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Meta-Immunological Profiling of Children With Type 1 Diabetes Identifies New Biomarkers to Monitor Disease Progression

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Type 1 diabetes is characterized by autoimmune destruction of pancreatic β-cells in genetically susceptible individuals. Triggers of islet autoimmunity, time course, and the precise mechanisms responsible for the progressive β-cell failure are not completely understood. The recent escalation of obesity in affluent countries has been suggested to contribute to the increased incidence of type 1 diabetes. Understanding the link between metabolism and immune tolerance could lead to the identification of new markers for the monitoring of disease onset and progression. We studied several immune cell subsets and factors with high metabolic impact as markers associated with disease progression in high-risk subjects and type 1 diabetic patients at onset and at 12 and 24 months after diagnosis. A multiple correlation matrix among different parameters was evaluated statistically and assessed visually on two-dimensional graphs. Markers to predict residual β-cell function up to 1 year after diagnosis were identified in multivariate logistic regression models. The meta-immunological profile changed significantly over time in patients, and a specific signature that was associated with worsening disease was identified. A multivariate logistic regression model measuring age, BMI, fasting C-peptide, number of circulating $CD3^+CD16^+CD56^+$ cells, and the percentage of $CD1c^+CD19^-CD14^-CD303^-$ type 1 myeloid dendritic cells at disease onset had a significant predictive value. The identification of a specific meta-immunological profile associated with disease status may contribute to our understanding of the basis of diabetes progression. ***Diabetes*** **62:2481–2491, 2013**

Type 1 diabetes is an autoimmune disease characterized by T cell–mediated destruction of insulin-secreting pancreatic β-cells in genetically susceptible individuals (1). The clinical onset is commonly preceded by a prodromal period characterized by seroconversion to islet autoantibody positivity and by subtle metabolic disturbances. The development of type 1 diabetes involves a complex interaction between pancreatic β-cells and both arms of the innate and adaptive immune system, which undoubtedly changes during the progression of the disease (1). Altered functions, an imbalance of immune cell subsets, or both, and metabolic/inflammatory molecules may contribute to the initiation and progression of the β-cell autoimmune responses resulting in the clinical onset of the disease (1).

In the past decades, we have witnessed a dramatic obesity epidemic from Western affluent countries into the developing world. It has been estimated that there are 1.5 billion overweight individuals worldwide, of which ~500 million are frankly obese (1,2). This rising trend has been paralleled by a similarly dramatic increase in the incidence of type 1 diabetes, which has doubled in the past 15 years. This increased incidence appears not to be uniformly distributed: type 1 diabetes associated with high risk HLA alleles has remained stable, whereas that associated with low-risk genotypes has increased significantly and has shown a tendency to associate with obesity and insulin resistance (3). Indeed, cohort studies have shown that children who developed type 1 diabetes are heavier in the first year of life compared with their peers who remain free of disease and that an increasing BMI strongly correlates with an earlier disease presentation (4–6). Also, insulin-resistance related to overweight not only often preceded clinical onset but was also the strongest predictor of type 1 diabetes, aside from HLA genotype (7,8).

The study of the pathogenesis of early type 1 diabetes in humans requires long follow-up of a large number of subjects, of which only a small fraction will progress to overt disease, due to the relatively low incidence of this disease in the general population and also among subjects at genetic risk.

Meta-immunology has been developing during the last 10 years and can be used in the study of type 1 diabetes to link metabolism with immunity, including immune tolerance. Most of the studies that have examined meta-immunologic parameters in type 1 diabetes have had discrepant results and did not yield definitive conclusions (9–12). Moreover, they typically involved relatively small numbers of subjects and analyzed single (or a few) parameters (12). More important, no study so far has examined meta-immunologic parameters for the prediction of residual β-cell function over time (e.g., via the monitoring of metabolic and immunologic functions characterizing type 1 diabetes).

Here we compared the meta-immunologic profiles of high-risk children, children with newly diagnosed type 1 diabetes, and control subjects. The analysis of 2 years of

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follow-up of meta-immunologic markers correlated with multiple parameters at disease onset and over time. A simple decision rule model based on a multivariate logistic regression analysis capable of predicting at diagnosis was developed, providing a way for discriminating, at diagnosis, children who will maintain good β-cell function from those who will lose their function up to 1 year from diagnosis.

RESEARCH DESIGN AND METHODS

Subjects. Children (n = 114, Supplementary Table 1) who received the diagnosis of type 1 diabetes were recruited after glycemic stabilization on exogenous insulin, achieved in 5 days. Diabetes was defined according to the Global International Diabetes Federation/International Society for Pediatric and Adolescent Diabetes Guidelines for Diabetes in Childhood and Adolescence and included symptoms of diabetes in addition to casual plasma glucose concentration ≥11.1 mmol/L (200 mg/dL), or fasting plasma glucose ≥7.0 mmol/L (≥126 mg/dL), or 2 h postload glucose ≥11.1 mmol/L (≥200 mg/dL) during an oral glucose tolerance test, and glycated hemoglobin (HbA_{1c}) ≥6.5 (13). Among the 114 diabetic children, 40 had ketoacidosis at disease onset, and 25 had at least another autoimmune disorder besides autoimmune diabetes—more frequently celiac disease (n = 12) or autoimmune thyroiditis (n = 13). The following criteria were used to select 34 healthy control subjects (Table 1): fasting blood glucose of <5.5 mmol/L (<100 mg/dL), negative personal and familial history of autoimmune disorders, and negativity for islet autoantibodies at the 99th percentile. The diabetic children and control subjects were recruited in the Department of Pediatrics at the University of Naples Federico II. A local ethical committee approved the study, and parents gave informed consent.

The third group, designed as high-risk subjects, included 29 individuals recruited from baseline of the Diabetes Prevention–Immune Tolerance study at the Skåne University Hospital, Malmö, Sweden. Children carrying HLA susceptibility alleles for type 1 diabetes were defined as high-risk based on the presence of GAD65 autoantibodies (GADA) and at least one more islets-specific autoantibodies (IA-2 antigen, zinc transporter type 8 autoantibodies, or insulin autoantibody). The Lund Regional Ethics Board approved the study, and informed consent was obtained from all subjects or their parents in accordance to the Declaration of Helsinki. There was no significant difference among the three study groups concerning sex and BMI standard deviation score (14). High-risk subjects were younger and consequently had lower BMIs than control subjects and patients with diabetes at onset. Characteristics of the study subjects are summarized in Supplementary Table 1.

Study design. At the beginning of the study, children from each group were analyzed for a wide range of immune (Supplementary Table 2) and metabolic parameters (Supplementary Table 3). Diabetic children were monitored for 12 (n = 60) to 24 (n = 30) months after disease onset and were then analyzed for the same parameters measured at the diagnosis. In addition, patients were dichotomized into two groups on the basis of residual fasting C-peptide (C-pep) evaluated 1 year after disease diagnosis. Specifically, diabetic children with C-pep <0.5 ng/mL were designed as "severe-disease patients," whereas patients with C-pep levels >0.5 ng/mL were defined "mild-disease patients."

A multivariate logistic regression analysis was performed to identify, at the time of diagnosis, biological parameters predictive of a maintained pancreatic function 12 months later. To exclude the possible influence of glucose levels on metabolic parameters, blood was drawn when glycemic values were in the range of 80–180 mg/dL (4.4–10 mmol/L). The actual glucose levels at time of draw were not considered in the analysis.

Flow cytometry. Immune cell profiling of cells from the subjects of the three groups was done at the time of the blood draw. Before flow cytometry to determine lymphocyte subsets, whole blood cells were analyzed with a clinical-grade hematocytometer to determine absolute lymphocyte numbers in each sample. For the control subjects and type 1 diabetes patients, 100 μL blood was incubated 30 min at room temperature with the specific antibodies combinations. Erythrocytes were lysed using BD FACS lysing Solution 2 (BD Bioscience) for 10 min and samples subsequently washed and resuspended in 300 μL PBS. Flow cytometry was carried out on cells gated on $CD45^+$ - Side Scatter (SSC). Immunophenotypic analysis was performed with an EPICS XL flow cytometer (Beckman Coulter, Milan, Italy) using the Beckman Coulter XL System II software program. Triple combinations of different human monoclonal antibodies (e.g., fluorescein isothiocyanate [FITC]– and phycoerythrin [PE]–anti-CD3, PE– and PC-5–anti-CD4, PC5–anti-CD8, PE–anti-CD16, PC5–anti-CD19, PE–anti-CD25, FITC–anti-CD45, and PE–anti-CD56; all from Coulter Immunotech, Marseille, France) were used to identify different cell populations. For high-risk subjects, whole-blood samples were stained with the following monoclonal anti-human antibodies in various combinations for flow cytometry: FITC-conjugated CD3; PE-conjugated CD19 and CD8; peridinin chlorophyll protein (PerCP)-conjugated CD4; CD16 and CD56 (PE; all from BD Bioscience, Becton, Dickinson, Franklin Lakes, NJ). Briefly, 100 μL blood was used for each staining, and samples were incubated 20–30 min at room temperature. Erythrocytes were lysed using BD FACS lysing Solution 2, and samples were washed and resuspended in 300 μL fluorescence-activated cell sorter buffer for flow cytometry. Lymphocytes cell analysis was done on gated $CD45^+$ SSC cells with a FACSCalibur (BD Bioscience, Franklin Lakes, NJ). Data were analyzed using CellQuest software (Becton Dickinson). No differences in the analysis of three studied groups can be ascribed to the procedures used because the same clones of monoclonal antibodies and similar experimental conditions were used in the flow cytometry setting.

Circulating myeloid (mDCs) and plasmacytoid (pDCs) dendritic cells were evaluated using the Blood Dendritic Cells Enumeration Kit (Miltenyi Biotec, Germany). Specifically, mDC1s, mDC2s, and pDCs were identified based on the expression of CD1c, CD141, and CD303 markers, after exclusion in the analysis of the $CD19^+$ and $CD14^+$ cells, respectively. The samples were analyzed by a CyAn flow cytometer and Summit software (Instrumentation Laboratory, Bedford, MA). Cell numbers are expressed as the percentage of a given cell population multiplied by number of lymphocytes/100, except for the number of DCs, for which the percentage was referred to white blood cells (WBCs).

Laboratory tests. For control subjects and high-risk subjects, a 4-mL blood sample was obtained at the time of recruitment. For patients, a 4-mL blood sample was obtained at diabetes onset, after 12 months, and at 24 months. An aliquot from each blood sample was used to perform immune cell profiling by flow cytometry, and the remaining part of the sample was used for serum-based assays. Sera were centrifuged and kept at –80°C until use. Fasting C-pep levels were measured in duplicate serum samples, at the same time in all samples, using a commercial ELISA kit (Millipore Corporation, Billerica, MA). Results for each assay were validated, and a high-level and a low-level control sample were included.

Circulating leptin (Lep) and soluble leptin receptor (sLepR) were determined in duplicate serum samples using human Leptin and human Leptin sR Immunoassays, respectively (R&D Systems Inc., Minneapolis, MN).

Soluble CD40 L (sCD40 L), soluble intracellular adhesion molecule (sICAM-1), monocyte chemoattractant protein-1 (MCP-1), myeloperoxidase (MPO), osteoprotegerin (OPG), resistin, and soluble tumor necrosis factor receptor (sTNFR) were analyzed using the bead-based analyte detection system Human Obesity 9plex kit (Bender MedSystem Inc, Burlingame, CA) in duplicate serum samples.

Plasma glucose levels were measured using enzymatic hexokinase method. HbA_{1c} was measured by high-performance liquid chromatography (HLC-723

TABLE 1
Estimated regression coefficients and adjusted odds ratio (95% CI) for predictors of residual C-pep secretion in the base and in the full logistic regression model

	Base model			Full model		
	Estimated coefficient	OR (95% CI)	P	Estimated coefficient	OR (95% CI)	P
Age at diagnosis	−0.175	0.84 (0.69–1.03)	0.091	−0.376	0.69 (0.51–0.93)	0.015
BMI at diagnosis	0.064	1.07 (0.92–1.24)	0.406	0.119	1.13 (0.92–1.39)	0.262
C-pep secretion at diagnosis ≤0.5 ng/mL	2.259	9.57 (1.83–50.11)	0.008	2.390	10.91 (1.81–67.00)	0.010
Percent of $CD1c^+CD19^-CD14^-CD303^-$ cells	—	—		−3.471	0.03 (0.01–0.59)	0.021
N of $CD3^+CD16^+CD56^+$ cells	—	—		−0.020	0.98 (0.96–0.99)	0.021

OR, odds ratio.

G7 TOSOH, Bioscience, Tokyo, Japan). Islet autoantibodies (GADA, IA-2A, and IAA) were measured by commercial ELISA (Pantec, Torino, Italy).

Statistical analysis. Normally distributed continuous variables are reported as mean ± SD, median (min–max) was used to describe not-normally distributed continuous variables, and categorical variables are reported as number of occurrences and percentages. For all study variables, comparison among control subjects, high-risk subjects, and patients was based on the nonparametric Kruskal-Wallis procedure, followed by the Mann-Whitney *U* test with Bonferroni correction. Paired comparisons between patients at baseline, after 12 months, and at 24 months from diagnosis were carried out using the Friedman test, followed by Wilcoxon signed-rank test with Bonferroni correction. The Spearman correlation coefficient was computed to investigate the biological correlations between the different variables.

Because of the large number of the variables examined and to control the family-wise error rate at level $\alpha = 0.05$, the significance of Kruskal-Wallis, Friedman, and Spearman correlation *P* values were judged by using the adaptive Bonferroni procedure (15,16).

A multivariable logistic regression model was fitted to predict the residual C-pep secretion (dichotomized as 1 if ≤0.5 ng/mL and 0 if >0.5 ng/mL) after 12 months from the diagnosis on the basis of patients' baseline measurements. The following two-step model strategy was adopted. All variables reported in Supplementary Tables 2 and 3 were tested in univariate analysis. Only the variables that showed a univariate association with the outcome ($P < 0.25$) were included in the subset of candidate predictors (17). Afterward, a backward elimination procedure (with a probability for removal equal to 0.10) was applied to identify those variables independently associated with the residual C-pep. In this second step, the model was further adjusted for the following covariates measured at diagnosis: age, BMI, and fasting C-pep secretion (dichotomized as 1 if ≤0.5 ng/mL and 0 if >0.5 ng/mL). This full model, including variables independently associated with residual C-pep, was compared with a base model including only the adjusting covariates of age, BMI, and C-pep secretion measured at diagnosis. Prognostic validity of the fitted models was evaluated by receiver operating characteristic (ROC) curve analysis and measured using the area under the ROC curve (AUC). Comparisons among different AUCs were carried out using the nonparametric approach (18). For all analyses, we used two-sided tests, with *P* values < 0.05 denoting statistical significance (unless otherwise specified). Modeling and statistical analyses were carried out using R 2.12.1 and SPSS 16 (SPSS Inc., Chicago, IL) software.

RESULTS

Meta-immunological profiling of type 1 diabetic patients at onset, high-risk subjects, and control subjects. To depict the immunological profile of type 1 diabetes at onset, we analyzed several immune cell populations in the

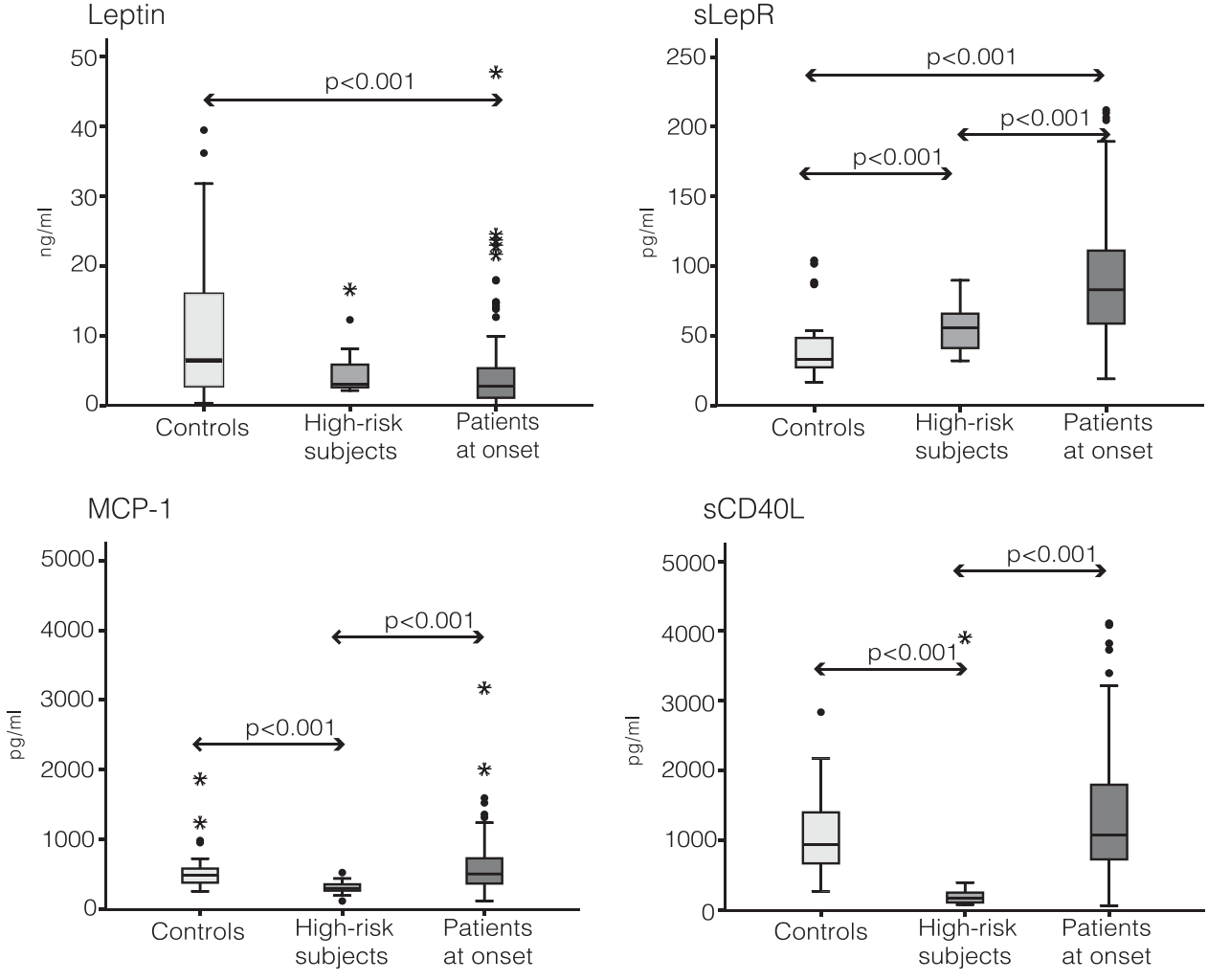

FIG. 1. Meta-immunological profiling in patients at onset, in high-risk subjects, and in control subjects. Box plots show the distribution of circulating level of leptin, sLepR, MCP-1, and sCD40 L in type 1 diabetic patients at onset, high-risk subjects, and control subjects. Dots represent outlier values (i.e., data points below Q1 − 1.5 × IQR or above Q3 + 1.5 × IQR), and asterisks represent extreme values (i.e., data points below Q1 – 3 × IQR or above Q3 + 3 × IQR). Q1 = 25th percentile; Q3 = 75th percentile; IQR (interquartile range) = Q3–Q1. Data are shown as median (horizontal line in the box) and Q1 and Q3 (borders of the box). Whiskers represent the lowest and the highest values that are not outliers (i.e., data points below Q1 − 1.5 × IQR or above Q3 + 1.5 × IQR) or extreme values (i.e., data points below Q1 − 3 × IQR or above Q3 + 3 × IQR). Patients had lower serum levels of Lep ($P < 0.001$) and higher serum levels of sLepR ($P < 0.001$) than control subjects. High-risk subjects had intermediate levels of sLepR between children with type 1 diabetes and control subjects. Patients and control subjects had higher levels of MCP-1 than high-risk subjects; the same was observed for sCD40 L levels.

peripheral blood of children at onset compared with high-risk subjects and control subjects (Supplementary Table 2). We found that patients at onset had a significantly lower percentage of circulating $CD8^+$ T cells compared with high-risk subjects ($P = 0.001$, after Bonferroni correction) but not compared with healthy control subjects. Furthermore, diabetic children also showed a significant higher number of $CD4^+$ T cells with a memory phenotype ($CD4^+CD45RO^+$) compared with the control subjects ($P = 0.03$, after Bonferroni correction). Finally, patients had a significantly higher percentage ($P = 0.03$, after Bonferroni correction) and total number ($P = 0.01$, after Bonferroni correction) of $CD303^+CD1c^-CD19^-CD14^-$ pDCs compared with healthy subjects (Supplementary Table 2).

To characterize the meta-immunological profile of type 1 diabetes, several indicators of inflammatory and metabolic activities (leptin, sLepR, MCP-1, sCD40 L, MPO, sICAM-1, resistin, OPG, and sTNFR) were analyzed (Fig. 1 and Supplementary Table 3). Diabetic children at onset had lower levels of Lep ($P < 0.001$, after Bonferroni correction) and higher concentrations of circulating sLepR than high-risk ($P < 0.001$, after Bonferroni correction) and control subjects ($P < 0.001$, after Bonferroni correction). High-risk subjects showed intermediate levels of sLepR between controls and patients ($P < 0.001$, after Bonferroni correction; Fig. 1 and Supplementary Table 3). We also observed that circulating levels of the proinflammatory chemokine MCP-1 were significantly higher in children affected by type 1 diabetes than in high-risk subjects ($P < 0.001$, after Bonferroni correction) and significantly lower in high-risk subjects than in controls ($P < 0.001$, after Bonferroni correction). The same trend was observed for

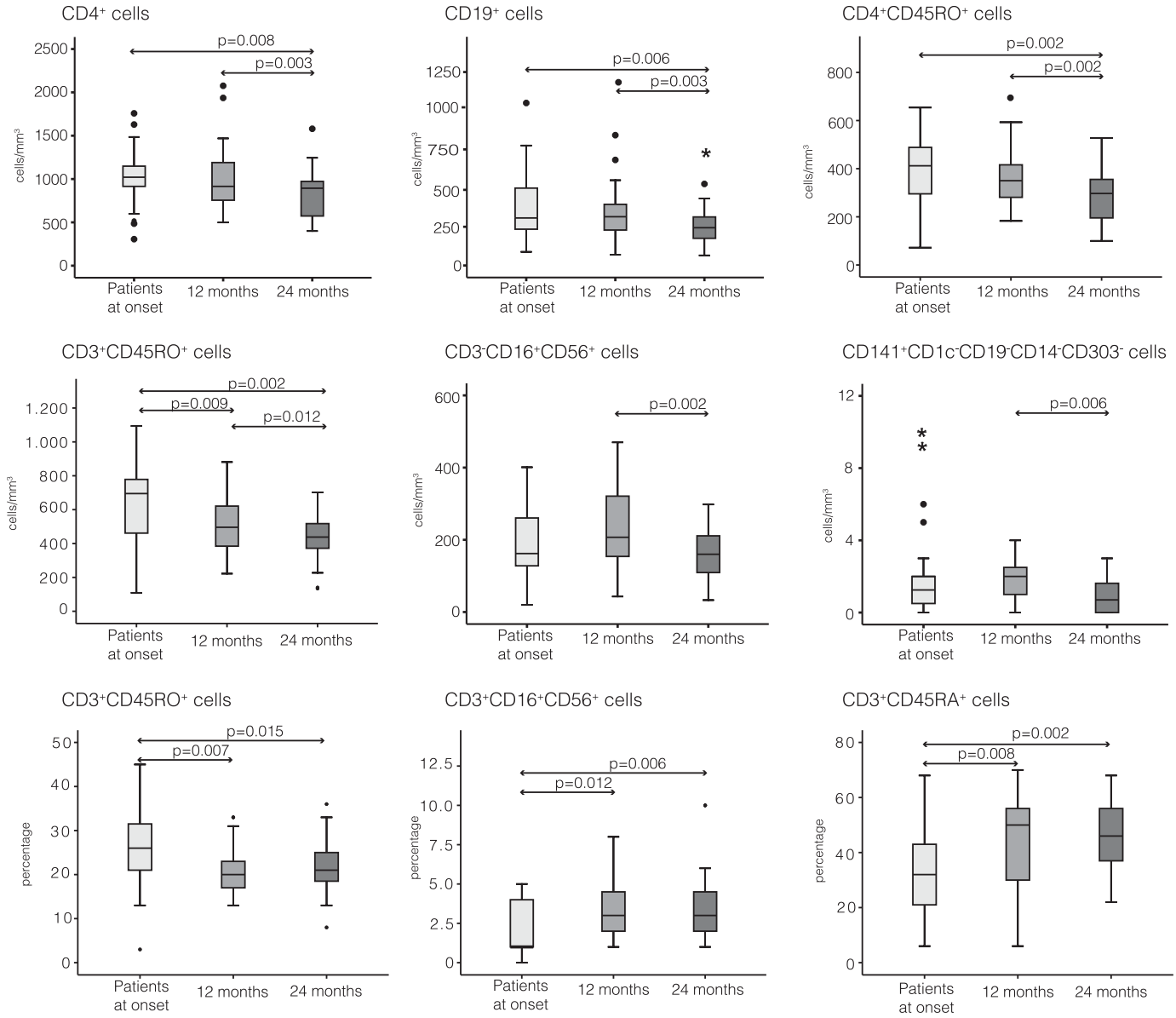

FIG. 2. Immunological follow-up in type 1 diabetes. Children affected by type 1 diabetes were studied at diagnosis and at 12 and 24 months after disease onset. Box plots show the distribution of $CD4^+$ T cells, $CD19^+$ B cells, $CD4^+CD45RO^+$ T cells, $CD3^+CD45RO^+$ T cells, $CD3^-CD16^+CD56^+$ cells, and $CD141^+$ cells and percentages of $CD3^+CD45RO^+$ T cells, $CD3^+CD16^+CD56^+$ cells, and $CD3^+CD45RA^+$ T cells in patients at onset and after 12 and 24 months. Data are shown as median (horizontal line in the box) and Q1 and Q3 (borders of the box). Whiskers represent the lowest and the highest values that are not outliers (i.e., data points below Q1 − 1.5 × IQR or above Q3 + 1.5 × IQR) or extreme values (i.e., data points below Q1 − 3 × IQR or above Q3 + 3 × IQR). Dots represent outlier values and asterisks represent extreme values. Q1 = 25th percentile; Q3 = 75th percentile; IQR (interquartile range) = Q3–Q1.

the circulating plasma levels of sCD40 L (Fig. 1 and Supplementary Table 3).

Meta-immunological follow-up profiling of type 1 diabetes. Sera from patients were also analyzed at follow-up, namely at 12 and 24 months after disease diagnosis. During disease progression, a significant progressive reduction was found in the number of $CD4^+$ T cells, with the lowest values at 24 months after diagnosis (Fig. 2). The same trend was observed for the absolute number of circulating $CD19^+$ B cells. In addition, during disease progression, there was a progressive reduction in the absolute number of circulating T cells with a memory phenotype ($CD4^+CD45RO^+$ or $CD3^+CD45RO^+$). Conversely, the percentage of naïve T cells ($CD3^+CD45RA^+$) was lower at disease onset compared with values observed at later time points. In addition, there were higher absolute numbers of natural killer (NK) cells ($P = 0.002$, after Bonferroni correction) and $CD141^+CD1c^-CD19^-CD14^-CD303^-$ mDC2s at 12 months compared with those observed at 24 months after disease onset ($P = 0.006$, after Bonferroni correction). The $CD3^+CD16^+CD56^+$ cells subset was lower at onset than at later time points. In serum, a progressive decline of circulating sLepR levels at 12 and at 24 months ($P < 0.001$ for both, after Bonferroni correction) and a progressive reduction of circulating OPG were observed from disease onset thereafter (Fig. 3). Finally, we also generated over-time analyses showing single trajectories at the individual level for immunological and metabolic parameters in type 1 diabetes patients (Supplementary Figs. 1 and 2).

Multiple correlations among meta-immunological markers. We performed multiple correlation analyses in diabetic patients at onset, in high-risk individuals, and in control subjects. In the group affected by type 1 diabetes, sLepR inversely correlated with circulating levels of fasting C-pep ($r = -0.39$, $P < 0.001$), which reflects β-cell function. This correlation was not present in control subjects or in high-risk subjects (Fig. 4 and data not shown). Moreover, in patients at onset, the circulating levels of resistin positively correlated with levels of sTNFR ($r = 0.47$, $P < 0.001$), MCP-1 (0.52 with $P < 0.001$), and MPO ($r = 0.71$, $P < 0.001$). Circulating MCP-1 positively correlated with MPO ($r = 0.62$, $P < 0.001$) and sTNFR levels ($r = 0.52$ with $P < 0.001$). All these correlations were not present in control subjects and in high-risk subjects (Fig. 4 and data not shown).

Several correlations in immune cell subsets were only found in patients and not in control subjects and high-risk subjects. In particular, only in the patients, the percentage of $CD3^+$ cells inversely correlated with the percentage of NK ($r = -0.41$, $P < 0.001$) and B cells ($r = -0.65$, $P < 0.001$). Moreover, the NK cells inversely correlated with $CD3^+CD45RA^+$ ($r = -0.51$, $P < 0.001$) and $CD4^+CD45RA^+$ ($r = -0.46$, $P < 0.001$) naïve T cells (Fig. 4).

One-year follow-up. Meta-immunological correlations in diabetic patients at onset and at 12 months after diagnosis were also studied. Significant differences were observed compared with the same patients at diabetes onset (Fig. 5). For example, the inverse correlation between sLepR and fasting C-pep observed at disease onset was lost after 12 months. A series of positive correlations at diagnosis, such as resistin versus MCP-1 ($r = 0.52$, $P < 0.001$) and MPO ($r = 0.70$, $P < 0.001$) as well as MCP-1 versus MPO ($r = 0.22$, $P < 0.01$) were observed. Only the correlation between resistin and MPO was maintained over time ($r = 0.51$, $P < 0.001$; Fig. 5). At the cellular level, the correlations among the percentage of $CD3^+$ T cells versus NK and B cells observed at diagnosis was lost after 12 months, whereas the percentage $CD4^+CD45RO^+$ T cells inversely correlated with that of $CD3^+CD45RA^+$ T cells and $CD4^+CD45RA^+$ T cells at disease onset. These correlations were not maintained 1 year later (Fig. 5).

Novel predictive tool of residual β-cell function over time. The possibility to predict residual C-pep levels after disease onset and the identification of biomarkers assessing therapeutic efficacy is a major goal in type 1 diabetes monitoring and prognosis. Multivariable logistic regression analysis showed that the number of $D3^+CD16^+CD56^+$ T cells and the percentage of

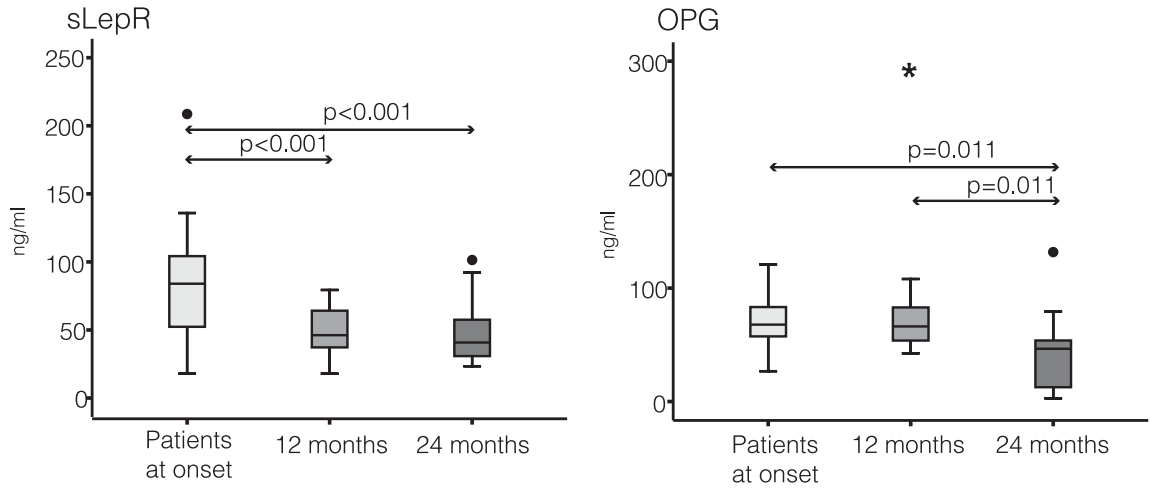

FIG. 3. Metabolic follow-up in type 1 diabetes. Box plots show the distribution of circulating levels of sLepR and OPG in diabetic children at onset and 12 and 24 months later. Patients had very high circulating levels of sLepR at disease onset. Serum levels of sLepR showed a trend toward reduction over time, with lower serum levels after 24 months from disease onset ($P < 0.001$). Serum levels of OPG progressively decreased over time ($P = 0.011$). Data are shown as median (horizontal line in the box) and Q1 and Q3 (borders of the box). Whiskers represent the lowest and the highest values that are not outliers (i.e., data points below Q1 − 1.5 × IQR or above Q3 + 1.5 × IQR) or extreme values (i.e., data points below Q1 − 3 × IQR or above Q3 + 3 × IQR). Dots represent outlier values and asterisks represent extreme values. Q1 = 25th percentile; Q3 = 75th percentile; IQR (interquartile range) = Q3–Q1.

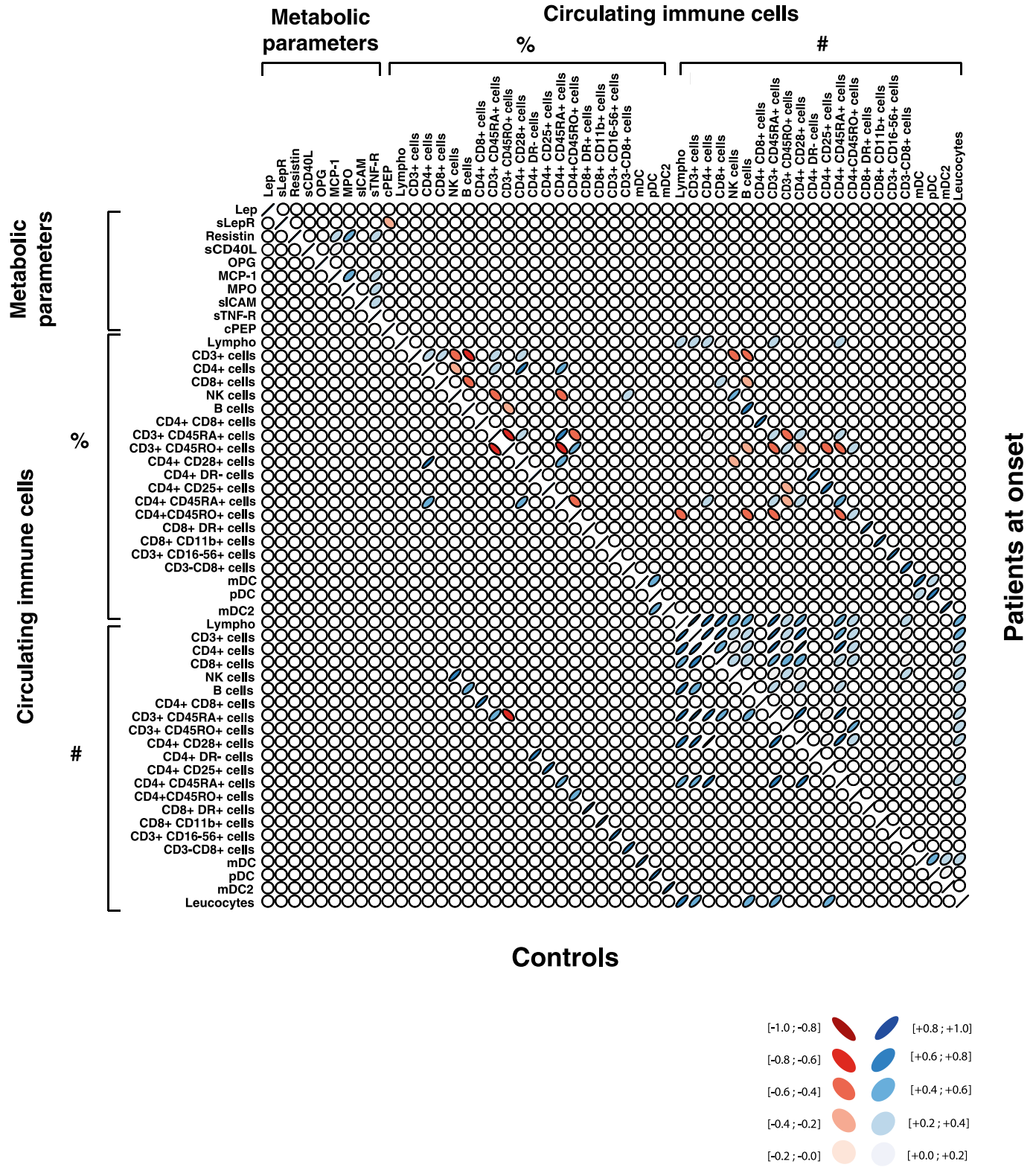

FIG. 4. Multiple biological correlations in type 1 diabetes. Two-dimensional graphical representation of the Spearman ρ nonparametric correlation matrix among the studied variables. The presence of a significant correlation between two variables is expressed by means of a red (negative correlation) or blue (positive correlation) ellipse, and an empty circle refers to a nonsignificant correlation. The color intensity and thickness of each ellipse are proportional to the correlation value (see graphic legend for numeric values). Correlation analysis, performed on all parameters analyzed, revealed a distinctive immune/metabolic profile for controls and new-onset diabetic patients. A negative correlation between sLepR and β-cell residual fasting C-pep secretion ($r = -0.39$, $P < 0.001$) was observed in individuals affected by type 1 diabetes but not in controls. #Absolute number of indicated cell populations with respect to the number of leukocytes; %percentage of indicated cell populations with respect to the number of leukocytes.

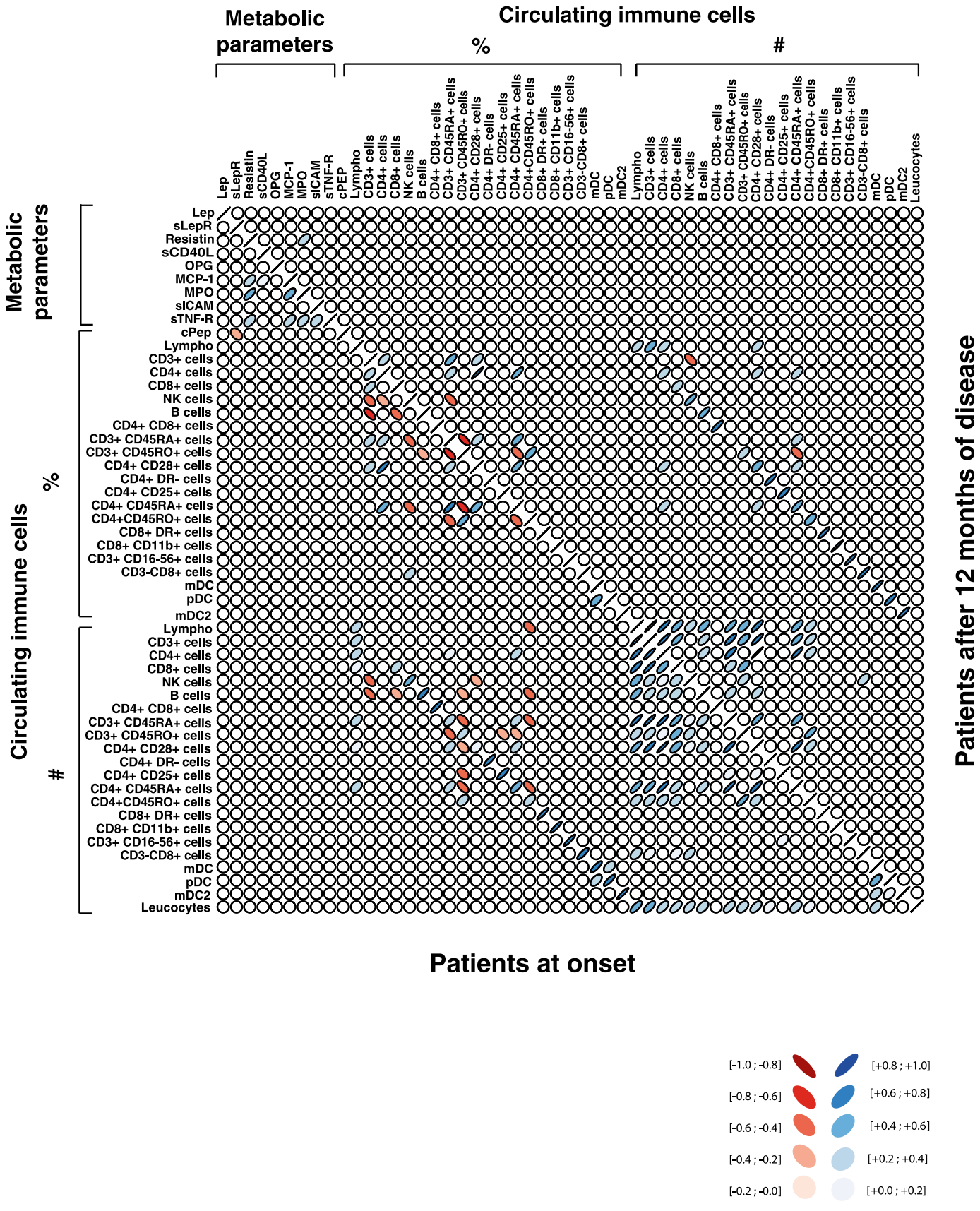

FIG. 5. Multiple biological correlations in type 1 diabetic children over time. Two-dimensional graphical representation of the Spearman ρ nonparametric correlation matrix among the study variables. The presence of a significant correlation between two variables is expressed by means of a red (negative correlation) or blue (positive correlation) ellipse, and an empty circle refers to a nonsignificant correlation. The color intensity and the thickness of each ellipse are proportional to the correlation value (see graphic legend for numeric values). Correlation analysis, performed on all parameters analyzed, revealed a distinctive immune/metabolic profile for children with type 1 diabetes over time. The inverse correlation between sLepR and fasting C-pep,

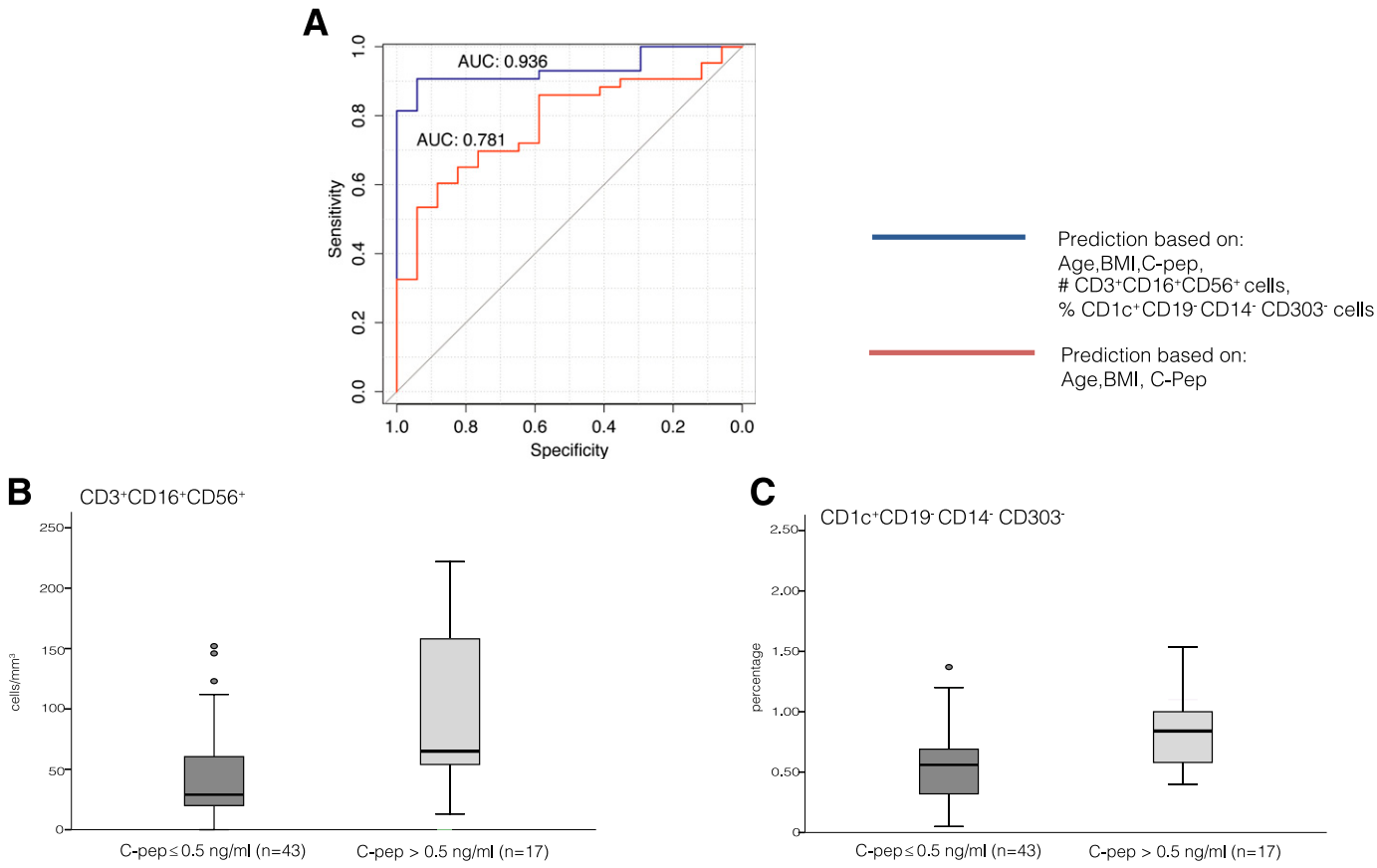

FIG. 6. Prediction analysis. *A*: ROC curves of the model-based prognostic scores for residual β-cell functioning. The base model included age, BMI, and fasting C-pep secretion measured at disease onset, whereas the full model added the number of $CD3^+CD16^+CD56^+$ T cells and the percentages of mDCs to the base model. *B*: Box plot shows that high numbers of $CD3^+CD16^+CD56^+$ T cells at onset are associated with a reduced β-cell activity 1 year later. Low numbers were associated with a residual β-cell function. *C*: Box plot shows that the high percentage of mDCs at onset was associated with a reduced β-cell activity 1 year later. Conversely, low numbers of these cells were associated with residual β-cell function. Data are shown as median (horizontal line in the box) and Q1 and Q3 (borders of the box). Whiskers represent the lowest and the highest values that are not outliers (i.e., data points below Q1 − 1.5 x IQR or above Q3 + 1.5 × IQR) or extreme values (i.e., data points below Q1 − 3 × IQR or above Q3 + 3 × IQR). Dots represent outlier values and asterisks represent extreme values. Q1 = 25th percentile; Q3 = 75th percentile; IQR (interquartile range) = Q3–Q1. #Absolute number of indicated cell populations with respect to the number of leukocytes; %percentage of indicated cell populations with respect to the number of leukocytes.

$CD1c^+CD19^-CD14^-CD303^-$ mDC1s at disease diagnosis were independent predictors of low (≤0.5 ng/mL) residual C-pep secretion after 12 months from diagnosis, after adjustment for age and BMI as covariates measured at diagnosis. The data in Table 1 report the regression coefficient and the corresponding odds ratio (95% CI) for the model and for a reduced base model including only the adjusting factors. In addition, a linear regression model confirmed the predictive role of $CD3^+CD16^+CD56^+$ T cells and $CD1c^+CD19^-CD14^-CD303^-$ mDC1s independently of age, BMI, and C-pep values at onset. The results of this model are reported in Supplementary Table 4. Figure 6 shows the ROC curves associated to the base model and to the full model. The AUC of the full model was 0.936 ($P <$ 0.001), and compared with the AUC of the base model (0.781, $P < 0.001$), was significantly higher ($P = 0.005$). Box plots in Fig. 6 indicate that low $CD3^+CD16^+CD56^+$ T cells and low mDC1s at disease onset associated with a reduced β-cell activity (fasting C-pep ≤0.5 ng/dL) in type 1 diabetes 1 year later.

To characterize further the patients with different residual β-cell function, we performed a correlation analysis of severe-disease patients (fasting C-pep ≤0.5 ng/dL) and mild-disease patients (fasting C-pep >0.5 ng/dL) at disease onset and 1 year later. We observed that in patients with a worse pancreatic function (fasting C-pep ≤0.5 ng/dL), resistin positively correlated with the levels of C-pep, MCP-1, and sTNFR. In addition, there was also a positive correlation in this group between MCP-1 and TNFR. These correlations were not observed in patients with residual β-cell function (fasting C-pep >0.5 ng/dL). An inverse correlation between the percentage of naïve T cells ($CD4^+CD45RA^+$) and memory T cells ($CD4^+CD45RO^+$) was observed in severe-disease patients but not in mild-disease patients. Many of the correlations observed at diabetes onset were not present 12 months after disease onset. $CD3^+$ positively correlated with $CD4^+$ (and $CD4^+CD28^+$) in severe-disease patients but not in mild-disease patients. Only in mild-disease patients did we observe an inverse correlation between $CD3^+CD45RA^+$ and $CD3^+CD45RO^+$ (Fig. 7).

observed at disease onset, was lost 12 months later. #Absolute number of indicated cell populations with respect to the number of leukocytes; %percentage of indicated cell populations with respect to the number of leukocytes.

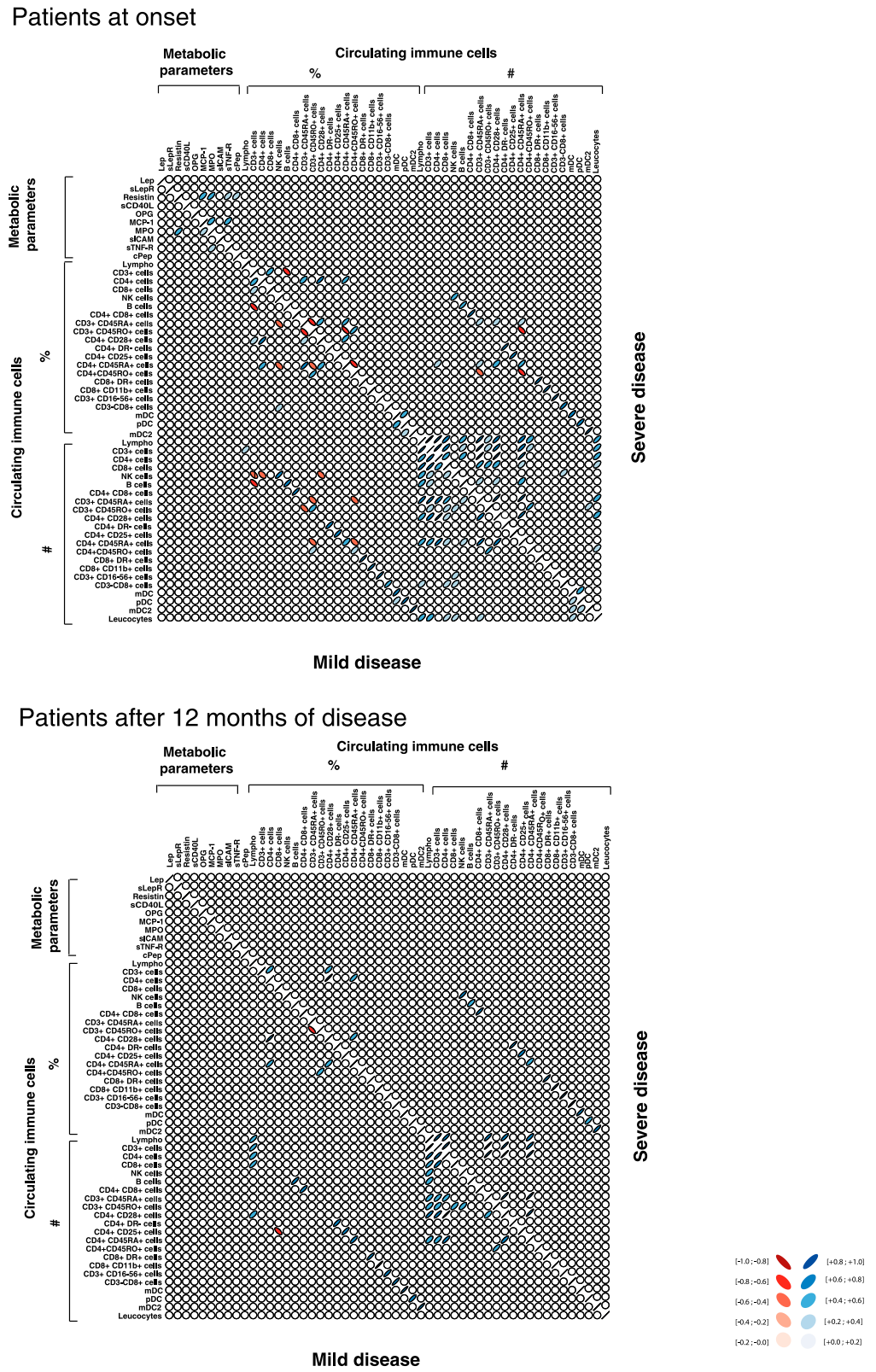

FIG. 7. Distinctive correlation profiles of patients affected by type 1 diabetes according to disease severity at onset and 12 months later. Graphical representation of the Spearman ρ nonparametric correlation matrix among the study variables. The presence of a significant correlation between two variables is expressed by means of a red (negative correlation) or blue (positive correlation) ellipse, whereas an empty circle refers to a nonsignificant correlation. The color intensity and thickness of each ellipse are proportional to the correlation value (see graphic legend for numeric values). The correlation profile between patients with mild and severe disease at onset and 12 months later is shown.

DISCUSSION

The comprehension of the pathogenesis of type 1 diabetes has improved considerably in recent years, yet there is a lack of predictive markers of risk for disease progression and pancreatic β-cell loss. Such markers could be very useful to monitor the disease, to ameliorate prognosis, and to evaluate therapeutic efficacy.

The meta-immunological profiling described here for type 1 diabetes at onset and during progression identified three circulating immune cell populations and four metabolic/inflammatory markers as significantly different among diabetic patients at onset, in high-risk subjects, and in control subjects. $CD8^+$ T lymphocytes were significantly higher in high-risk subjects than in patients in accordance with a previous report (19), a finding that is reminiscent of the known increased proliferation of this cell subset during insulitis, before the onset of hyperglycemia and overt diabetes. Higher numbers of memory T cells in patients at onset than in controls were also found. Interestingly, in the 24-month follow-up, there was a progressive decline in the number of memory T cells in diabetic patients, in accordance with the findings that GAD65-specific T cells in diabetic patients bear a memory phenotype, whereas they are naïve T cells in healthy individuals (20). We hypothesize that the progressive decrease of memory T cells might be secondary to a gradual exhaustion of islet-specific T-cell clones after initial epitope spreading. Moreover, pDCs were higher in children with type 1 diabetes at onset compared with healthy children. This aspect is interesting, because in the presence of β-cell–specific autoantibodies, pDCs process and present islet autoantigens to CD4 T cells to amplify and maintain T-cell responses favoring epitope spreading (21). Previous investigations studied the frequency of this cell subset with discrepant results, showing a reduced pDCs number in the blood of patients compared with healthy controls (10,22,23) or an increased frequency of pDCs at diagnosis of type 1 diabetes (24). These conflicting results could depend on the procedures for the analysis, the timing of sampling, and the size of the studied population. Our study is the first to analyze pDCs longitudinally for 24 months in a large cohort of patients after disease diagnosis.

Metabolically, the leptin/sLepR axis was altered in newly diagnosed diabetic children compared with high-risk subjects and control subjects. Leptin profoundly affects metabolism and immune functions (25) and promotes the development of type 1 diabetes in NOD mice (26,27). On the contrary, it has been reported that leptin improves insulin-deficient type 1 diabetes, reverting catabolism through the suppression of hyperglucagonemia, resembling the anabolic action of insulin monotherapy, and normalizing HbA_{1c} (27). sLepR, the main leptin-binding protein in human blood, can modulate leptin effects on target organs by influencing leptin action in two ways: *1*) by inhibiting the binding of leptin to its membrane receptors, and *2*) by increasing the availability of Lep, thus delaying its clearance. We found that children affected by type 1 diabetes at diagnosis had lower Lep and higher sLepR levels compared with high-risk and control subjects, which is consistent with the report of elevated serum sLepR in diabetic children with metabolic decompensation (28). Low levels of leptin in subjects with type 1 diabetes could be partially explained by the high amount of sLepR in the same patients. sLepR could mask leptin epitopes and hamper detection of this adipokine. The behavior of sLepR levels during diabetes progression suggests a potential of sLepR as an early novel marker of type 1 diabetes. This is linked to the finding that sLepR levels decreased over time during diabetes progression but still remained higher than in healthy control subjects. In addition, sLepR circulating levels inversely correlated with β-cell function, measured as levels of circulating fasting C-pep, at disease onset (Fig. 4). This finding was not confirmed in high-risk and control groups, although the lack of significance could be attributed to the reduced power due to smaller sample size for those groups. A better understanding of the mechanisms controlling the leptin/sLepR axis may lead to speculation on the possibility of using leptin as a substitute for or a combination therapy with insulin in type 1 diabetes.

The importance of our study relies on the current lack of markers able to predict diabetes progression and severity in type 1 diabetes. Such markers could help clinicians in choosing the most appropriate therapeutic intervention, in disease monitoring, and in the evaluation of therapeutic efficacy, ultimately improving prognosis. Because type 1 diabetes is a complex multifactor disease with a strong genetic component and significant environmental influences, a broad immunological assessment and multifactor algorithm to pick patterns is likely needed to dissect the complex pathogenesis and identify key patterns of disease progression. By using a multivariate logistic regression analysis adjusted for age, BMI, and the value of fasting C-pep at disease onset, we identified here two specific immune cell populations, both measured at disease onset, that were capable to predict C-pep secretion as a surrogate measure of β-cell mass in humans with type 1 diabetes (29). Indeed, the number of $CD3^+CD16^+CD56^+$ T cells and the percentage of mDC1s were independent predictors of residual C-pep secretion 12 months after diagnosis.

Our prognostic score of the fitted model reached an AUC that was significantly higher than the AUC of a prognostic base model that included only age, BMI, and fasting C-pep measured at disease onset ($P < 0.001$). We also provided a wide range of biological correlates of disease compared with healthy and disease-progressing individuals. The biological correlations between meta-immunological parameters in subjects with mild and severe type 1 diabetes indicated that in mild-disease patients, the T cells negatively correlated with NK and B cells, both correlations not detectable in subjects with severe disease. An advantage of the current study is the multiparametric analysis on a large, well-characterized cohort of newly diagnosed patients with type 1 diabetes monitored prospectively and longitudinally for 24 months. As such, this is the first comprehensive study associating β-cell secretion capability with circulating immune cell subsets. The number and percentage of mDC1s and $CD3^+CD16^+CD56^+$ T cells were prognostic factors of pancreatic insulin secretion up to 1 year after disease onset. Finally, our approach could also be of valuable help to understand the molecular basis of slow versus fast progressors to disease and to monitor high-risk subjects toward the development of β-cell failure in conjunction with tests measuring the acute insulin response to glucose (i.e., AIR-glucose), which could reflect very early an ongoing immune attack against insulin producing β-cells.

In conclusion, our experimental model could represent a novel tool to monitor the staging of type 1 diabetes patients and may be useful, together with previously known parameters (i.e., HbA_{1c}, insulin requirement), to assess disease progression and also contribute to define the most appropriate therapeutic approach according with

the aggressiveness of the disease at onset. Our results could lead to the discovery and/or validation of biomarkers that represent early events in the disease as well as those that may serve as surrogate read-outs or end points for efficacy of specific interventions.

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M.G. wrote the manuscript and researched data. R.N. enrolled patients, wrote the manuscript, and researched data. D.B. performed statistical analyses. F.P., V.D.R., C.P., E.M., and H.E.L. researched data. C.M.C. enrolled patients and reviewed and edited the manuscript. A.L. reviewed and edited the manuscript. A.L.C. contributed to discussion and reviewed and edited the manuscript. A.F. enrolled patients and contributed to discussion. G.M. designed and coordinated research and wrote the manuscript. G.M. 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.

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