.00 (−0.03 to 0.03)	0.00 (−0.03 to 0.04)
IP-10 × follow-up time	0.01 (−0.01 to 0.04)	−0.01 (−0.04 to 0.01)
TNF- $\alpha$	<b>−0.05 (−0.08 to −0.03)</b>	0.01 (−0.05 to 0.06)
TNF- $\alpha$ × follow-up time	0.00 (−0.02 to 0.02)	0.02 (−0.01 to 0.05)
HbA <sub>1c</sub>		
CRP	0.01 (−0.01 to 0.03)	−0.02 (−0.05 to 0.02)
CRP × follow-up time	0.00 (−0.02 to 0.02)	−0.02 (−0.04 to 0.01)
Adiponectin	<b>0.02 (0.00 to 0.04)</b>	<b>0.03 (0.00 to 0.05)</b>
Adiponectin × follow-up time	<b>0.02 (0.00 to 0.03)</b>	<b>0.03 (0.01 to 0.05)</b>
Leptin	<b>0.07 (0.05 to 0.09)</b>	0.03 (0.00 to 0.07)
Leptin × follow-up time	0.01 (0.00 to 0.03)	0.01 (−0.02 to 0.03)
IL-1Ra	0.01 (−0.01 to 0.03)	0.01 (−0.04 to 0.03)
IL-1Ra × follow-up time	−0.01 (−0.03 to 0.00)	0.00 (−0.02 to 0.02)
IL-6	0.01 (−0.02 to 0.01)	0.00 (−0.02 to 0.02)
IL-6 × follow-up time	0.00 (−0.02 to 0.02)	0.00 (−0.03 to 0.03)
IL-8	0.01 (−0.01 to 0.02)	−0.03 (−0.08 to 0.01)
IL-8 × follow-up time	<b>−0.02 (−0.03 to 0.00)</b>	0.00 (−0.04 to 0.05)
IL-15	<b>−0.03 (−0.05 to −0.01)</b>	−0.02 (−0.06 to 0.01)
IL-15 × follow-up time	−0.01 (−0.03 to 0.00)	0.00 (−0.02 to 0.02)
IP-10	−0.02 (−0.04 to 0.00)	−0.02 (−0.05 to 0.02)
IP-10 × follow-up time	0.00 (−0.01 to 0.02)	0.02 (0.00 to 0.04)
TNF- $\alpha$	0.02 (0.00 to 0.03)	0.03 (0.00 to 0.06)
TNF- $\alpha$ × follow-up time	<b>−0.02 (−0.03 to 0.00)</b>	−0.01 (−0.04 to 0.02)

Note: The  $\beta$  coefficient represents the  $\beta$  unit change in HbA<sub>1c</sub>/HOMA-IR z-scores per unit increase in z-scores of inflammatory markers, whereas the interaction  $\beta$  coefficient represents the rate of change in the association between inflammation (z-scores) and HbA<sub>1c</sub>/HOMA-IR (z-scores) per 2-year increase in follow-up time. Associations at  $p < 0.05$  are shown in bold. Significant associations after Bonferroni correction are shown in bold and italics. Abbreviations: CRP, C-reactive protein; IL-1Ra, interleukin-1 receptor antagonist; IP-10, interferon gamma inducible protein.

<sup>a</sup>Adjusted for age and sex with follow-up time as a random slope.

<sup>b</sup>Additionally adjusted for study region, waist-to-height ratio, lifetime smoking and alcohol status, pubertal status, birthweight, healthy diet adherence score, family history of diabetes, membership in sport club, screen time/week and other inflammatory markers (minimal sufficient adjustment set).



**(A) HOMA-IR****(B) HbA<sub>1c</sub>**

**FIGURE 1** Marginal effects of pro- and anti-inflammatory markers on HbA<sub>1c</sub> and HOMA-IR. Abbreviations: CRP, C-reactive protein; IP-10, interferon gamma inducible protein; IL-1Ra, interleukin-1 receptor antagonist; Marginal effects are presented as regression coefficient for HbA<sub>1c</sub>/HOMA-IR per unit increase in z-scores of inflammatory markers at baseline and different years of follow-up with negative coefficients showing protective associations and positive coefficients showing increased risk. Lines represent regression coefficients and shaded areas represent 95% confidence intervals. The effects have been adjusted for age, sex, study region, waist-to-height ratio, lifetime smoking and alcohol status, pubertal status, birthweight, healthy diet adherence score, family history of diabetes, membership in sport club, screen time/week and other inflammatory markers with follow-up time as a random slope

IL-1Ra in the circulation is linked to an increased risk of type 2 diabetes.<sup>38-42</sup> This may be due to a counterregulation to proinflammatory and/or metabolic stimuli and can be interpreted as a futile response to the presence of multiple diabetes risk factors, thus not conferring a

sufficient degree of protection against the onset of the disease.<sup>43</sup> However, when the cohort studies were pooled for a joint genotyping analysis along with gene expression, genetically raised levels of IL-1Ra seemed to protect against increased insulin resistance.<sup>43</sup> Similarly, our



TABLE 3 Association of the combined effect of inflammatory markers on HbA<sub>1c</sub> and HOMA-IR

		Crude <sup>a</sup> $\beta$ (95% CI)	Adjusted <sup>b</sup> $\beta$ (95% CI)
HOMA-IR	Combined effect	<b>0.38 (0.31 to 0.46)</b>	<b>0.20 (0.11 to 0.28)</b>
	Combined effect $\times$ follow-up time	<b>0.12 (0.06 to 0.17)</b>	<b>0.11 (0.05 to 0.17)</b>
HbA <sub>1c</sub>	Combined effect	0.02 (−0.05 to 0.08)	−0.04 (−0.11 to 0.04)
	Combined effect $\times$ follow-up time	−0.04 (−0.09 to 0.00)	−0.04 (−0.09 to 0.01)

Note: Combined effect = sum of z-scores of proinflammatory markers (CRP, leptin, TNF- $\alpha$ , IP-10, IL-8, IL-6) - sum of z-scores of anti-inflammatory markers (IL-1Ra, IL-15, adiponectin). The continuous variable of combined effect was categorised to sex- and survey-specific quartiles.  $\beta$  coefficients calculated for upper quartile versus lower three quartiles (reference) of the combined effect of inflammatory markers. The  $\beta$  coefficient represents the  $\beta$  unit change in HbA<sub>1c</sub>/HOMA-IR z-scores per unit increase in z-scores of inflammatory markers, whereas the interaction  $\beta$  coefficient represents the rate of change in the association between inflammation (z-scores) and HbA<sub>1c</sub>/HOMA-IR (z-scores) per 2-year increase in follow-up time. Significant associations at  $p < 0.05$  are shown in bold. Significant associations after Bonferroni correction are shown in bold and italics.

Abbreviations: CRP, C-reactive protein; IL-1Ra, interleukin-1 receptor antagonist; IP-10, interferon gamma inducible protein.

<sup>a</sup>Adjusted for age and sex with follow-up time as a random slope.

<sup>b</sup>Additionally adjusted for study region, waist-to-height ratio, lifetime smoking and alcohol status, pubertal status, birthweight, healthy diet adherence score, family history of diabetes, membership in sport club and screen time/week.

observation of the protective association of IL-1Ra in children may have been influenced by genetic susceptibility marker, however, this needs to be further confirmed by in-depth genetic association analysis.

The literature is inconsistent for CRP. A large Mendelian randomisation study concluded that associations between CRP and insulin resistance, glycaemia, and diabetes are most likely noncausal<sup>8</sup> whereas, most prospective studies have reported an independent aggravating effect of CRP on diabetes incidence, which was confirmed in meta-analyses.<sup>4,44</sup> The absence of an association between CRP and FG, HbA<sub>1c</sub>, HOMA-IR in the present study can be explained by the fact that average CRP values change only marginally in younger children, whereas they seem to rise during late adolescence.<sup>45,46</sup> Therefore the present study may need a longer follow-up to assess the association between CRP and markers of glycaemia/insulin resistance.

Though there have been studies showing protective effects of adiponectin on prediabetic markers,<sup>3,7,47</sup> we did not observe any protective association after Bonferroni correction for multiple comparisons with adiponectin which is consistent with results from Mendelian randomisation studies that did not support a causal role for reduced circulating adiponectin levels in type 2 diabetes.<sup>6,48</sup>

Our results confirms the role of leptin as a proinflammatory marker for insulin resistance and adds to the existing evidence on protective role of IL-15 for insulin resistance in a large prospective cohort of children. We also observed an association with the combined effect of inflammatory markers which was largely driven by leptin (Table S5). The development of such scores or a diabetes panel may prove to be beneficial in the identification of high-risk group individuals resulting in early diagnosis. There have been some studies that have targeted in preparation of such panels, however, more detailed studies are required.<sup>14</sup> Further, our observation of increased correlations between some of the proinflammatory markers (IL-6, 8 with TNF- $\alpha$ ) at T<sub>3</sub>, comprising of older children and adolescents compared to T<sub>0</sub>, comprising mainly of younger children (Table 1; Figure S3) is consistent with the theory that the levels of proinflammatory markers increase with age<sup>49,50</sup> and may thus have higher correlations between them.

The strength of this study is the large sample size and the population-based longitudinal design with two repeated measurements, which enabled us to take into account changes in risk factors for type 2 diabetes over time as potential confounders. Our study is one of the few population-based cohort studies conducted in children<sup>51</sup> which has the advantage of not being largely influenced by other diseases, medications, or active tobacco smoking. Nevertheless, the inclusion of more than two-time points would have enriched our analysis for example by enabling us to look at trajectories in more detail. Though the assumptions for linear associations were fulfilled, a non-linear association between inflammatory markers and markers of glycaemia/insulin resistance was not assessed. The serum samples from T<sub>0</sub> and T<sub>3</sub> were not assessed in the same plate which may have led to some batch effects. Sports club membership have been reported to be positively associated with moderate-to-vigorous physical activity,<sup>22</sup> however, we expect some residual confounding. Further, though the children taking anti-diabetic drugs or anti-inflammatory drugs or corticosteroids were excluded, the residual confounding effect of these drugs cannot be completely ruled out. Nevertheless, we were able to confirm the observed association with series of sensitivity analyses, (i) by excluding study participants with high levels of HOMA-IR/HbA<sub>1c</sub>/FG (>90th percentile) at baseline (ii) by checking robustness of DAG MSAS and (iii) by limiting the analysis to children with no overall, abdominal obesity and normal triglycerides. Future large scale prospective studies are warranted for assessing the protective role of IL-15 and its interactive effects on TNF- $\alpha$ .

## 5 | CONCLUSIONS

The associations observed in the present study provide observational evidence suggesting that systemic inflammation may potentially contribute to the aetiology of prediabetes. Our findings imply a potential clinical value of these inflammatory factors as early stage markers for type 2 diabetes. Particularly, leptin may hold the promise

of early detection and IL-15 should be evaluated further as preventive target of prediabetes via their association with marker of insulin resistance in children.

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## CONFLICT OF INTEREST

None.

## ETHICS STATEMENT

Ethical approval was obtained from the relevant local or national ethics committees by each of the study centres. We certify that all applicable institutional and governmental guidelines and regulations concerning the ethical use of human volunteers were followed during this research. Before children entered the study, parents provided written informed consent. Additionally, children aged 12 years and older gave simplified written consent. Younger children gave verbal assent for examinations and sample collection.

## AUTHOR CONTRIBUTIONS

Rajini Nagrani contributed to the conceptualisation, data analysis, data interpretation and writing of the manuscript. Ronja Foraita and Maike Wolters contributed to the data interpretation, writing and reviewing of the manuscript. Manuela Marron contributed to the conceptualisation and reviewing of the manuscript. Stefaan De Henauw, Staffan Marild, Dénes Molnár, Luis A Moreno, Paola Russo, Michael Tornaritis, Toomas Veidebaum and Wolfgang Ahrens critically reviewed the manuscript. All authors approved the final version for publication. Rajini Nagrani is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

## DATA AVAILABILITY STATEMENT

All data generated or analysed during this study are included in this published article or in the data repositories listed in References.

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## PEER REVIEW

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