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SHORT COMMUNICATION

High levels of blood circulating immune checkpoint molecules in children with new-onset type 1 diabetes are associated with the risk of developing an additional autoimmune disease

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Abstract

Aims/hypothesis We assessed the levels of blood circulating immune checkpoint molecules (ICMs) at diagnosis of type 1 diabetes, and determined their association with the risk of developing an additional autoimmune disorder over time.

Methods Children with new-onset type 1 diabetes (n = 143), without biological and/or clinical signs of additional autoimmune disorders, and healthy children (n = 75) were enrolled, and blood circulating levels of 14 ICMs were measured. The children with type 1 diabetes were divided into two groups on the basis of the development of an additional autoimmune disease in the 5 years after diabetes onset. Differences in soluble ICM levels between the groups were assessed, and a Cox regression analysis was used to evaluate their association with the risk of development of an additional autoimmune disease over time. To validate the data, circulating ICMs were measured in an independent cohort of 60 children with new-onset type 1 diabetes stratified into two groups.

Results We found that the levels of circulating ICMs were significantly higher in children with new-onset diabetes compared with healthy children. Further, we observed that children with type 1 diabetes who developed a second autoimmune disease over time (T1D-AAD$^+$ children) had higher levels of soluble ICMs than children with type 1 diabetes who did not (T1D-AAD$^-$ children). Cox regression models revealed that high circulating levels of CD137/4-1BB and PD-1 molecules at diabetes diagnosis were associated with the risk of developing an additional autoimmune disease in both type 1 diabetes cohorts.

Conclusions/interpretation Our findings suggest that soluble CD137/4-1BB and PD-1 molecules may be used as prognostic biomarkers in children with type 1 diabetes, and may pave the way for novel immunological screening at diabetes onset, allowing early identification of children at higher risk of developing other autoimmune conditions over time.

Keywords Autoimmune diseases · Autoimmune thyroiditis · Biomarkers · Coeliac disease · Soluble immune-checkpoint molecules · Type 1 diabetes

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Research in context

What is already known about this subject?

- Immune checkpoint molecules (ICMs) are pivotal factors involved in control of immune responses
- The role of soluble ICMs has only been partially explored in children with new-onset type 1 diabetes

What is the key question?

- Are levels of circulating ICMs dysregulated in type 1 diabetes at onset, and are they associated with the risk of developing additional autoimmune diseases?

What are the new findings?

- Children with new-onset type 1 diabetes showed higher levels of circulating ICMs than healthy children
- Children with new-onset type 1 diabetes who developed an additional autoimmune disorder over time showed higher levels of soluble ICMs than children with type 1 diabetes who did not develop an additional autoimmune disorder
- Soluble forms of CD137/4-1BB and PD-1 molecules were prognostic factors for the risk of developing an additional autoimmune disorder in children with type 1 diabetes

How might this impact on clinical practice in the foreseeable future?

- Our findings pave the way to novel immunological screening at diabetes onset for early identification of children with type 1 diabetes who are at risk of developing other autoimmune conditions

Abbreviations

AIT	Autoimmune thyroiditis
BTLA	B and T lymphocyte attenuator
CD	Coeliac disease
CTLA-4	Cytotoxic T lymphocyte antigen-4
FP	Fractional polynomial
GITR	Glucocorticoid-induced TNFR-related
HVEM	Herpes virus entry mediator
ICMs	Immune checkpoint molecules
IDO	Indoleamine 2,3-dioxygenase
LAG-3	Lymphocyte activating-3
PD-1	Programmed death-1
PDL-1	Programmed death ligand-1
PDL-2	Programmed death ligand-2
T1D-AAD$^-$	Type 1 diabetes without additional autoimmune disorder
T1D-AAD$^+$	Type 1 diabetes with additional autoimmune disorder
TIM-3	T cell immunoglobulin and mucin-domain containing 3

Introduction

Type 1 diabetes is regarded as an autoimmune condition characterised by dysregulated immune responses culminating in T cell-mediated destruction of insulin-producing beta cells in the pancreas [1]. Excessive immune responses in individuals with type 1 diabetes may also determine the development of additional autoimmune diseases, most commonly autoimmune thyroiditis (AIT) and coeliac disease (CD) [2, 3]. The processes of tissue destruction and loss of immunological self-tolerance involve both immune cells and molecular factors, which have not been completely elucidated. In this context, it has been reported that immune checkpoint molecules (ICMs) are pivotal for modulating the length and magnitude of auto-reactive T cell responses, in order to minimise the tissue damage and maintain immune homeostasis [4, 5]. ICMs are ligand–receptor pairs, expressed by cells of both the innate and adaptive immune systems, that have inhibitory or stimulatory effects on immune responses [4, 5]. Furthermore, soluble forms of ICMs, produced by cleavage of their membrane-bound counterparts or alternative splicing of mRNA, act as competitive regulators of their membrane-bound proteins [6]. Although the key role of ICMs in the control of immune responses is well documented [4, 5], a limited number of studies have investigated soluble ICMs in the context of type 1 diabetes [7, 8]. Over recent years, several immune alterations have been shown to be associated with the loss of immunological tolerance and development of autoimmune diabetes; however, additional unexplored immune determinants, including ICMs, may be involved in the development of type 1 diabetes in children and their high risk of developing additional autoimmune disorders [9].

In this study, we investigated whether the circulating levels of soluble ICMs are altered in children with new-onset type 1 diabetes, and whether these changes are associated with the development of non-islet autoimmune disorders over time.

Methods

Study design and participants Between 2010 and 2015, we enrolled 143 children with new-onset type 1 diabetes diagnosed according to the International Society for Pediatric and Adolescent Diabetes Guidelines, including positivity for at least two anti-islet autoantibodies [10]. At diabetes diagnosis, children enrolled in the study were not affected by other autoimmune disorders and were negative for thyroid peroxidase autoantibodies or thyroglobulin autoantibodies for diagnosis of AIT and negative for tissue transglutaminase autoantibodies for diagnosis of CD. Recruitment of children with type 1 diabetes was achieved within 10 days after disease diagnosis, upon glycaemic stabilisation by treatment with exogenous insulin, as previously reported [11]. Children with type 1 diabetes were followed up for 5 years after diabetes diagnosis to monitor the development of a second autoimmune disease such as AIT or CD (see Fig. 1a). Of the 143 children with type 1 diabetes, 45 (31.5%) developed an additional autoimmune condition (see Fig. 1a and electronic supplementary material [ESM] Table 1). AIT was diagnosed by the presence of thyroid peroxidase autoantibodies, and/or thyroglobulin autoantibodies with normal or low thyroid function free thyroxine 4 [FT4], thyroid-stimulating hormone [TSH]) and heterogeneity and hypo-echogenicity of thyroid parenchyma at ultrasound examination [12]. Diagnosis of CD was based on the presence of tissue transglutaminase autoantibodies and biopsy in accordance with the European Society for Pediatric Gastroenterology Hepatology and Nutrition guidelines [13]. As a control group, we recruited 75 healthy children without type 1 diabetes and/or other autoimmune conditions. The children with type 1 diabetes and healthy children in the main cohort were recruited at the Regional Centre of Pediatric Diabetology, University of Naples 'Federico II', Italy. For the validation cohort, we analysed plasma from 60 children with new-onset type 1 diabetes, from the Regional Centre for Pediatric Diabetes, University of Verona, Italy. In this cohort, 20 out of 60 children with diabetes (33%) developed an additional autoimmune disorder (ESM Table 2). The study protocol was approved by the Institutional Review Board of the Ethics Committee of the University of Naples 'Federico II' and the University of Verona. Informed written consent was obtained from the legal guardians of the children; assent was obtained from children recruited in the study.

Analysis of circulating ICMs The soluble ICMs included in this study were B and T lymphocyte attenuator (BTLA), CD27, CD28, CD80, CD137/4-1BB, cytotoxic T lymphocyte antigen-4 (CTLA-4), glucocorticoid-induced TNFR-related (GITR), herpes virus entry mediator (HVEM), indoleamine 2,3-dioxygenase (IDO), lymphocyte activating-3 (LAG-3), programmed death-1 (PD-1), programmed death ligand-1 (PDL-1), programmed death ligand-2 (PDL-2) and T cell immunoglobulin and mucin-domain containing-3 (TIM-3). Levels of these ICMs were measured in plasma samples from children with new-onset type 1 diabetes and healthy children, using a bead-based multianalyte immunoassay (Invitrogen/Thermo Fisher Scientific, Waltham, MA, USA), and Luminex 200 multiplex technology (Luminex Instruments, Austin, TX, USA), according to the manufacturer's recommendations. xPONENT 3.1 software (Luminex Instruments, Austin, TX USA) was used for data acquisition.

Statistical analyses The characteristics of enrolled children with type 1 diabetes and healthy children are reported using standard descriptive statistics: mean ± SD, or median (Q1–Q3) in the case of numerical variables, and absolute frequencies and percentages for categorical factors (ESM Tables 1 and 2). Differences between groups were assessed by ANOVA, two-tailed Student's t test for unpaired samples, the Mann–Whitney U test or Fisher's exact test. Parametric ANOVA and non-parametric ANOVA (Kruskal–Wallis test) were used as the omnibus test for BMI and age at enrolment.

Association between single ICM and development of a second autoimmune disorder was further assessed using Cox regression analysis. Analysis of ICM differential expression in the discovery cohort was not corrected for multiple testing in order to reduce the risk of ignoring true findings that have been instead independently tested in the validation cohort. To account for the potential non-linear relationship between soluble ICMs and the hazard function, a fractional polynomial (FP) approach was used [14]. The approach allows exploration of non-linear and asymmetric relationships between continuous predictors and the hazard function, moving from the linear relationship between predictor and outcome ($\beta_1 X$) to a power transformation ($\beta_1{}^{p} X$), where the power p is chosen from the restricted set −2, −1, −0.5, 0, 0.5, 1, 2, with a power of 0 implying logarithmic transformation. For each predictor, first- and second-degree FPs were allowed according to the algorithm as previously reported [15]. The analysis was performed on the main cohort, and then the associations were tested in an independent validation cohort in which the same algebraic transformation was applied.

All Cox regression models were adjusted by sex and age. Due to the strong collinearity among the distribution of soluble ICMs, no attempt was made to build a multivariable model. The results of the Cox regression models are expressed

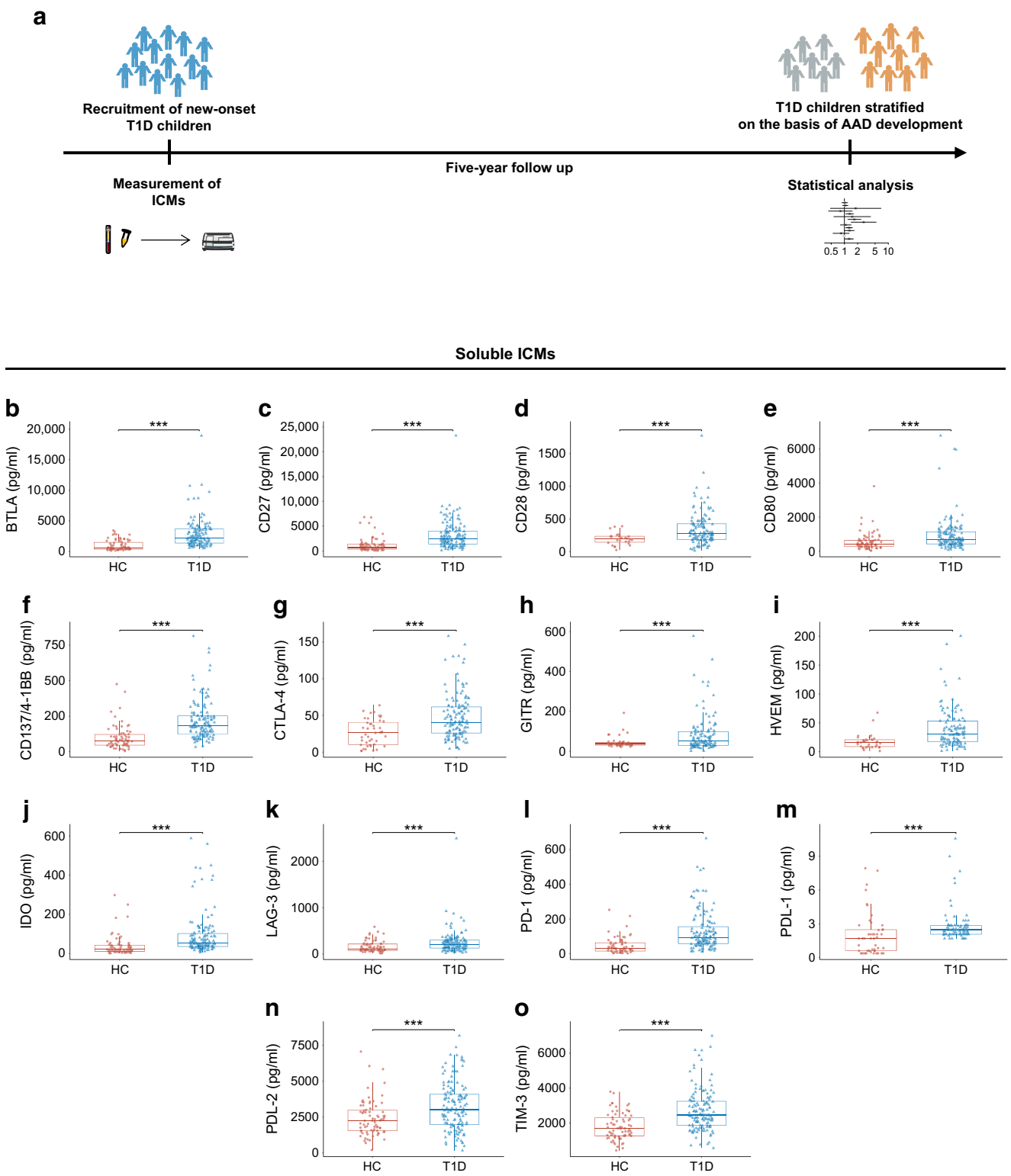


Fig. 1 Altered levels of circulating ICMs in children with new-onset type 1 diabetes. (**a**) Schematic representation explaining the study design. T1D, type 1 diabetes; AAD, additional autoimmune disorder. (**b–o**) Box plots showing the distribution of plasma levels (pg/ml) of soluble ICMs in healthy children (HC, red) and children with new-onset type 1 diabetes (T1D, blue). Data are shown as the median (horizontal line in the box) and Q1 and Q3 (borders of the box). Whiskers show the lowest and highest values that are not outliers (i.e. data points below Q1–1.5 × IQR or above Q3–1.5 × IQR). Dots outside the whiskers represent outlier values. The statistical significance of the differences between groups was assessed using the Mann–Whitney *U* test (***$p<0.001$)

as HRs with the corresponding 95% CI. The correlation between soluble CD137/4-1BB and PD-1 was assessed using Pearson's test. All statistical analyses were performed in R (version 4.0.3) using the *mfp* package to model FP. A p value <0.05 indicates statistical significance.

Results

The circulating levels of 14 soluble ICMs were analysed in 143 children with new-onset type 1 diabetes and 75 healthy children. We observed significantly higher levels of the soluble ICMs analysed in children with new-onset diabetes compared with healthy children (Fig. 1b–o). The observed differences were maintained (except for soluble CD27 molecules) after controlling for age and BMI (data not shown).

Next, we evaluated differences in ICM levels between type 1 diabetes children who developed an additional autoimmune disorder (T1D-AAD$^+$) and those who did not (T1D-AAD$^-$) during 5 years of follow-up (see Fig. 1a). We found that, at diabetes onset, T1D-AAD$^+$ children had significantly higher levels of soluble CD27, CD28, CD137/4-1BB, CTLA-4, GITR, HVEM, PD-1, PDL-2 and TIM-3 compared with T1D-AAD$^-$children (Fig. 2a–n). No significant difference between ICM plasma levels and the development of either AIT or CD in T1D-AAD$^+$ children was observed (ESM Table 3).

Finally, we used Cox regression models, adjusted for age and sex, to identify blood ICMs associated with the risk of developing another autoimmune disorder in children with type 1 diabetes (Fig. 2o). An independent cohort of 60 children with new-onset type 1 diabetes was used to validate the model (ESM Table 2); 20 of these children subsequently developed an additional autoimmune disorder. We found that among the 14 soluble ICM analysed, only high circulating levels of soluble CD137/4-1BB and PD-1 were associated with a risk of developing an additional autoimmune disorder in the 5 years of follow-up in both cohorts of children with type 1 diabetes (Fig. 2o and ESM Table 4). A strong positive correlation between the levels of soluble CD137/4-1BB and PD-1 was also observed in T1D-AAD$^+$ children (r = 0.80; p<0.0001).

These data suggest that soluble forms of CD137/4-1BB and PD-1 may be used as specific prognostic factors associated with the risk of developing an additional autoimmune disease in children with type 1 diabetes.

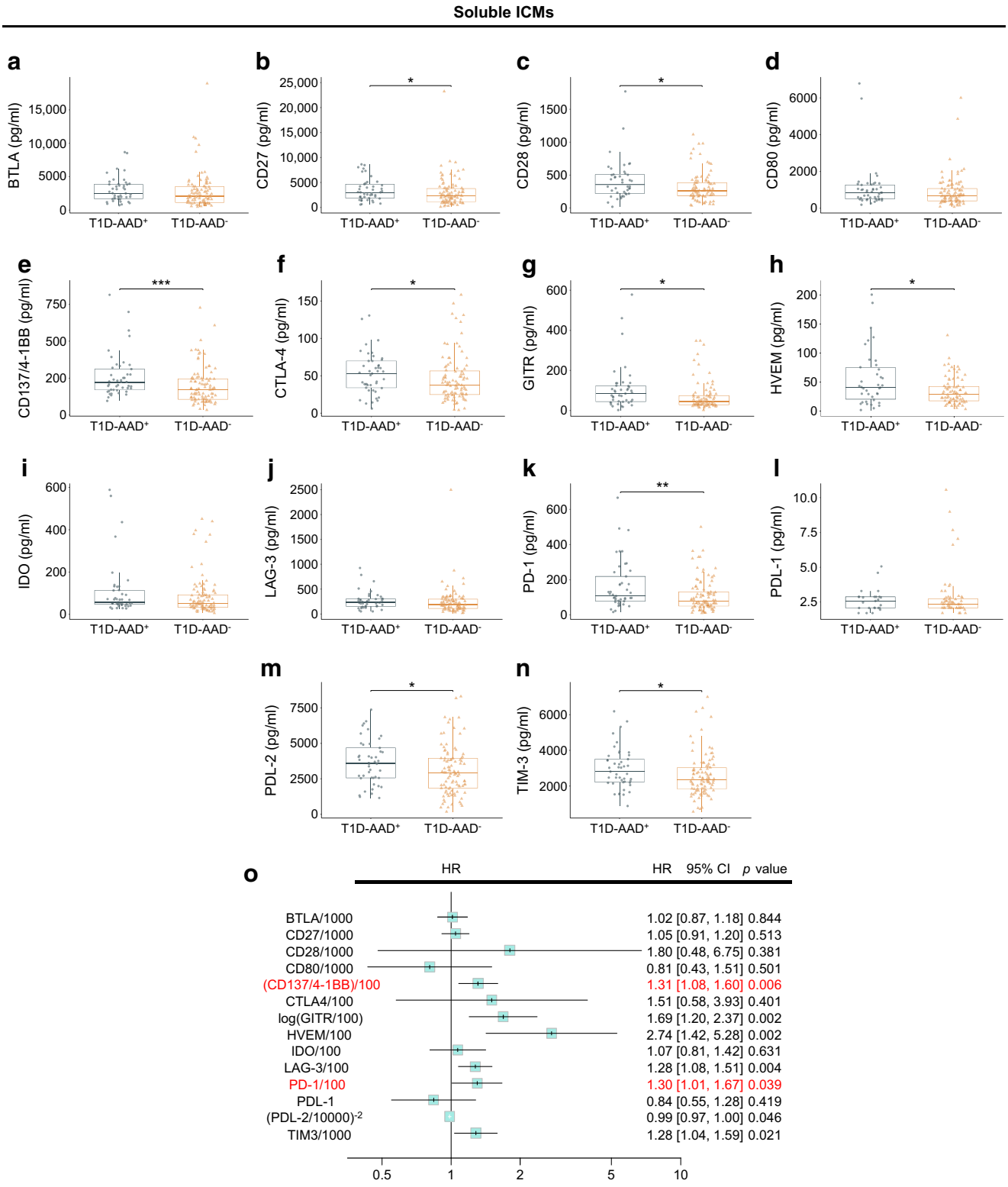
Discussion

The involvement of circulating ICMs in the immune derangement that characterises the pathogenesis of type 1 diabetes is poorly explored. Here we analysed the soluble ICM profile in children with new-onset type 1 diabetes, and found that their circulating levels are up-regulated in comparison with healthy children. The highest levels of specific soluble ICMs were observed at onset of type 1 diabetes in children who developed an additional autoimmune disease in the following 5 years. Through a round of discovery and validation phases, we identified soluble CD137/4-1BB and PD-1 as high-risk predictive factors related to the development of extra-pancreatic autoimmune diseases. This study provides proof of concept that obtaining an ICM signature at the time of diabetes diagnosis may reveal pathophysiological changes accompanying development of a second autoimmunity, before the actual surge of non-islet autoantibodies.

Individuals with type 1 diabetes have a higher risk of developing other autoimmune diseases, in particular AIT and CD [9], probably due to an excessive activation of immune cells that favours autoimmune reactivity in other tissues. To balance altered immune responses, a fine-tuned regulation by co-stimulatory and co-inhibitory molecules, including ICMs, is required. Changes in the concentrations of soluble ICMs observed in type 1 diabetes at onset may represent a novel immune signature, reflecting the hyperactivation of the immune system.

In this context, our findings highlight the presence of a soluble ICM storm in children with type 1 diabetes at diagnosis, revealing soluble CD137/4-1BB and PD-1 as early biomarkers, higher levels of which anticipate the development of an additional autoimmune disorder. It is well known that these molecules play contrasting roles in the immune response: while soluble CD137/4-1BB suppresses effector T cell responses, the soluble form of PD-1 acts as a decoy receptor to limit T cell exhaustion [7, 16]. Unexpectedly, we found increased levels of both molecules associated with a higher risk of developing a second autoimmunity. This probably reflects an attempt at self-regulation in individuals with type 1 diabetes, whereby the increased levels of soluble CD137/4-1BB balance the high levels of soluble PD-1 in order to maintain immune homeostasis. This is also supported by our results showing a positive strong correlation between circulating levels of soluble CD137/4-1BB and PD-1 molecules. It is possible that loss of this equilibrium over time leads to the development of other autoimmune reactions in other tissues of children with type 1 diabetes.

Most cases of type 1 diabetes-related autoimmune diseases are asymptomatic or paucisymptomatic, without non-islet autoantibodies [3], thus delaying the diagnosis of AIT and/or CD in children with type 1 diabetes. A direct consequence of delayed diagnosis may be a lower ability to prevent and control complications related to additional autoimmune diseases. Hence, the identification of early biomarkers that anticipate the development of



additional autoimmune conditions is an important unmet clinical need.

Although our findings highlight use of soluble CD137/4-1BB and PD-1 levels as high-risk prognostic factors, one of the limitations of this study is that we were not able to pinpoint a specific cut-off value that unequivocally predicts the risk of an additional autoimmune condition, possibly due to the small and limited number of T1D-AAD$^+$ children analysed. Further studies on larger cohorts are required in order to build a predictive statistical model. However, our approach may

◀ **Fig. 2** High levels of circulating ICMs in children with new-onset type 1 diabetes are associated with a risk of developing an additional autoimmune disorder over time. (**a**–**n**) Box plots showing the distribution of plasma levels (pg/ml) of soluble ICMs in children with type 1 diabetes who developed (T1D-AAD$^+$, grey) or did not develop (T1D-AAD$^-$, orange) an additional autoimmune disorder during 5 years of follow-up. Data are shown as the median (horizontal line in the box) and Q1 and Q3 (borders of the box). Whiskers show the lowest and highest values that are not outliers (i.e. data points below Q1–1.5 × IQR or above Q3–1.5 × IQR). Dots outside the whiskers represent outlier values. The statistical significance of the differences between groups was assessed using the Mann–Whitney *U* test (*p<0.05, **p<0.01, ***p<0.001). (**o**) Forest plots showing HR with the corresponding 95% CI for the risk of developing an additional autoimmune condition, estimated for each soluble ICM in children with new-onset type 1 diabetes. Due to the very wide range of ICM values, the original values were scaled by a factor of 10/100/1000/10,000, as appropriate, in order to avoid numerical problems in the estimation of HRs. HRs >1 indicate that the higher the value for the ICM, the higher the risk of developing an additional autoimmune disorder. In the case of GITR, due to the logarithmic transformation, the HR of 1.69 implies that a doubling of the level of this ICM indicates an approximately threefold increase in the risk of developing an additional autoimmune disorder [i.e. HR: exp.(1.69 × $\log_e$[2]) = 3.23]. Red text indicates CD137/4-1BB and PD-1, which have been identified as potential prognostic biomarkers

represent a useful novel immunological tool in the management of individuals with type 1 diabetes to identify early those with higher susceptibility of developing additional autoimmune disorders over time.

Our data reveal a novel immune signature in children with type 1 diabetes who developed an additional autoimmune condition. These findings open the way to future studies elucidating the mechanistic involvement of ICMs in type 1 diabetes-associated autoimmune diseases, and provide new insight into processes leading to the development of additional autoimmunity in individuals with type 1 diabetes.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00125-022-05724-3.

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Data availability The full datasets or part thereof will be made available upon reasonable request.

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Authors' relationships and activities The authors declare that there are no relationships or activities that might bias, or be perceived to bias, their work.

Contribution statement MG designed the study. SB performed most of the experiments, analysed and interpreted the data. EP, FC and MTL acquired and analysed part of the data. DB performed the statistical analysis. EM, MM, CM and AF were responsible for the recruitment of the children and collection of clinical data. SB, DB, PdC, AP, RS, JL, MB, GM and MG were involved in the data discussion, and critically revised the work for its intellectual content. MG performed the final data interpretation and wrote the manuscript, and all authors contributed to its editing and gave final approval of the version to be published. MG accepts full responsibility for the work and the conduct of the study, had access to the data, and controlled the decision to publish.

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