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Time-dependent effects on circulating cytokines in patients with LADA: A decrease in IL1-ra and IL-1 beta is associated with progressive disease

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ABSTRACT

Background: Cytokines and chemokines participate in autoimmune processes at cellular targets which include insulin-producing beta cells. To which extent such participation is reflected in the circulation has not been conclusively resolved.
Aim: We compared the time course of cytokines/chemokines in Latent Autoimmune Diabetes in Adults (LADA) patients heterogeneous for high or low autoimmune activity as determined by levels of antibodies against glutamic acid decarboxylase (GADA).
Methods: Serum samples to be measured were from a two-armed randomized controlled trial (RCT) in 68 LADA patients. The study encompassed 21 months with C-peptide as primary endpoint. We measured 27 immune mediators at baseline, at 9 and at 21 months (end of study). Results of measurements were analyzed by multiple linear regression.
Results: At baseline, a high body mass index (BMI) (>26 kg/m^2) was associated with elevated levels of the interleukins (IL) IL-1 beta, IL-1ra, IL-2, IL-5, IL-6 and IL-13. Treatment during RCT (sitagliptin vs. insulin) did not affect the time course (21 months) of levels of cytokines/chemokines (by univariate analyses). However, levels of the cytokines IL-1ra and IL-1 beta decreased significantly ($p < 0.04$ or less) in patients with high vs. low GADA when adjusted for BMI, age, gender (male/female), treatment (insulin/sitagliptin) and study site (Norwegian/Swedish).
Conclusions: In LADA, high levels of GADA, a proxy for high autoimmune activity and linked to a decline in C-peptide, was associated with a decrease of selected cytokines over time. This implies that the decline of IL-1ra and IL-1 beta in the circulation reflects autoimmune activity and beta cell demise in LADA.

1. Introduction

The term LADA (Latent Autoimmune Diabetes in Adults) is generally used to classify adult-onset diabetes in patients who have antibodies like in type 1 diabetes, in particular antibodies to glutamic acid decarboxylase (GADA), but do not at diagnosis of diabetes have a clinical need for insulin treatment. It is established that LADA is at least partly an autoimmune disease. But it is also clear that the autoimmune activity varies greatly between patients in concert with the progression to develop need for insulin treatment.

The prognostic importance of levels of GADA for progression was highlighted in a recently published study of ours [1]. This was a randomized clinical treatment study in LADA patients, comparing the effects of early insulin treatment with that of sitagliptin as add-ons to treatment with metformin. The primary endpoint of the study was the evolution of C-peptide after 21 months of study. We did not find any difference between the two arms of the study. However, we found a strong coupling between high levels of GADA and a major decline of

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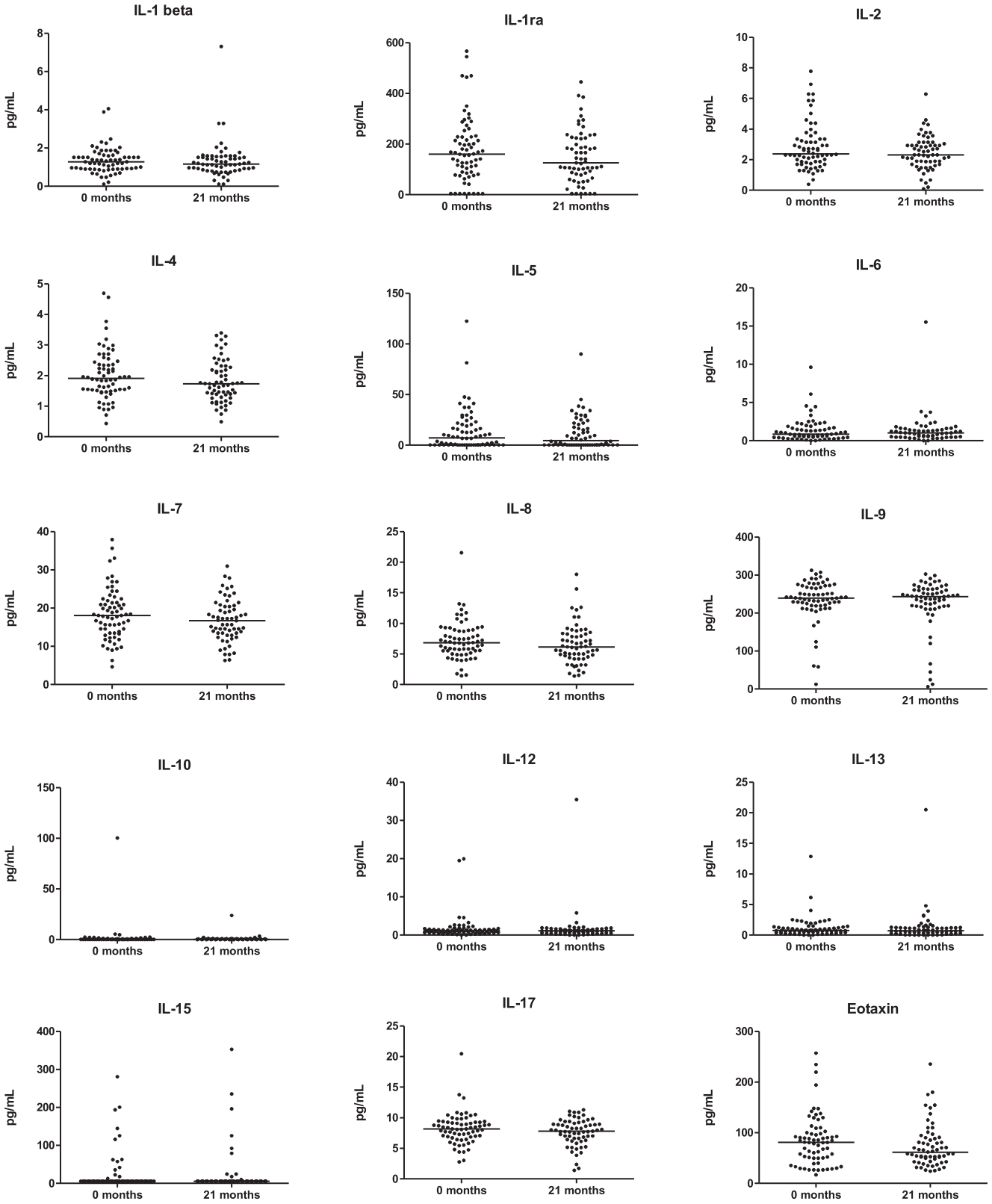

Fig. 1. Serum concentrations of immune mediators at 0 months (baseline) and 21 months after baseline (end of study). Each point represents results from one individual. At 0 months n = 68 and at 21 months n = 63. Horizontal lines depict median values. Abbreviations as follows: IL-1 beta = Interleukin 1 beta, IL-1ra = Interleukin 1 receptor antagonist, IL-2 = Interleukin 2, IL-4 = Interleukin 4, IL-5 = Interleukin 5, IL-6 = Interleukin 6, IL-7 = Interleukin 7, IL-8 = Interleukin 8, IL-9 = Interleukin 9, IL-10 = Interleukin 10, IL-12 = Interleukin 12, IL-13 = Interleukin 13, IL-15 = Interleukin 15, IL-17 = Interleukin 17, FGF = Fibroblast Growth Factor, GCSF = Granulocyte Colony Stimulating Factor, GMCSF = Granulocyte Macrophage Colony Stimulating Factor, IFN gamma = Interferon gamma, IP10 = Interferon gamma-induced Protein, MCP-1 = Monocyte Chemotactic Protein 1, MIP-1 alpha = Macrophage Inflammatory Protein 1 alpha, MIP-1 beta = Macrophage Inflammatory Protein 1 beta, PDGF = Platelet-Derived Growth Factor, TNF alpha = Tumor Necrosis Factor alpha.

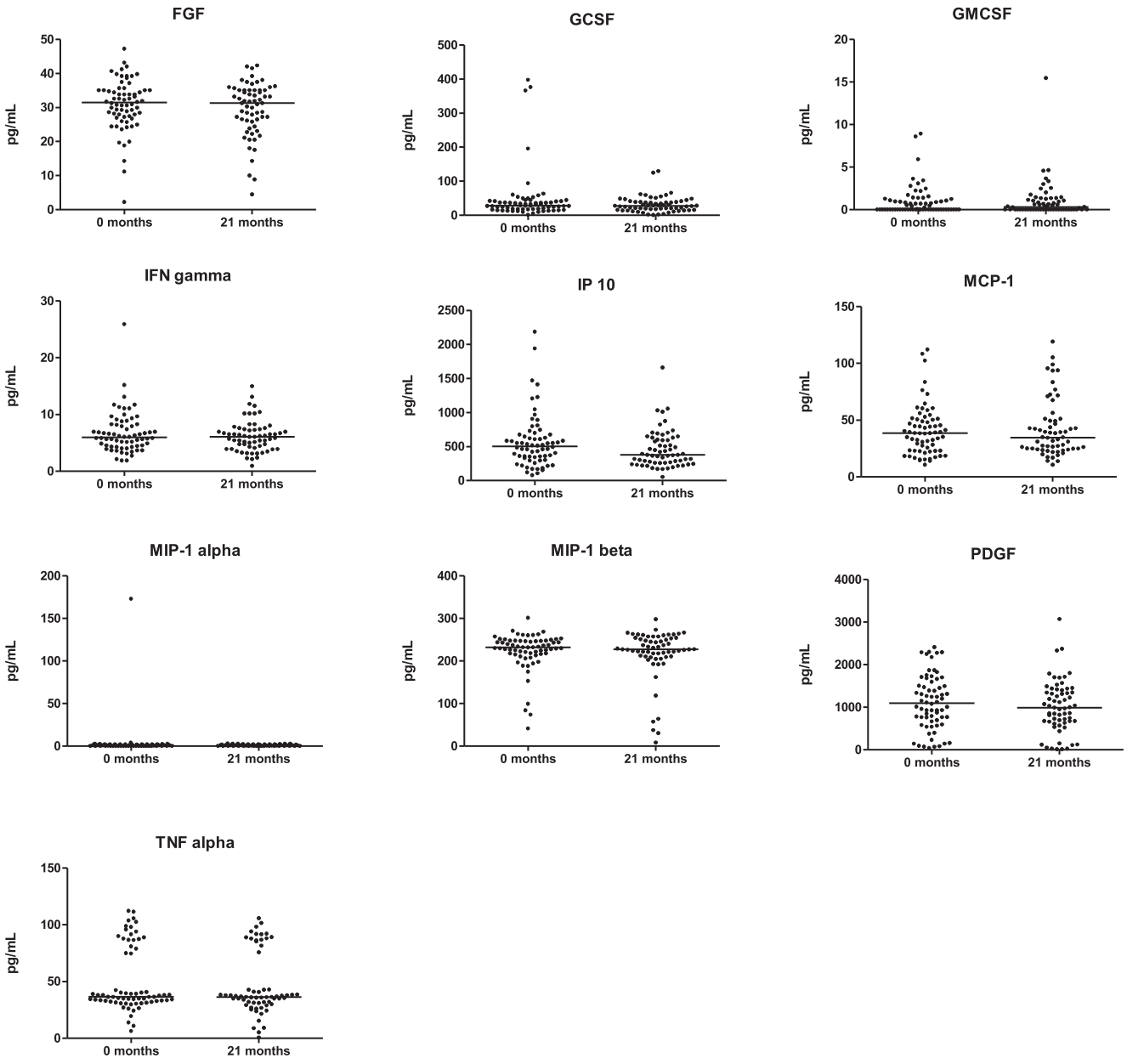

Fig. 1. (*continued*).

stimulated C-peptide; in contrast no such coupling was seen in patients with low levels of GADA [1].

Cytokines and chemokines participate in autoimmune processes at cellular targets which include insulin-producing beta cells in LADA [2]. To which extent is such participation reflected in the circulation? Studies on this subject have been performed cross-sectionally [3,4] demonstrating a different profile of cytokines vs. type 2 diabetes but no clear differences of LADA vs. type 1 diabetes However, previous studies in LADA have not followed the evolvement of circulating cytokines/chemokines longitudinally in parallel to an ongoing assault on beta cells. Hence, whether an ongoing and progressive autoimmune process is reflected by levels of specific circulating cytokines/chemokines has not been conclusively resolved in the case of LADA.

We reasoned that measurements of cytokines/chemokines in our published LADA study could shed light on the question of reflection of beta-cell directed autoimmunity. The design of the study thus enabled us to compare profiles of cytokines/chemokines at defined levels of autoimmunity (by GADA titer) during ongoing beta cell deterioration. Accordingly, we focused our attention on possible differences in circulating cytokines/chemokines over time between patients displaying high vs. low levels of GADA.

2. Methods

2.1. Study population

The study includes serum samples from 68 individuals with newly diagnosed LADA type of diabetes. Sixty-four individuals were participants in our previous two-armed 21 months randomized controlled trial (RCT) that tested for the treatment effect of early insulin vs. sitagliptin on beta cell function (NCT01140438) [1]. The additional four participants were post RCT recruited individuals exposed to the same treatment regime as in the RCT. Participants were from three study sites in Norway (25/5/1) and two in Sweden (36/1). Four of the RCT participants withdrew from the study between 3 and 9 months of intervention and two between 9 and 21 months [1]; available data from the

withdrawals were included in our analyses. The study was conducted according to the Code of Ethics of the World Medical Association (Declaration of Helsinki). Informed consent was obtained from all participants.

2.2. Measurements

Fasting serum samples were collected at baseline and after 3, 9 and 21 months of intervention and frozen at −80°C for later analyses at the study site in Trondheim, Norway. For the four participants that left the trial between 3 and 9 months of intervention, serum samples collected at 3 months were used as replacement for 9 months serum samples. Serum levels of 27 immune mediators (all depicted in Fig. 1 and Fig. 1**S**) were measured by use of the Bio-Plex Pro Human Cytokine, Chemokine and Growth factor Assay 27-Plex Panel (Bio-Rad, Oslo, Norway). Measurements performed were within the measurable range, except for all measurements of RANTES (Regulated upon Activation Normal T-cell Expressed and presumably Secreted) and VEGF (Vascular Endothelial Growth Factor); these immune mediators were therefore excluded from the presentation of measurements given in Fig. 1 and Fig. 1**S** and from further analyses. Levels of IL-10 (Interleukin 10), IL-15 (Interleukin 15), MIP-1a (Macrophage Inflammatory Protein 1 alpha) and GM-CSF (Granulocyte Macrophage Colony Stimulating Factor) were below the limits of detection in >34% of total samples and were therefore excluded from significance testing. Intra-assay mean %CV was 2.3 and mean inter-assay %CV was 15.

2.3. Statistics

Analyses were performed using IBM SPSS Statistics 26. Serum concentrations of the immune mediators were not normally distributed (and log10 transformation did not normalize data). The "study site" variable was classified as "Norwegian" or "Swedish". Mann-Whitney U tests were used to test for differences in independent variables at baseline. Changes of levels of immune mediators with time (9 and 21 months after baseline) were analyzed using multiple linear regression with levels of GADA (high/low, defined as $>$ 190 or $\leq$ 190 IU/ml), body mass index (BMI) (kg/m^2), age (years), gender (male/female), treatment (insulin/sitagliptin) and study site (Norwegian/Swedish) as independent variables. Results are presented as dot blots, means ± SD and medians if not otherwise indicated.

3. Results

3.1. Clinical characteristics

The median age of participants was comparable to other LADA populations as was also BMI (Table 1) [4–6]. The levels of GADA did not change significantly over 21 months of observation [1].

3.2. Levels at baseline

As anticipated from a previous study [7] the levels of immune mediators at baseline varied much between participants (Fig. 1). A high BMI ($>$ 26 vs. $\leq$ 26 kg/m^2) was associated with elevated levels of interleukin 1 beta (IL-1 beta), interleukin 1 receptor antagonist (IL-1ra), interleukin 2 (IL-2), interleukin 5 (IL-5), interleukin 6 (IL-6) and interleukin 13 (IL-13) (Table 1**S**). Levels of IL-6 were positively associated with age ($\leq$ 53 vs. $>$ 53 years). Levels of IL-12 and MIP-1b were higher in males than in females. Levels of IL-7 were higher in low compared to high GADA individuals (Table 1**S**).

Table 1
Clinical characteristics of the study population at the time of randomization (baseline).

	Baseline values
Total number (*n*)	68
Study site, Norwegian/Swedish (*n*)	31/37
Treatment, insulin/sitagliptin (*n*)	34/34
Autoimmunity category, Low/high GADA (*n*)	26/42
Female/male	32/36
Age at randomization, Years (median (IQR))	53 (45–59)
Age at diabetes diagnosis, Years (median (IQR))	52 (44–58)
Time, diagnosis to randomization, Months (median (IQR))	10 (5–18)
BMI, kg/m^2 (mean ± SD)	26.7 ± 5.1
BMI, kg/m^2 (range)	18–45
Fasting C-peptide, nmol/L (mean ± SD)	0.6 ± 0.4
HbA_{1c}, mmol/mol (mean ± SD)	51 ± 8
HbA_{1c}, % (mean ± SD)	6.9 ± 0.7

Low GADA, ≤190 IU/ml; high GADA, >190 IU/ml. IQR = interquartile range (25th and 75th percentile), BMI = body mass index, HbA_{1c} = glycated haemoglobin.

3.3. Time course

A decline over time of levels of IL-1ra and IL-1 beta was independently and significantly associated with high levels of GADA. The decline of IL-1ra was apparent both at 9 and 21 months from baseline while the decline of IL-1 beta was significant at 21 months from baseline (Table 2 and Fig. 2**)**. Levels of IFN gamma also tended to associate with decline with time in individuals with high levels of GADA (Table 2 and Fig. 2). Treatment (sitagliptin vs. insulin) did not affect the time course of cytokines/chemokines ($p > 0.05$ for comparisons with all measured cytokines/chemokines).

4. Discussion

Our main finding is that high GADA associates with a decline over 21 months of the cytokines IL-1ra, IL-1 beta and perhaps also IFN gamma. Evidence indicates that these findings reflect beta cell directed autoimmune activity. High GADA was thus strongly associated with a decline of C-peptide-glucagon responses over time in our LADA patients [1]. High GADA could therefore be regarded as a proxy for high autoimmune activity leading to beta cell demise.

A decrease over time of IL-1ra in conjunction with beta cell demise, has also been found in newly diagnosed type 1 diabetes [8,9]. Notably IL-1ra was the only cytokine tested that was associated with beta cell demise in the mentioned studies.

IL1-ra is well documented as an anti-inflammatory cytokine; also it has been proposed to be a central mediator directly linked with many pro-and anti-inflammatory mediators [10]. Accordingly, IL-1ra analogues has been used therapeutically in autoimmune diseases [11]. A beneficial response has been reported in type 2 diabetes in response to the IL-1 receptor antagonist, anakinra [12]. However, RCTs with IL-1ra analogues in type 1 diabetes have failed to affect progression of the disease [13]. Thus, in the present setting, no conclusions on causality can be drawn; the relevance of our finding is restricted to a possible use

Table 2
Change in levels of immune mediators at 9 and 21 months from baseline in study participants with high vs. low levels of GADA.

	Change in levels of immune mediator (pg/ml) from baseline (0 months)		Difference in change in levels of immune mediator (pg/ml) between GADA categories (high vs. low)		
	Low GADA	High GADA	p-value	Beta coefficient	95% Confidence interval
IL-1ra					
Δ9-0 months[a]	11.2 ± 71.1 (21.8)	-38.5 ± 95.4 (-18.2)	**0.025** 0.055[b]	-49.7 -44.1[b]	-93.1, -6.4 -89.3, 1.1[b]
Δ21-0 months	10.1 ± 85.6 (1.5)	-61.5 ± 118.9 (-39.7)	**0.015** **0.040**[b]	-60.6 -52.8[b]	-108.8, -12.3 -103.0, -2.6[b]
IL-1 beta					
Δ9-0 months[a]	0.10 ± 0.74 (0.00)	-0.17 ± 0.53 (-0.12)	0.084 0.090[b]	-0.27 -0.29[b]	-0.58, 0.04 -0.62, 0.05[b]
Δ21-0 months	0.35 ± 1.38 (0.07)	-0.24 ± 0.53 (-0.76)	**0.020** **0.020**[b]	-0.59 -0.64[b]	-1.09, -0.10 -1.17, 0.10[b]
IFN gamma					
Δ9-0 months[a]	0.51 ± 2.71 (0.56)	-0.56 ± 2.47 (-0.40)	0.098 **0.043**[b]	-1.07 -1.40[b]	-2.35, 0.20 -2.75, 0.04[b]
Δ21-0 months	-0.03 ± 2.82 (0.47)	-1.24 ± 2.55 (-0.76)	0.080 0.074[b]	-1.21 -1.32[b]	-2.58, 0.15 -2.76, 0.13[b]

Results are calculated for delta values at 9 months with n = 68 participants and at 21 months with n = 63 participants. Data on concentrations are given as mean ± SD (median). Multiple linear regression was performed for the effects of GADA category low (≤190 IU/ml) and high (>190 IU/ml) on change in levels of immune mediators at 9 and 21 months after baseline.

[a] Calculations include 3 months data from the four study participants that left the RCT between 3 and 9 months of intervention. Removing these data from the analyses did not change the results of significance.

[b] Adjusted for BMI (kg/m^2), age (years), gender (male/female), treatment (insulin/sitagliptin) and study site (Norwegian/Swedish). Significant p-values are depicted in bold and remained significant also after removing one or two outliers. IL-1ra = Interleukin 1 receptor antagonist, IL-1 beta = Interleukin 1 beta, IFN gamma = Interferon gamma.

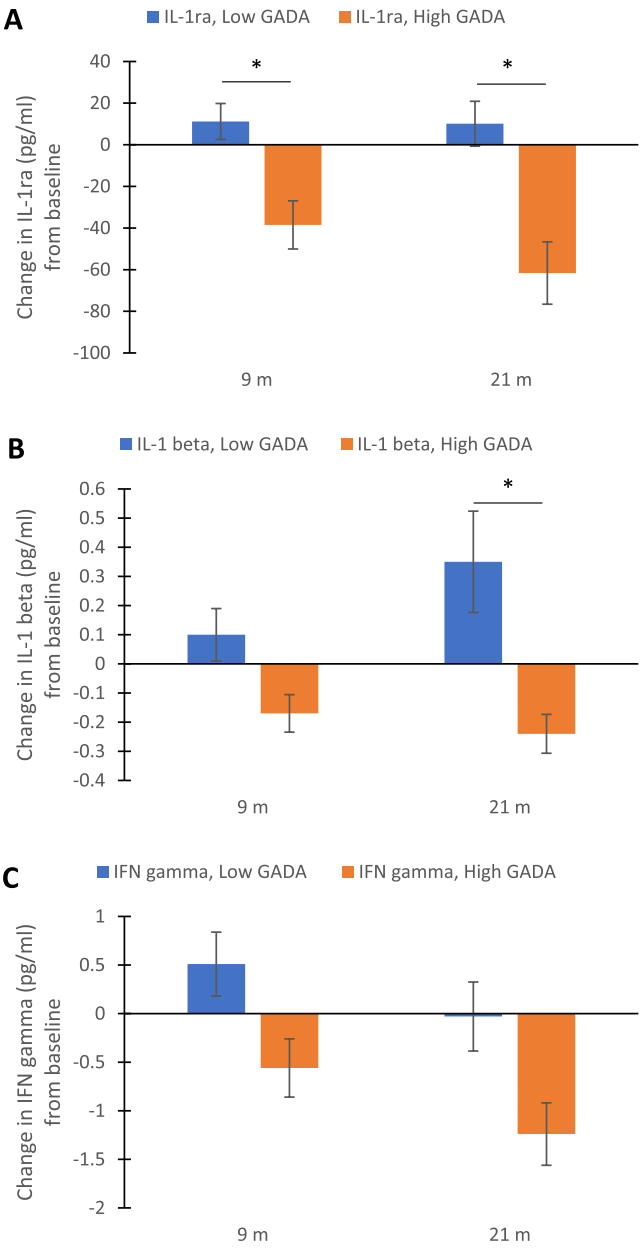

Fig. 2. The figure shows change in levels of **A**) IL-1ra, **B**) IL-1 beta and **C**) IFN gamma in the low and high GADA categories at 9 and 21 months from baseline. Data are mean ± SEM. *P < 0.015–0.025 for difference in change in levels of immune mediators between GADA categories (high vs. low). Adjustment for BMI (kg/m^2), age (years), gender (male/female), treatment (insulin/sitagliptin) and study site (Norwegian/Swedish) reduced the level of significance depicted in **A**) from p = 0.025 to 0.055 at 9 months and from p = 0.015 to 0.040 at 21 months. Adjustment for the mentioned variables did not affect results of significance depicted in **B**) (p = 0.02) while in **C**) a non-significant difference between GADA categories as to change of IFN gamma at 9 months (p = 0.098) became significant (p = 0.043). IL-1ra = Interleukin 1 receptor antagonist, IL-1 beta = Interleukin 1 beta, IFN gamma = Interferon gamma, m = months.

of IL-1ra as marker of beta cell directed autoimmune activity in LADA. Such a conclusion is reinforced by the seemingly paradoxical finding of a decrease of the proinflammatory cytokine IL-1 beta being associated with GADA-defined autoimmune activity. This paradox extends to the findings of increased expression of the IL-1 beta gene in islets from LADA patients [2].

Beta cells constitute a very small fraction of body tissues; hence it is difficult to envisage a direct link between circulating cytokines and cytokine release from islets. In fact, circulating levels of cytokines/chemokines are known to be influenced by several non-islet factors. Obesity is known to elevate levels of certain cytokines possibly due to low grade inflammation in adipose tissue. This is a probable reason for elevated levels of cytokines in type 2 diabetes vs. type 1 diabetes and LADA [4]. An effect of increased body weight could be distinguished also in the present study and was duly adjusted for as was also for effects by age and gender.

Sitagliptin, a DPP-4 inhibitor, has been reported to affect immunological parameters of potential importance for insulin sensitivity and/or beta cell function [14]. However, as stated in the Results section no effect on cytokines/chemokines by sitagliptin vs. insulin could be discerned in our study. Hence, the design of the RCT, which encompasses the present study population, appears not to bias our results.

5. Conclusion

We conclude that in LADA, high levels of GADA, a proxy for high autoimmune activity and linked to a decline in C-peptide, are associated with a decrease of IL1-ra and IL-1 beta cytokines over time. These effects could be used in the clinical evaluation of autoimmune-directed beta cell demise in LADA patients.

CRediT authorship contribution statement

Ingrid K Hals: Formal analysis, Investigation, Data curation, Validation, Writing – review & editing. **Anneli Björklund:** Resources, Validation, Writing – review & editing. **Hanne Fiskvik Fleiner:** Resources, Validation, Writing – review & editing. **Valdemar Grill:** Conceptualization, Validation, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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