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Clinical Research Article

Blood Lymphocyte Subsets and Proinflammatory Cytokine Profile in ROHHAD(NET) and non-ROHHAD(NET) Obese Individuals

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Abstract

Context: Rapid-onset obesity with central hypoventilation, hypothalamic dysfunction, and autonomic dysregulation with neural crest tumors (ROHHAD-NET) syndrome pathophysiology remains elusive. Acquired neuroimmunological dysfunction has been proposed as a possible pathogenetic pathway.

Objective: The aim of our study was to characterize lymphocyte subpopulations subsets in peripheral blood (PB) and to evaluate a panel of proinflammatory cytokines/chemokines in ROHHAD(NET) patients vs controls.

Methods: We included 11 ROHHAD(NET) patients, 7 ROHHAD and 4 ROHHAD-NET, selected by clinical criteria. Controls were 11 simple obese children, matched for age and sex. Flow cytometric analysis and enzyme-linked immunosorbent assay were performed on PB and serum samples of the 2 groups.

Results: Analysis revealed that T lymphocytes are significantly increased in ROHHAD(NET) patients ($P = .04$) with a prevalence of CD4-T cells ($P = .03$) and a lower number of activated CD8-T cells ($P = .02$). With regard to regulatory subset, patients displayed increased regulatory B cells ($P = .05$) and type-1 regulatory T cells ($P = .03$). With regard to CD8-T cells, a lower number of T effector memory was observed ($P = .02$). In contrast, among CD4-T cells, we found a higher number of T naive ($P = .04$) and T effector ($P = .0008$). Interleukin-8 (IL-8) levels and monocyte chemotactic protein-1 were increased in patients vs controls ($P = .008$ and $P = .01$, respectively). Furthermore, IL-8 levels were higher in the subgroup with neural tumor ($P = .0058$) (ROHHAD-NET) than in patients without neural tumor (ROHHAD). Soluble HLA-G was significantly lower in patients vs controls ($P = .03$).

Conclusion: Our findings contribute to support the hypothesis of immune dysregulation, which may underlie this complex, often fatal disease. Because ROHHAD(NET) syndrome is an ultra-rare disease, multicentric studies are needed to improve the effect of our data in the management of this condition.

Key Words: ROHHAD, ROHHAD-NET, immune phenotype, cytokines, chemokines

Abbreviations: BMI, body mass index; Breg, regulatory B cells; BSA, bovine serum albumin; CNS, central nervous system; CSF, cerebrospinal fluid; F, female; FT4, free thyroxine; HLA, human leukocyte antigen; IL, interleukin; M, male; MCP1, monocyte chemotactic protein-1; NET, neural crest tumors; NK, natural killer; NT1, type 1 narcolepsy; OSAS, obstructive sleep apnea syndrome; PB, peripheral blood; PBS, phosphate-buffered saline; ROHHAD, rapid-onset obesity with central hypoventilation, hypothalamic dysfunction, and autonomic dysregulation; RT, room temperature; s, soluble; T1DM, type 1 diabetes mellitus; TNFα, tumor necrosis factor α; Tr1, type 1 regulatory T cells; Treg, regulatory T cells; TSH, thyrotropin.

Rapid-onset obesity with central hypoventilation, hypothalamic dysfunction, and autonomic dysregulation (ROHHAD) is a very complex disease, definitively characterized by Ize-Ludlow et al in 2007 [1, 2]. Since the description of the first case in 1965 [3], ROHHAD syndrome remains challenging in diagnosis and treatment due to the systemic nature of the disease and its still unknown etiology. It requires a multidisciplinary approach, and life-long follow-up is recommended, considering the high morbidity and mortality rate of up to 60% [1].

The earliest sign of ROHHAD syndrome is usually hyperphagic obesity [1] with a rapid weight gain occurring between

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ages 2 and 4 years in otherwise healthy children. Changes in the nocturnal respiratory pattern can also be an early and worrying sign of this disease [1, 4]. Endocrine disorders (hyperprolactinemia, central hypothyroidism, central adrenal insufficiency, precocious or delayed puberty, growth hormone deficiency, water and electrolyte imbalance) and autonomic dysregulation generally appear later [5]. Neurological and psychiatric disorders like seizures, psychomotor retardation, or aggressive behavior may also be present in these patients [6, 7]. In addition, strabismus and other ophthalmologic disorders are often reported.

Up to half of patients develop neural crest tumors (ROHHAD-NET), especially mediastinal ganglioneuroma, ganglioneuroblastoma, and neuroblastoma [5, 6, 8].

Genetic, paraneoplastic, and dysimmune pathogenic mechanisms have been proposed, but a certain and conclusive etiology has never been confirmed for ROHHAD(NET) syndrome [9-11]. Genes involved in the regulation of neural development have been investigated as possible causes of ROHHAD(NET) syndrome, with no definite result so far [9, 12]. Alternative hypotheses encompass paraneoplastic mechanisms and immune dysfunctions [13-15]. The autoimmune/paraneoplastic hypothesis arises from the observation of the association between ROHHAD(NET) clinical phenotype and neural crest tumors, similar to what occurs in opsoclonus-myoclonus ataxia syndrome. Furthermore, postmortem examinations in ROHHAD(NET) patients have shown neuropathological findings similar to those observed in autoimmune encephalitis [10, 16, 17], thus supporting the autoimmune pathogenesis hypothesis, while in other cases the hypothalamus, brainstem, and whole brain were normal at autopsy [5, 18]. Gharial and colleagues [19] reported the involvement of the hypothalamus and brainstem tegmentum, with a specific pattern of inflammation consisting of both dense perivascular cuffs and patchy nodular parenchymal infiltrates. In one case report, focal inflammation in the periaqueductal gray matter and a bulky pituitary stalk with gadolinium enhancement on magnetic resonance imaging scan corroborate autopsy evidence of lymphocytic hypothalamic infiltration [20].

Table 1. Criteria for rapid-onset obesity with central hypoventilation, hypothalamic dysfunction, autonomic dysregulation (ROHHAD) or ROHHAD–neural crest tumor diagnosis in our cohort

Major criteria	Minor criteria
Rapid onset obesity after age 2 y	Autonomic dysfunction
Central hypoventilation	One or more endocrinopathies (GHD, central hypothyroidism, central precocious puberty, hypogonadotropic hypogonadism, Cushing disease, central adrenal insufficiency, central diabetes insipidus)
Neural crest tumor (ganglioneuroma, ganglioneuroblastoma, neuroblastoma)	Hyperprolactinemia
	Strabismus
	Behavior disorders
	Water-balance abnormalities

Abbreviation: GHD, growth hormone deficiency.

The finding of intrathecal synthesis of oligoclonal bands (generally immunoglobulin G type) [21, 22], elevated levels of neopterin, and the discovery of antihypothalamus and antipituitary autoantibodies suggest activation of B lymphocytes and their differentiation in antibody-secreting cells, as seen in other central nervous system (CNS) immune diseases. Prolonged immunotherapies, such as intravenous steroids, cyclophosphamide, rituximab, intravenous immunoglobulins, and mycophenolate, have been proposed [23-25], resulting in partial, transient improvement of behavior disturbances, sleep, and dysautonomic disorders in isolated reports.

Recently, Mandel-Brehm et al [26] detected autoantibodies against ZSCAN1—an antigen expressed in neural tissue and the hypothalamus—in ROHHAD-NET patient serum samples and cerebrospinal fluid (CSF), thus suggesting that ZSCAN1 autoantibodies may represent a marker for tumor-associated pediatric ROHHAD.

Identifying the underlying mechanisms of the syndrome is still a challenge. Through the better knowledge of the pathogenetic mechanisms, we would be able to improve the management of this rare condition, optimize follow-up, and increase survival rates.

The aim of this study is to characterize the immune phenotype of lymphocyte subpopulations, to evaluate the proinflammatory cytokine and chemokine pattern in patients with ROHHAD(NET) syndrome, and to compare them with the ones found in obese controls, matched for age and sex.

Materials and Methods

We evaluated retrospectively patients with a clinically defined diagnosis of ROHHAD and ROHHAD-NET syndrome followed at the Pediatric Endocrine Unit, IRCCS Istituto Giannina Gaslini, Genoa, Italy, between January 2008 and December 2020. The diagnosis of ROHHAD(NET) syndrome was made on the basis of clinical features, as reported in Table 1.

The diagnosis of ROHHAD(NET) syndrome was considered clinically definite in the presence of 3 major criteria, or 2 major plus 2 minor criteria, and probable in the presence of 1 major plus 4 minor criteria or 2 major criteria plus 1 minor criteria.

We define as ROHHAD patients the ones who met the clinical criteria but did not show a neural tumor, and ROHHAD-NET the ones that met the clinical criteria and were diagnosed with a neural tumor as described in Table 1. We refer to ROHHAD(NET) for the whole ROHHAD and ROHHAD-NET cohort.

Obesity—both in patients and in controls—was defined as body mass index (BMI) above the 97th percentile (for children older than 5 years) or above the 99th percentile (for children aged 2-5 years) based on 2006 and 2007 World Health Organization standards [27].

Genetic analysis of 15q11. 2-q13 did not reveal paternal deletion of this region, maternal uniparental disomy 15, or an imprinting defect, allowing us to exclude Prader-Willi syndrome. Comparative genomic hybridization array and/or standard karyotype confirmed the absence of major chromosomal alterations; pathological conditions due to structural impairment of the hypothalamic-pituitary axis secondary to brain lesions were ruled out.

PHOX2B mutations associated with hypoventilation due to congenital central hypoventilation syndrome was excluded in all patients.

We recorded anamnestic data (birth weight, age at onset of rapid weight gain, physical and neurological development, medical history) and anthropometric data at diagnosis (height, weight, BMI SD score [SDS], and pubertal Tanner stage).

Nocturnal polygraphy was performed by using the Embletta® MPR sleep system, which records nasal airflow, respiratory movements through thoracic and abdominal belts, transcutaneous oxygen saturation and pulse rate, patient position, and activity. Transcutaneous partial pressure of carbon dioxide was recorded during sleep by means of a SenTec® digital monitoring system. Nocturnal hypoventilation, obstructive sleep apnea syndrome (OSAS), and central sleep apneas were diagnosed based on the 2007 to 2012 American Academy of Sleep Medicine criteria [28]. All patients underwent brain magnetic resonance imaging with evaluation of the brain and sellar region.

Controls

We recruited children with simple obesity, without clinical features of ROHHAD(NET) and no associated genetic or syndromic conditions, and no associated endocrine disease, who were followed at our center, as a control group. We refer to this group as "non-ROHHAD obese." ROHHAD(NET) patients were matched to control individuals by age range (6-9.99 y, 10-13.99 y, >14 y) and sex.

The study was approved by the local ethics committee (protocol No. IGG-MOMA-007); parents or guardians of eligible children signed an informed consent according to the Declaration of Helsinki. All patients underwent fasting venous blood sampling between 8 AM and 9 AM. Samples were processed within 1 hour as described next.

Table 2. List of all antibodies and the corresponding identification

Manufacturer	Name	Antibody ID
eBiosciences	CD45 FITC	RRID:AB_10852703
eBiosciences	CD8 PE	RRID:AB_1724104
eBiosciences	HLA-DR FITC	RRID:AB_2572544
eBiosciences	TCR αβ FITC	RRID:AB_10544398
eBiosciences	TCR γδ PE	RRID:AB_1603300
eBiosciences	CD3 PE-Cyanine 7	RRID:AB_2637478
eBiosciences	CD16 FITC	RRID:AB_10805747
eBiosciences	CD56 PE	RRID:AB_10598200
eBiosciences	NKp46 PE-Cyanine 7	RRID:AB_2573444
eBiosciences	NKp44 APC	RRID:AB_2573202
eBiosciences	CD19 APC	RRID:AB_10804519
eBiosciences	CD24 FITC	RRID:AB_10854886
eBiosciences	CD38 PE	RRID:AB_10667740
eBiosciences	CD127 FITC	RRID:AB_1907342
eBiosciences	CD25 PE	RRID:AB_2043825
eBiosciences	CD45RO APC	RRID:AB_1907398
eBiosciences	LAG-3 PE	RRID:AB_2572597
eBiosciences	CD49b FITC	RRID:AB_1907400
eBiosciences	CD27 FITC	RRID:AB_10669045
Beckman Coulter	CD3 APC	RRID:AB_130788
Beckman Coulter	CD69 APC	RRID:AB_2941905
Beckman Coulter	CD4 PE-Cyanine 7	RRID:AB_10641616
BD	Human Inflammatory CBA Kit	RRID:AB_2868941
BD	Human Chemokine CBA Kit	RRID:AB_2868970
eBiosciences	HLA-E unconjugated	RRID:AB_1210774
Exbio	HLA-G unconjugated	RRID:AB_10735448
Exbio	Anti-β2 microglobulin HRP	RRID:AB_10734874

Abbreviations: CBA, BD Cytometric Bead Array; FITC, fluorescein isothiocyanate; HRP, horseradish peroxidase.

Flow Cytometry

Flow cytometric analysis was performed on whole peripheral blood (PB) samples from patients (n = 8) and controls (n = 6) —2 controls are missing due to preanalytical errors—using 50 µL of whole blood per tube. The antibodies used for flow cytometric analysis and their identification are listed in Table 2.

Samples were incubated with specific antibodies (15′ at room temperature [RT] in the dark) and then subjected to erythrocytes lysis using BD FACS Lysing Solution (BD Biosciences) by incubating 20′ at RT in the dark.

Cells were then washed with phosphate-buffered saline (PBS) 0.5% bovine serum albumin (BSA), resuspended in PBS 0.5% BSA, and then run on a Gallios cytometer (Beckman Coulter), acquiring at least 10^5 events. Data were analyzed using Kaluza software (Beckman Coulter). Gating analysis was performed on lymphocytes for both cell populations. Data were expressed as cells per microliter (µL), considering lymphocyte counts for each individual. Natural killer (NK) cells were identified as $CD3^-CD16^+CD56^{dim}$ or $CD3^-CD16^-CD56^{bright}$, and the expression of NKp44, NKp46 and CD69 was evaluated on both NK cell subsets. Regulatory T cells (Treg), type 1 regulatory T cells (Tr1), and regulatory B cells (Breg) were identified as $CD4^+CD25^{high}CD127^{-/low}$, $CD4^+CD45R0^+CD49b^+LAG\text{-}3^+$, and $CD19^+CD24^{high}CD38^{high}$ cells, respectively. CD4 and CD8 T cells were identified as $CD45RO^+CD27^+$ (T central memory, T_{CM}), $CD45RO^+CD27^-$ (T effector memory, T_{EM}), $CD45RO^-CD27^+$ (T naive), and $CD45RO^-CD27^-$ (T effector, T_{EFF}).

Evaluation of Cytokines and Chemokines

The concentration of a panel of proinflammatory cytokines and chemokines was evaluated on serum samples from patients (n = 9) and controls (n = 9) using BD Cytometric Bead Array Kits, following the manufacturer's protocol (identified in Table 2). Samples were acquired using the FACS Canto cytometer (BD) and analyzed using FCAP Array software. Data were expressed as pg/mL.

HLA-G and HLA-E Enzyme-linked Immunosorbent Assay

Concentration of soluble HLA-G (sHLA-G) or sHLA-E was evaluated in serum samples from patients (n = 11) and controls (n = 11). Enzyme-linked immunosorbent assay was performed as previously described [29]. Briefly, MaxiSorp Nunc-Immuno 96 microwell plates (Nunc A/S) were coated overnight at 4° C with 3D12 mAb, specific for HLA-E HC (eBioscience) or MEM-G9 (Exbio), which recognizes the HLA-G molecule, in β2-microglobulin–associated form, at a concentration of 10 µg/mL. After 3 washes with PBS 0.05% Tween 20 (washing buffer), plates were saturated with

200 µL/w of PBS 2% BSA (Sigma) for 30 minutes at RT. A total of 100 µL of test samples (serum) or standard (serial dilutions of total extract from normal PB mononuclear cells or supernatants from the 721.221.G1 cell line) were added to each well and incubated at RT for 1 hour. After 3 washes, 100 µL of detection reagent (horseradish peroxidase–conjugated anti-β2 microglobulin mAb, Exbio) was added, and the plates were incubated for 1 hour at RT. After 3 washes, 100 µL of TMB (substrate for horseradish peroxidase), was added, and the reaction was stopped after approximately 30 minutes by adding H_2SO_4 5N. Absorbance at 450 nm was measured using an Infinite® 200 PRO spectrometer (Tecan Group Ltd). Results are expressed as i) arbitrary units/mL for HLA-E (1 unit = quantity of sHLA-E in 1 µg of total extract) and ii) ng/mL for HLA-G. All antibodies are listed in Table 2.

Statistical Analysis

Statistical analysis was performed using Prism Software v. 5.03 (GraphPad Software). The normality of each variable was checked by using the Kolmogorov-Smirnov test. When normality of data distribution was found for all variables, statistical analysis was performed by a parametric approach. Conversely, when normality of data distribution was rejected for several variables, a nonparametric analysis was applied. Accordingly, the *t* test or Mann-Whitney test was used. The statistical significance value was reported as follows: $*P < .05$ (significant), $**P < .01$, and $***P < .001$.

Results

Study Participants

Median age at the time of enrollment was 9.1 years for patients (1^{st}-3^{rd} quartile 8.5-13.1 y) and 10.5 years for controls (1^{st}-3^{rd} quartile 9.6-13.4 y), median patient follow-up at the time of blood sampling was 7.8 years (1^{st}-3^{rd} quartile 4.1-8.8 y).

All patients were born at term, with an adequate weight for gestational age. The first symptom reported was rapid weight gain followed by obesity—in previously normal-weight children—for all patients. Other clinical characteristics are summarized in Table 3. Median age at disease onset was 3.0 years for all groups.

Table 3. Prevalence of clinical manifestations in our cohort

Main clinical aspects	No. of patients/% (N = 8)
Obesity	N = 8 (100%)
Central hypoventilation	N = 7 (88%)
Hypothalamic dysfunction	N = 8 (100%)
Hyperprolactinemia	N = 8 (100%)
GHD	N = 7 (88%)
Central hypothyroidism	N = 6 (75%)
Central adrenal insufficiency	N = 5 (63%)
Puberty disorders	N = 6 (75%)
Na^+ disorders	N = 2 (25%)
Diabetes insipidus	N = 2 (25%)
Autonomic dysfunction	N = 5 (63%)
Strabismus	N = 5 (63%)
Psychiatric disorders	N = 5 (63%)
OSAS	N = 3 (38%)
Neural crest tumor	N = 4 (50%)

Abbreviations: GHD, growth hormone deficiency; OSAS, obstructive sleep apnea syndrome.

Median BMI SDS in ROHHAD(NET) patients was +3.8 (1^{st}-3^{rd} quartile 3.0-4.5), median BMI SDS in non-ROHHAD obese patients was +3.1 (1^{st}-3^{rd} quartile 2.8-3.5). Median BMI did not differ significantly between the 2 groups ($P = .17$), and we observed no difference between ROHHAD and ROHHAD-NET individuals.

We included a total of 11 participants, 7 ROHHAD (4 female [F] and 3 male [M]) and 4 ROHHAD-NET (all F) patients. Samples from 8 of these patients (4 ROHHAD, 2 F and 2 M, and 4 ROHHAD-NET, all F) underwent phenotypical analysis. In this group, the median age at 1^{st} visit at our center was 4.6 years, with a median BMI of +4.1 SDS. The levels of proinflammatory cytokines and chemokines were investigated in samples obtained from 9 out of 11 patients (5 ROHHAD, 2 F and 3 M, and 4 ROHHAD-NET, all F). Enzyme-linked immunosorbent assay for sHLA-G or sHLA-E was performed on samples from all patients. For the last 2 groups, the median age at 1^{st} visit at our center was 4.8 years, with a median BMI of +3.9 SDS.

Differences in Lymphocyte Subsets Between ROHHAD(NET) Patients and Non-ROHHAD Obese Children

As shown in Fig. 1, phenotypical analysis of PB samples revealed an increase of T lymphocytes in patients (cells/µL, mean ± SD: 1859 ± 450.5) compared to controls (1544 ± 441.8; $P = .04$). Such an increase was related to a significantly higher number of $CD4^+$ T cells in patients (1129 ± 394.4) than in controls (749.3 ± 316.2; $P = .03$). In contrast, the number of B cells and NK cells was similar between the 2 groups. With regard to regulatory cell subsets, patients displayed a significant increase of Breg (30.61 ± 11.75) and Tr1cells (4.880 ± 2.74) compared to controls (19.07 ± 14.44; $P = .05$; and 2.205 ± 1.735; $P = .03$; respectively).

We next analyzed the expression of activation markers on T and NK cells. As shown in Fig. 2, the number of activated CD8 T cells ($CD8^+$ $HLA\text{-}DR^+$) was significantly lower in patients (90.90 ± 47.15) than in controls (204.1 ± 113.1; $P = .02$). In contrast, the number of CD4 T cells and NK cells expressing activation markers (HLA-DR, NKp44, NKp46, and CD69) was similar between the 2 groups.

Finally, we analyzed the number of $CD45RO^+CD27^+$ (T_{CM}), $CD45RO^+CD27^-$ (T_{EM}), $CD45RO^-$ $CD27^+$ (T naive), and $CD45RO^-CD27^-$ (T_{EFF}) among CD4 and CD8 T cells in patients and controls. As shown in Fig. 3, patients displayed a lower number of T_{EM} (20.56 ± 15.15) than controls (57.66 ± 35.47; $P = .02$) among CD8 T cells. With regard to CD4 T cells, patients displayed a higher number of T naive (826.1 ± 413.9) and T_{EFF} (34.32 ± 26.53) than controls (383.1 ± 304.1; $P = .04$; and 7.59 ± 3.82; $P = .008$; respectively).

Interleukin-8 and Monocyte Chemotactic Protein-1 Are Increased in Patient Serum Samples

We next investigated whether the serum levels of proinflammatory cytokines and chemokines are different between patients and controls. Among cytokines, interleukin-8 (IL-8) levels were increased in patients (pg/mL, mean ± SD: 58.35 ± 120.7) compared to controls (30.03 ± 72.43; $P = .008$).

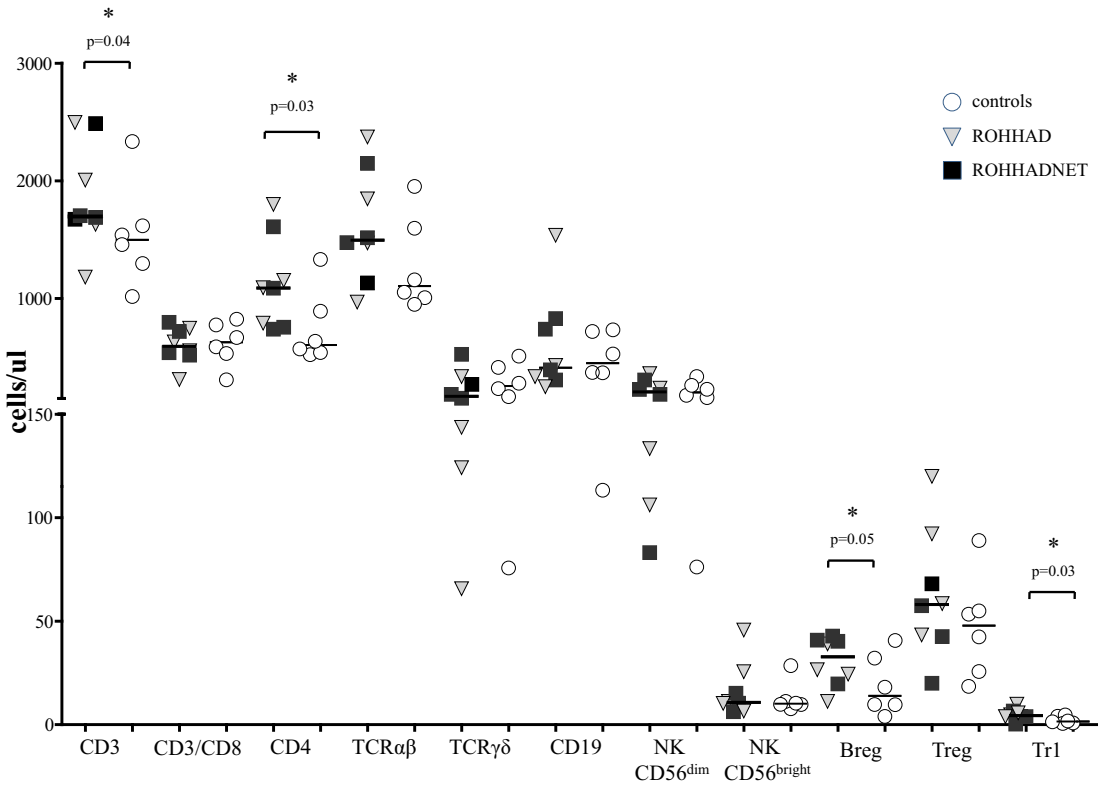

Figure 1. Immunophenotype of PBMNC. The percentage of cells expressing different lineage markers for T cells (CD3, CD3/CD8, CD4, TCRαβ, TCRγδ), B cells (CD19), NK cells (CD16, CD56), regulatory B cells (Breg, CD19, CD38, and CD24), regulatory T cells (Treg, CD4, CD25, and CD127) and type 1 regulatory T cells (Tr1, CD4, CD45RO, LAG-3, and CD49b) has been evaluated by flow cytometry on PBMNC cells from patients affected by ROHHAD (n = 4, black squares), ROHHAD-NET (n = 4, gray triangles), and control individuals (n = 6, white dots). Results are expressed as number of cells/μL, which has been calculated as follows: (number of lymphocytes/μL × percentage of positive cells)/100. Horizontal bars indicated medians. *P* values are indicated where differences are statistically significant.

Notably, IL-8 levels were higher in ROHHAD-NET patients (pg/mL, mean ± SD: 116.7 ± 168.1) than in ROHHAD patients (11.68 ± 10.14; $P = .0058$). The levels of the other molecules tested (IL-12, tumor necrosis factor α [TNFα], IL-10, IL-6, and IL-1β) were similar between patients and controls (Fig. 4). The serum levels of monocyte chemotactic protein-1 (MCP-1) were higher in patients (86.91 ± 68.74) than in controls (28.15 ± 16.59; $P = .01$), whereas the levels of all the other chemokines tested (IP-10, MIG, and RANTES) were similar between the 2 groups (see Fig. 4).

HLA-G Is Decreased in Patient Serum Samples

Finally, we analyzed the concentration of the soluble (s) isoform of immunosuppressive molecules HLA-G and -E in patients and controls. As shown in Fig. 5, sHLA-G concentration was significantly lower in patients (ng/mL, mean ± SD: 1.31 ± 3.09) than in controls (9.56 ± 14.83; $P = .03$), whereas sHLA-E concentration was similar between the 2 groups.

Discussion

In our participants, the immunological phenotype of ROHHAD-NET patients and obese, non-ROHHAD controls, matched for age and sex, showed that the PB lymphocyte population was significantly different in ROHHAD(NET) patients compared with non-ROHHAD obese controls. In particular, our patients displayed a higher number of total T lymphocytes and, among these, CD4+ T cells. In addition, activated CD8-T cells were lower in patients. Patients also displayed increased regulatory B cells and Tr1 cells, while lower CD8 T-effector memory were observed in this group. Among CD4-T cells, T naive and T effector were higher in patients. Regarding cytokines, IL-8 and MCP-1 levels were increased in patients, with a higher IL-8 level in patients with neural tumor (ROHHAD-NET) than in patients without neural tumor (ROHHAD); sHLA-G was also significantly lower in patients vs controls.

These findings altogether support the hypothesis that a systemic immune dysregulation may underlie this disease. This hypothesis has been raised in a previous report by Gharial et al [19], who performed a postmortem examination in brain tissue from one ROHHAD-NET patient and found perivascular cuffs consisting predominantly of B cells ($CD20^+$) and helper T cells ($CD4^+$), with relatively fewer plasma cells ($CD138^+$), cytotoxic T cells ($CD8^+$), and macrophages/microglia ($CD68^+$). While the latter findings and our own demonstrated that helper T cells seem to have a relevant role in the pathogenesis of this condition, brain parenchymal infiltrates in the hypothalamus and pontine tegmentum contained all the aforementioned cell types, but with relatively more cytotoxic T cells in Gharial's report. Other authors [10, 16, 17, 30] found mainly perivascular lymphocytic-histiocytic infiltrates with some parenchymal involvement affecting the hypothalamus, periaqueductal gray matter, ventral superior colliculi, and brainstem nuclei in patients with a clinical

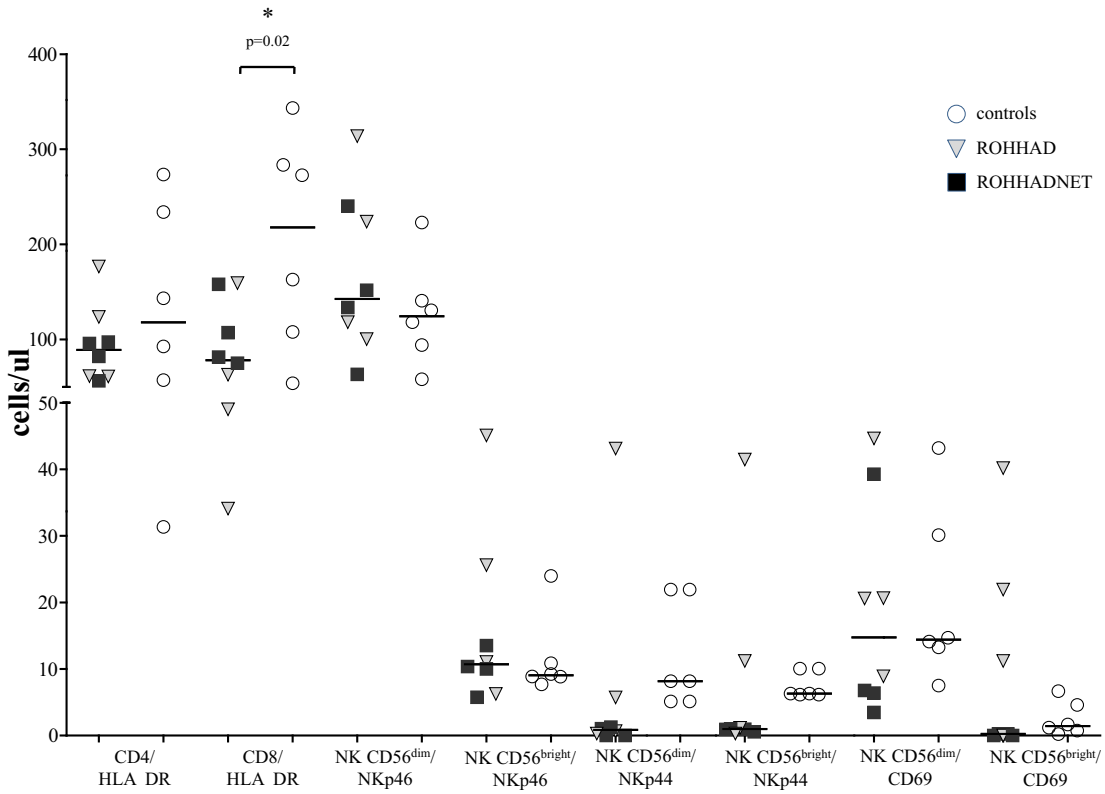

Figure 2. Expression of activation markers on T lymphocytes and natural killer (NK) cells. The expression of activation markers for $CD4^+$ and $CD8^+$ T cells (HLA-DR) or $CD56^{dim}$ and $CD56^{bright}$ NK cells (NKp46, NKp44, and CD69) has been evaluated by flow cytometry on PBMNC cells from ROHHAD patients (n = 4, black squares), ROHHAD-NET patients (n = 4, gray triangles), and control individuals (n = 6, white dots). Results are expressed as number of cells/μL, which has been calculated as follows: (number of lymphocytes/μL × percentage of positive cells)/100. Horizontal bars indicated medians. *P* values are indicated where differences are statistically significant.

phenotype consistent with ROHHAD(NET) syndrome who died because of disease complications. In most cases, those lymphocytes stained as T cells (often with no further subtype characterization), with occasional B cells demonstrated [19, 30]. Furthermore, in the affected area, reactive gliosis and moderate neural loss, as nonspecific signs of neuroinflammation, have been reported.

Our data, for the first time, and, unlike previous studies that described immune cell subsets infiltrating well-defined brain areas, focus on alterations in circulating lymphocyte pool. Although the observed variability across the topographic distribution of brain infiltrates and blood lymphocyte phenotype has not been fully explained yet, we believe that the interaction between these two compartments plays an important role in shaping the clinical spectrum and the degree of severity of ROHHAD phenotype.

Similarly to our findings, a higher percentage and absolute count of PM T lymphocytes—entirely due to a larger $CD3^+CD4^+$ fraction—has been reported in children with autoimmune polyglandular syndrome type 1 compared to healthy individuals, as well as a lower $CD8^+$ count [31], and this pattern has also been reported in other autoimmune diseases in children, such as type 1 diabetes mellitus (T1DM) [32]; these authors also detected higher $CD4^+$ naive levels in patients and lower numbers of T_{EM} than controls among CD8 T cells, as we did in our ROHHAD(NET) group. Other studies showed an unbalanced activation of $CD4^+$ and $CD8^+$ lymphocytes with predominant activation of the $CD8^+$ lymphocyte subset in T1DM [33], while our ROHHAD(NET) patients showed lower activated $CD8^+$ cells. A more recent study showed low Treg levels in children with T1DM [34]. As a matter of fact, low Treg counts and/or impairment of their function have been interpreted as a possible risk factor for autoimmune diseases [35]; nevertheless, in our study, Treg levels were similar in ROHHAD(NET) and non-ROHHAD obese individuals, whereas Breg cells and Tr1, a particular subset of Treg cells that inhibit the autoimmune response through production of the immunosuppressive cytokine IL-10 and other mechanisms, were higher in ROHHAD(NET) patients. Tr1 increase in our ROHHAD(NET) cohort could represent a consequence of the autoimmune activation in our patients, to be interpreted as an ineffective attempt to control such response, but this hypothesis needs to be confirmed. In contrast to what we found in ROHHAD(NET) patients, reduced PB $CD4^+$ and increased $CD8^+$ counts have been reported in some autoimmune conditions such as systemic lupus erythematosus in children, while low $CD8^+$ cells have been linked to vasculitis [36] and increased number of specific $CD4^+$ and $CD8^+$ subpopulations have been detected in the PB of children with juvenile idiopathic arthritis [37]. Furthermore, low Breg subsets have been reported in other autoimmune conditions, such as idiopathic thrombocytopenic purpura [38], opposite to what we found in our patients.

Interestingly, the clinical history of all our ROHHAD(NET) patients enrolled in the present study, including autoimmunity and recurrent infections or higher susceptibility to infectious disease, was unremarkable (only one of our ROHHAD patients has celiac disease). In addition, total immunoglobulin

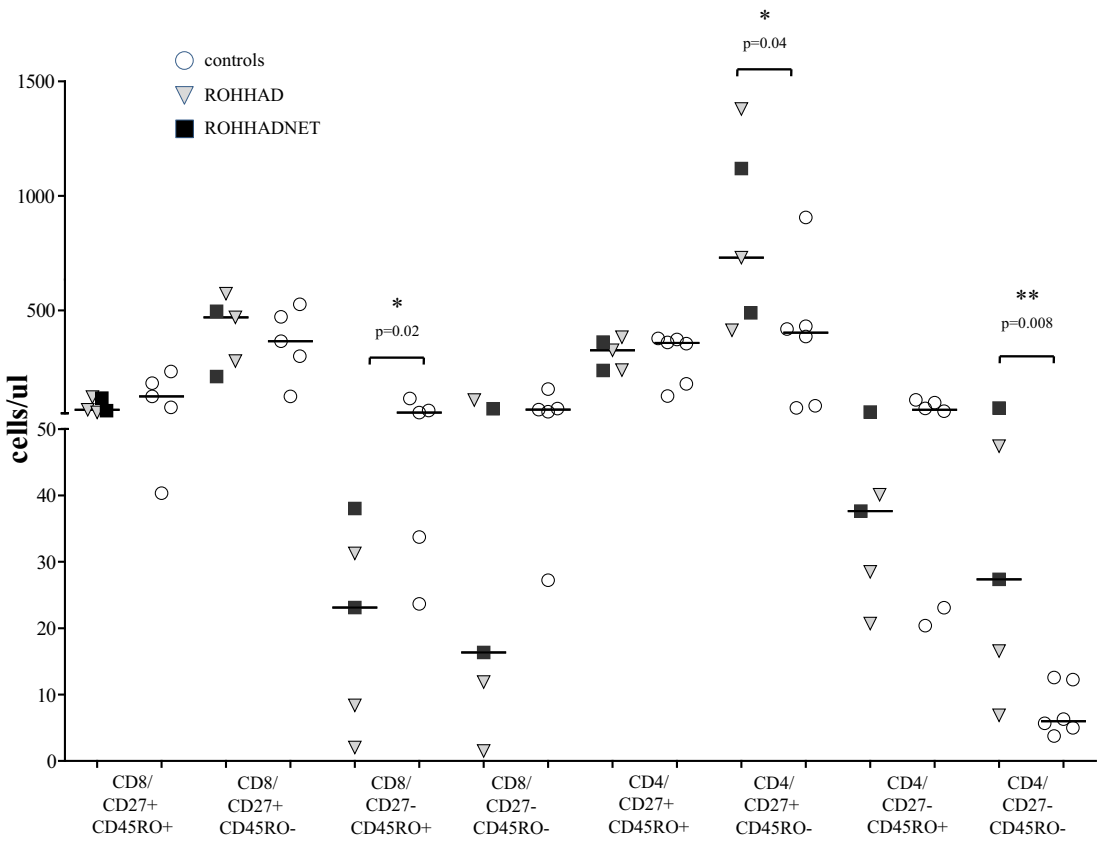

Figure 3. Evaluation of effector and memory T-cell subsets. The percentage of CD45RO$^+$CD27$^+$ (central memory T cells), CD45RO-CD27$^+$ (naive T cells), CD45RO$^+$CD27$^-$ (effector memory T cells), and CD45RO-CD27$^-$ (effector T cells) CD3$^+$CD4$^+$ or CD3$^+$CD8$^+$ T cells was evaluated by flow cytometry on PBMNC cells from ROHHAD patients (n = 4, black squares), ROHHAD-NET patients (n = 4, gray triangles), and control individuals (*n* = 6, white dots). Results are expressed as number of cells/μL, which has been calculated as follows: (number of lymphocytes/μL × percentage of positive cells)/100. Horizontal bars indicated medians. *P* values are indicated where differences are statistically significant.

levels were normal according to age-related intervals (data not shown). In type 1 narcolepsy (NT1), an autoimmune condition that affects the hypothalamus, Lecendreux et al [39] found no significant differences in CD3$^+$, CD4$^+$, CD8$^+$ T cell, B cell, and NK cell numbers in PB between NT1 patients and healthy donors; they demonstrated an increased number of PB central memory CD4$^+$ T cells (CD62L$^+$CD45RA$^-$) associated with an activated phenotype. In our study, this subset was similar in the two groups, and high percentage and absolute count of Tregs, while we only found an increase in Tr1 in ROHHAD(NET), and no difference in Treg; cytokine production by CD4$^+$ and CD8$^+$ T cells—including IL-8 levels—was not modified in NT1 patients in this study. Other authors [40] found no significant difference in PB CD4$^+$ or CD8$^+$ between 14 NT1 patients and healthy individuals. Even though NT1 has some clinical and biochemical features in common with ROHHAD(NET), namely the hypothalamic involvement, the presence of obesity, central precocious puberty, altered sleep pattern, and reduced CSF hypocretin levels [41-44], PB lymphocyte subsets do not show the same alterations in these 2 diseases.

Little information is available about PB lymphocyte subpopulations, IL-8, and MCP-1 levels in pediatric autoimmune encephalitis—another condition that shows clinical overlap with ROHHAD, while an increased neutrophil/lymphocyte ratio has been reported in adult patients [45], and a higher CD4/8$^+$ T-cell ratio in PB was identified in specific subsets of patients [46].

The cause for altered immune phenotype in our ROHHAD(NET) patients could also be partially linked to the unique endocrine phenotype these patients present, since it is well known that the endocrine and the immune system interact with each other. In particular, thyroid function affects immune regulation [47]. In a large cohort of healthy volunteers, in fact, Jaeger et al [47] found that NK–T-cell counts were positively associated with thyrotropin (TSH) levels, and a significant positive correlation was found also between TSH and T-cell counts, while a positive correlation was observed between free thyroxine (FT4) and B-cell counts. The same authors did not find any correlation between IL-8 levels and TSH or FT4 concentrations in healthy adults. In our opinion, the prevalence of central hypothyroidism—that is, low TSH levels—in our patients does not explain the observed differences in T-cell counts in our cohort, since ROHHAD(NET) patients, with a 75% prevalence of central hypothyroidism, actually have a higher number of T cells, quite unexpectedly, given the positive correlation between TSH levels and T-cell counts. On the other hand, the effect of FT4 on B-cell count described by Jaeger et al [47] may have been masked by levothyroxine therapy—which normalizes FT4 levels—in our cohort. In fact, all our TSH-deficient ROHHAD(NET) patients were on replacement levothyroxine therapy and had optimal FT4 levels at the time of blood sampling, except for a single ROHHAD patient, with low FT4.

The influence of adrenal replacement therapy was ruled out by the fact that no significant difference was found either in

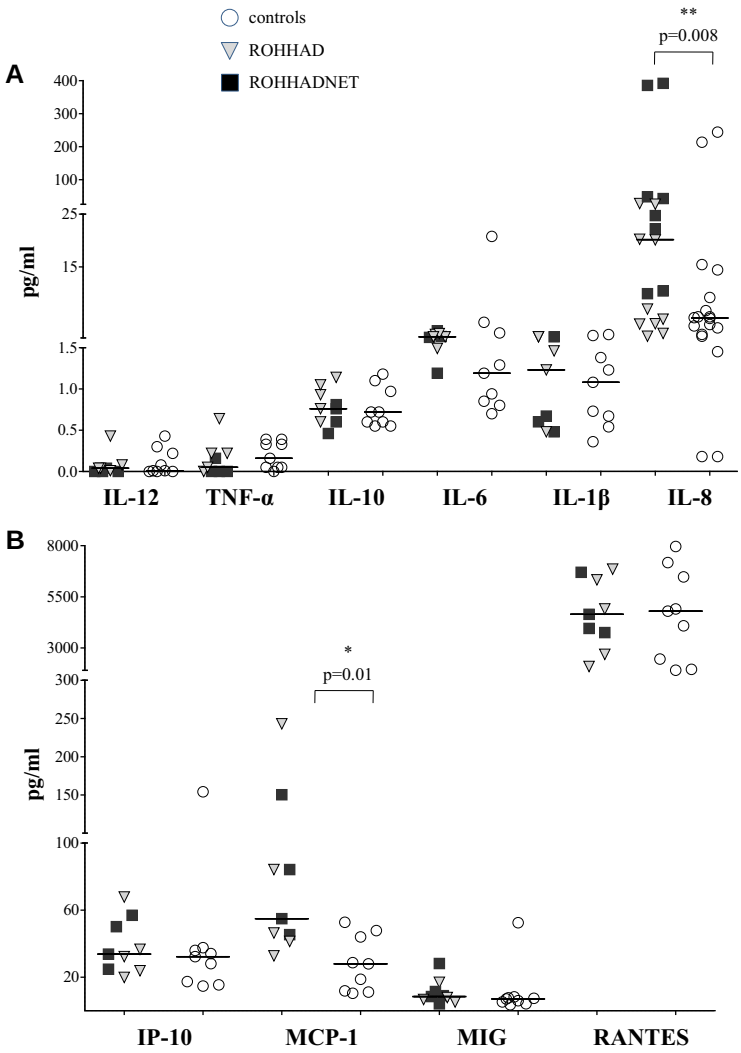

Figure 4. Evaluation of cytokines and chemokines concentration in serum samples. The concentration of cytokines (A, IL-12; TNF-α; IL-10; IL-6; IL-1β; and IL-8) and chemokines (B, IP-10; MCP-1; MIG; and RANTES) was evaluated by flow cytometry on serum samples from patients affected by ROHHAD ($n = 4$, black squares), ROHHAD-NET (n = 5, gray triangles), and control individuals (n = 9, white dots) using cytometric bead array kits. Results are expressed as pg/mL. Horizontal bars indicated medians. *P* values are indicated where differences are statistically significant.

lymphocyte subtype or in cytokine/chemokine profile in patients who were on oral hydrocortisone therapy vs those who were not treated at the time of the study (data not shown). Regardless, steroid therapy was prescribed in the replacement range for central adrenal insufficiency (ie, oral hydrocortisone 8-10 mg/m^2 body surface area/d) for all treated patients.

IL-8 levels were increased in our patients. IL-8, a proinflammatory cytokine secreted mainly by mononuclear macrophages, is increased in infectious diseases such as tuberculosis, urinary tract infections, and others. Elevated IL-8 levels have also been found in adults and children with OSAS [48, 49]. Since OSAS prevalence is higher in our ROHHAD(NET) group (data not shown), this could partially explain the difference we observed, thus strengthening the hypothesis that inflammation, obesity, and sleep-related breathing disorders are linked in ROHHAD(NET) as well as in the general population. However, our ROHHAD(NET) patients with OSAS or other sleep-related breathing disorders were all being treated with noninvasive ventilation at the time of blood sampling, with complete resolution or improvement of respiratory phenotype. IL-8 levels are related to apnea/hypopnea index, and usually IL-8 and other proinflammatory cytokines return within normal range with OSAS treatment [48, 50]; for this reason, it is likely that some other inflammation mechanisms are involved in ROHHAD(NET). In vitro studies [51] have shown that human astrocyte exposure to hypoxia resulted in upregulation of IL-1β, TNFα, and IL-8 cytokine production, with cerebral inflammation and upregulation of intercellular adhesion molecule-1 and MCP-1, the latter also being elevated in ROHHAD(NET) patients compared to obese controls in our study. Moreover, OSAS has been demonstrated to carry a high risk for the development of autoimmune disorders in adults [52]. ROHHAD(NET) patients usually develop obesity and other hypothalamic manifestations before the onset of OSAS or of nocturnal hypoventilation—or at the same time—they show sleep-related breathing disorders even in the presence of mild obesity, and their respiratory phenotype does not resolve with weight loss, contrary to what occurs in other subsets of obese patients [3, 4]. Therefore, the potential autoimmune mechanisms seem to precede—and not follow—OSAS onset in these patients.

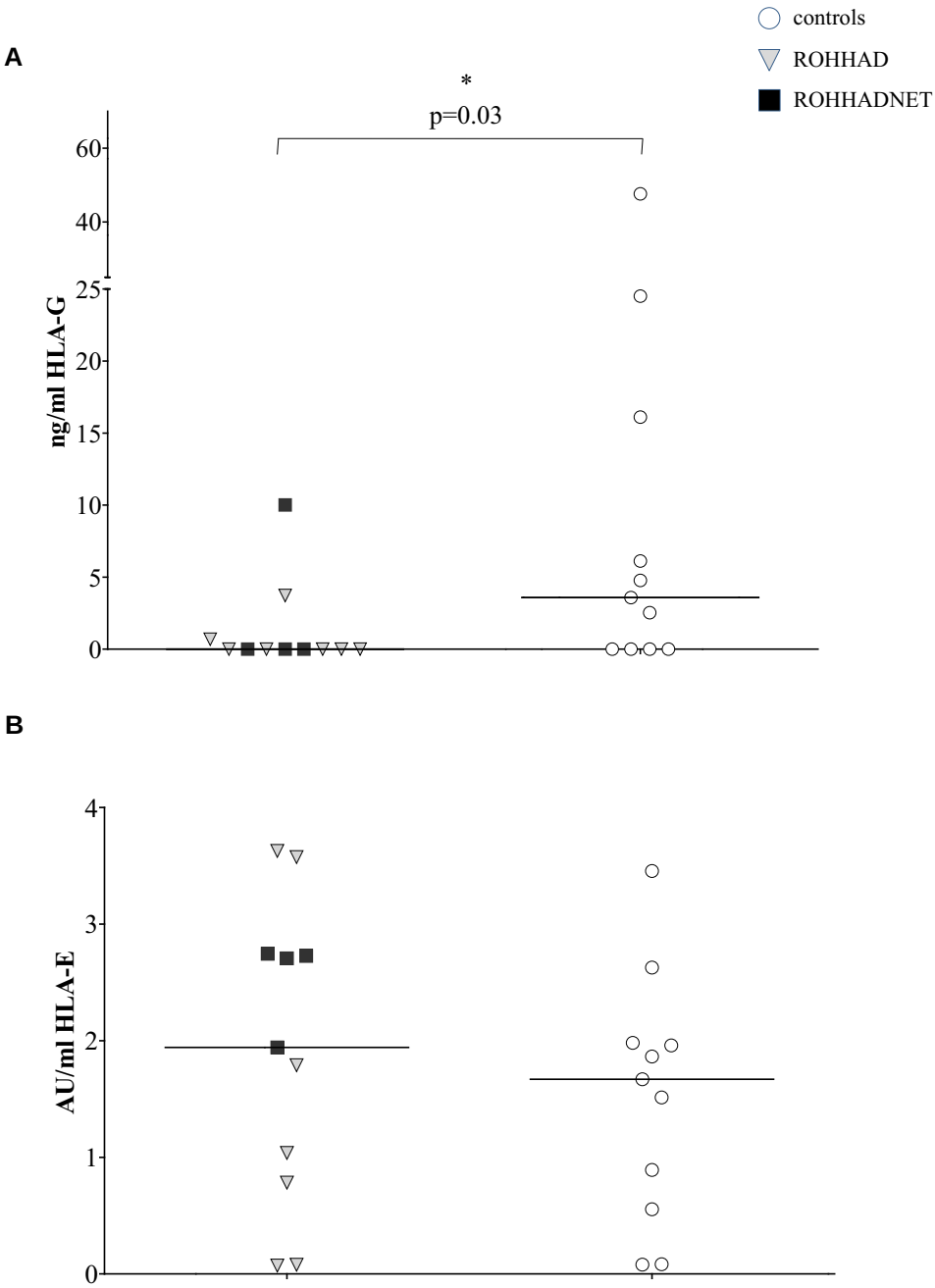

Figure 5. Evaluation of HLA-Ib molecules concentration in serum samples. The concentration of A, HLA-G, and B, HLA-E, was evaluated by enzyme-linked immunosorbent assay on serum samples from patients affected by ROHHAD (n = 4, black squares), ROHHAD-NET (n = 7, gray triangles), and control individuals (n = 11, white dots). Results are expressed as ng/mL for HLA-G and arbitrary units (AI)/mL for HLA-E. Horizontal bars indicated medians. *P* values are indicated where differences are statistically significant.

Additionally, we found that IL-8 levels were higher in ROHHAD-NET than in ROHHAD patients, thus suggesting that the increased serum levels of this cytokine may be related to the presence of tumor cells. Indeed, it has been reported that IL-8 was expressed in tumor specimens [53] and released by neuroblastoma cells [54]. Additionally, IL-8 expression may be related to a worse prognosis in patients with neuroblastoma [55].

MCP-1 is one of the main chemokines that regulate migration and infiltration of monocytes/macrophages. IL-8/MCP-1 act as neutrophil/monocyte chemoattractants, respectively, and have been demonstrated to be associated with obesity [56]. MCP-1 is linked to insulin resistance and metabolic syndrome in children [57]. The prevalence of insulin resistance (defined as basal insulin >15 µUI/mL or insulin >150 µU/mL at any time point during the oral glucose tolerance test, or total insulin after oral glucose tolerance test >300 µUI/mL, or pathologic homeostatic model assessment of insulin resistance) was similar between ROHHAD(NET) patients and controls: In fact, we found insulin resistance in 8 patients (89%) and 7 controls (77.8%) ($P = .48$). Hence, we believe that the cause of increased proinflammatory cytokines in our ROHHAD(NET) cohort remains unclear. Briefly, a trend toward higher BMI, insulin resistance,

and the presence of sleep-related breathing disorders could partially explain the difference in proinflammatory cytokines and inflammatory status we found in ROHHAD(NET) patients, but these differences, in our opinion, are too evident to be linked only to patients' metabolic and respiratory disorders. HLA-G, a nonclassical human leukocyte antigen (HLA) class I molecule that has some important tolerogenic functions in various physiological and pathological situations, is reduced in our ROHHAD(NET) cohort. The development of insulin resistance in obese adolescents is associated with strong downregulation of the expressions of HLA-G and other regulatory molecules [58]. Again, the fact that in our study controls themselves were obese and insulin resistant implies that —in ROHHAD(NET) patients—immune regulation is affected more deeply than in "simple" obesity.

Some authors have recently underlined that the ROHHAD(NET) phenotype overlaps with the one found in autoimmune adipsic hypernatremia [59], in which an autoimmune response develops against some parts of the CNS—specifically the ones where the blood-brain barrier is weaker, such as circumventricular organs. These areas, which have afferent and efferent neurons with the surrounding hypothalamic nuclei, are easily invaded by autoantibodies from the bloodstream. Though adipsic hypernatremia and the ROHHAD(NET) clinical phenotype differ in some features —namely the absence of adipsia and the often transient nature of hypernatremia in ROHHAD (NET) patients—this similarity needs further attention. The identification of novel CNS antigens and autoantibodies associated with pediatric paraneoplastic ROHHAD [26] is puzzling and may be helpful to clarify the pathogenetic mechanisms and to identify a potential treatment for this rare, fatal disease. Up to now, in fact, only anecdotal reports of successful—or partially successful —immune-suppressing therapy have been published [23-25, 60], and no treatment protocol is available to date.

The different age and disease duration at blood sampling and the limited number of patients and controls are, in our view, limitations that do not affect our conclusions. ROHHAD(NET) is in fact a very rare condition, possibly underdiagnosed due to lack of diagnostic markers and to the timeline of symptom appearance, and therefore a single-center, or even single-nation, cohort will not meet the necessary numerosity criteria. We believe it would be useful to confirm the hypothesis of a peculiar ROHHAD(NET) immune phenotype by extending the investigations to larger cohorts and by taking into consideration other factors, either genetic/epigenetic or acquired, that may influence the immune response in these patients; specifically, given the $CD4^+$ expansion we found, T-cell cytokine production such as IL-17—that have been shown to be altered in other autoimmune conditions and in metabolic syndrome-related inflammation [61] —should be investigated. To our knowledge, the strength of our data consists in the absence of prior research reports on immune and cytokine/chemokine phenotype in ROHHAD(NET); by extending these investigations to a larger, international cohort, we would be able to confirm the peculiarity of this pattern and to hypothesize a disease model that could imply initial inflammatory activation with cytokine secretion followed by an adaptive response, or vice versa. In a recent case report, the ROHHAD phenotype appeared a few weeks after SARS-CoV2 infection [62]; this may imply an infectious—possibly viral—trigger for inflammatory activation. As neuroinflammation has been suggested, CSF immune phenotype characterization is also warranted to gain further insight into the pathophysiology of this disease.

Conclusions

Our preliminary findings support the hypothesis of immune dysregulation as a possible underlying pathogenetic/ contributor mechanism for the development of ROHHAD(NET) syndrome. The extension of these investigations to larger cohorts of patients and to further immunological markers may provide the basis for the identification of a disease model and, moreover, of a treatment protocol for this severe condition.

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Author Contributions

D.F. and A.A. collected data, examined patients' clinical phenotypes, took care of patients' follow-up, drafted and revised the manuscript. F.N. designed the study, examined patients' clinical phenotypes, took care of patients' follow-up, collected data, drafted and revised the manuscript. I.P. and F.M. performed the tests, analyzed the results, drafted and revised the manuscript. P.B. performed the tests and analyzed data. C.P. took care of patients and collected data. S.V. and G.d.A. revised the manuscript. G.P., E.C., A.E.M.A., and N.D.I. took care of patients and revised the manuscript. A.P. and M.M. designed the study and revised the manuscript. All authors approved the final manuscript as submitted and agreed to be accountable for all aspects of the work.

Disclosures

The authors report no competing interest.

Data Availability

Most original data generated and analyzed during this study are included in this published article. Some data sets generated during and/or analyzed during the current study are not publicly available but are available from the corresponding author on reasonable request.

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