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ORIGINAL ARTICLE

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Hyperprolactinemia is associated with a high prevalence of serum autoantibodies, high levels of inflammatory cytokines and an abnormal distribution of peripheral B-cell subsets

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Abstract

Purpose Hyperprolactinemia (HPRL) has been reported in many autoimmune diseases. However, the serum autoantibody profile and peripheral B-cell subset distribution in women with HPRL are largely unknown. The current study aimed to investigate the autoantibody prevalence and cytokine levels as well as to further explore the B-cell subset distribution in women with HPRL.

Methods Sera from 202 women with HPRL and 97 healthy women were included in this study. All sera were examined for prolactin (PRL), anti-nuclear antibody (ANA), rheumatoid factor, anticardiolipin (ACL), immunoglobulin G, immunoglobulin M, complement 3, complement 4, interleukin 4 (IL-4) and interleukin 6 (IL-6). Peripheral blood was collected from 22 women with HPRL and 19 healthy women, and B-cell subsets were measured by flow cytometry.

Results At least one autoantibody was found in 47 out of 202 women with HPRL compared with 9 of 97 healthy women ($p < 0.001$). The levels of IL-4 ($p < 0.0001$) and IL-6 ($p < 0.0001$) were significantly higher in women with HPRL than in healthy women. The percentages of naive IgD^+IgM^- B cells (B_{ND} cells, $p < 0.0001$), antibody-secreting cells ($p = 0.007$) and unswitched memory B cells ($p = 0.004$) among the total B cells from HPRL women were significantly higher than those from healthy women.

Conclusions Women with HPRL had a higher prevalence of autoantibodies, higher serum levels of IL-4 and IL-6, and more B_{ND} cells, antibody-secreting B cells and unswitched memory B cells than healthy women. These data imply that a high level of PRL is associated with autoimmune diseases.

Keywords Hyperprolactinemia · Prolactin · Autoantibodies · Anti-nuclear antibodies · Rheumatoid factor · B-cell subsets

Introduction

Autoimmune diseases are well known to be more common in women than in men, suggesting that female sex hormones may regulate immune responsiveness [1, 2]. Prolactin (PRL)

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is a peptide hormone produced by lactotroph cells in the anterior pituitary gland. In addition, PRL synthesis has been detected from extra pituitary sources including immune cells. PRL receptors have been identified on monocytes as well as B and T lymphocytes. Therefore, PRL has the ability to promote cell growth or differentiation in several tissues and functions as an immune-stimulator [3, 4].

Over the years, the role of PRL in T and B cells modulation, cytokine regulation and antibody production has been studied in vivo and in vitro [5]. Many studies have shown that PRL has an important role in rheumatic diseases, such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), reactive arthritis, Sjögren's syndrome, systemic sclerosis, psoriasis, Bechet's disease and polymyositis [6, 7].

Hyperprolactinemia (HPRL) is characterised by high expression levels of serum PRL, and the expected rate of HPRL is 0.4–3% in a healthy population [8]. Hashimoto's thyroiditis, which is the most common autoimmune disease associated with HPRL, was detected more frequently in patients with prolactinoma than in patients with acromegaly [9]. In line with this observation, recent data from a cross-sectional case–control study showed that autoimmune hypothyroidism is three times more common in female prolactinoma patients than in healthy women [10].

In fact, many autoantibodies may present a few years before the onset of symptomatic autoimmune disease [11]. Therefore, exploring whether autoimmune markers are altered in HPRL patients without autoimmune symptoms is of interest. Some early studies have reported an increasing prevalence of various autoantibodies in patients with HPRL [12, 13]. However, these studies enroled rather small cohorts of patients. Notably, a recent study revealed an association between increased levels of IL-4 and anti-TPO with HPRL [14]. At present, limited data are available in the literature regarding the prevalence of various autoantibodies in HPRL. The aim of the current study was to compare the prevalence of autoantibodies, the levels of cytokines, and the distribution of B-cell subsets between women with HPRL and healthy controls.

Materials and methods

Patients and clinical information

Sera were obtained from 202 women with HPRL and 97 healthy women. Peripheral blood was obtained from 22 women with HPRL and 19 healthy women. Samples were collected from female outpatients in the Department of Reproduction, the Department of Endocrinology, the Department of Head and Neck Surgery or the Center of Health Examination from 2015 to 2018 at Changzheng Hospital. HPRL was defined as a serum PRL level >26.72 ng/ml. The healthy women were recruited from the Center of Health Examination from 2015 to 2018, and age-matched donors with normal examination results and normal PRL levels (<26.72 ng/ml) were selected. All patients and controls underwent a full medical history and complete physical examination. Women who were breastfeeding, using oral contraceptives, undergoing menopause and those who were pregnant were excluded from the study. Women on medications or with a clear diagnosis of renal or liver failure, hypothyroidism, chronic inflammatory diseases or autoimmune disease were also not included. Written informed consent was obtained from each individual in agreement with the Declaration of Helsinki. The study was reviewed and approved by the Medical Ethics Committee of Shanghai Changzheng Hospital, Second Military Medical University.

Laboratory analyses

Venous blood samples were drawn between 8 am and 9 am, whereas the participants were fasting. PRL was detected by the Department of Laboratory Diagnosis, Shanghai Changzheng Hospital on a DXI 800 analyser (Beckman Coulter Inc., USA).

All sera were screened by ANA Screen ELISA (EUROIMMUN, Lübeck, Germany), which can detect the presence of autoantibodies against 10 different antigens, including double-stranded DNA (dsDNA), histones, ribosome ribonucleoprotein (rRNP), Smith (Sm), nuclear ribonucleoprotein (nRNP), SSA, SSB, Scl-70, Jo-1 and centromere B (CENP-B). The sera those were positive for anti-nuclear antibody (ANA) were then confirmed for the specific types by a Euroline ANA profile test (EUROIMMUN, Lübeck, Germany). Anticardiolipins (ACL) were measured by immunoassay using an ACL ELISA (IgA/G/M), (EUROIMMUN, Lübeck, Germany). Rheumatoid factor (RF), immunoglobulin (Ig), complement 3 (C3), complement 4 (C4) were detected by the Department of Laboratory Diagnosis, Shanghai Changhai Hospital on an IMMAGE 800 analyser (Beckman Coulter Inc., USA). The serum levels of interleukin 4 (IL-4) and interleukin 6 (IL-6) were measured by immunoassay using commercial kits from R&D (Minneapolis, MN, USA).

Flow cytometry

Peripheral blood mononuclear cells (PBMCs) were isolated from freshly collected blood samples using a Ficoll density gradient. Cells were washed in Iscove's Modified Dulbecco's Medium supplemented with 5% foetal bovine serum and incubated with Fc block (Biolegend) for 30 min at 4 °C. After washing, cells were stained with APC-anti-human

CD19 (HIB19, Biolegend), PE-anti-human IgD (IA6-2, BD), PE-Cy7-anti-human CD38 (HIT2, Biolegend), PE-Cy5.5-anti-human CD27 (M-T271, Biolegend), BV421-anti-human IgM (G20-127, BD), BV605-anti-human CD10 (HI10a, Biolegend), and Fixable Viability Dye eFluor 520 (eBioscience). Fluorescence-activated cell sorting analysis was performed using BD LSRFortessa, and results analysis was performed with FlowJo Cytometric software (Tree Star, Inc.).

Statistical analyses

Comparisons between women with HPRL and healthy women were performed using the Mann–Whitney *U* test. Relationships were calculated using Spearman's correlation. Fisher's exact test was used to detect group differences in the prevalence of autoantibodies. Data were analysed using SPSS 16.0 software or GraphPad Prism 7. Values of $p < 0.05$ were considered indicative of statistical significance.

Results

General characteristics of participants

Sera from 202 women with HPRL and 97 healthy women were collected in this study (Table 1); no significant difference in ages was found between the two groups (37.04 ± 11.16 and 38.76 ± 13.09 years, respectively). The women with HPRL had a significantly higher level of PRL (49.11 ± 34.62 ng/ml) compared with healthy women (15.15 ± 6.73 ng/ml). The highest PRL level among the patients was 201 ng/ml. All 202 women with HPRL were premenopausal. In this group, 50 of 202 (24.75%) women had evidence of pituitary adenoma on computerised tomography (CT) or magnetic resonance imaging evaluation. All of the tumours had a diameter <10 mm. In the remaining 75.25% of patients with HPRL, two had craniopharyngioma, three had ovarian cysts, 14 had polycystic ovarian syndrome, and 133 had idiopathic HPRL. No allergic history was found in their medical records. All patients had normal thyroid, liver and renal function tests. None of the women were taking drugs known to cause HPRL, and none of the women with HPRL presented with symptoms or signs related to autoimmune disorders.

Autoantibody profile in women with HPRL

Sera from the 202 women with HPRL and the 97 healthy women were tested for the presence of ANA, RF and ACL (Table 1). Of the 202 HPRL serum samples tested for the presence of autoantibodies, 47 (23.27%) were found to be positive for at least one autoantibody, whereas only 9 (9.28%) samples from healthy women were found to be positive ($p < 0.001$). The autoantibody profile distribution in women with HPRL is shown in Table 2. Notably, the most frequently detected autoantibodies in HPRL women were RF (17.82%), SSA (5.45%), Ro-52 (3.47%), nRNP/Sm (3.47%) and CENP-B (1.98%) (Fig. 1). Surprisingly, among the HPRL women who were autoantibody positive, 78.72% (37/47) of them had a PRL level between 26.72 and 50 ng/ml, and 13 of the 15 ANA-positive women (86.67%) had a PRL level between 26.72 and 50 ng/ml. The mean level of PRL in HPRL women who were ANA-positive was 41.13 ± 2.62 ng/ml (35.51–46.75, 95% CI). Therefore, we divided women with HPRL into two groups: a group showing a mild increase with PRL levels between 26.72 and 50 ng/ml, and one exhibiting a moderate-to-severe increase with PRL levels over 50 ng/ml. However, the results showed that the autoantibody-positive rate in the group with a mild increase in PRL was not significantly higher than that in the group with a moderate-to-severe increase (23.9% vs. 21.3%, $p = 0.712$).

Table 1 Demographic characteristics and laboratory parameters of 97 healthy women and 202 women with HPRL

	Healthy women	Women with HPRL	*P* value
No. of subjects	97	202	
Age (years)	38.76 ± 13.09	37.04 ± 11.16	0.4621
PRL (ng/ml)	15.15 ± 6.73	49.11 ± 34.62	<0.0001
IgG (g/l)	13.61 ± 2.47	13.34 ± 2.86	0.0624
IgM (g/l)	1.49 ± 0.59	1.48 ± 059	0.7664
C3 (g/l)	1.09 ± 0.28	1.03 ± 0.15	0.0863
C4 (g/l)	0.236 ± 0.084	0.225 ± 0.071	0.3538
IL-4 OD	0.056 ± 0.049	0.155 ± 0.110	<0.0001
IL-6 OD	0.053 ± 0.111	0.171 ± 0.197	<0.0001
RF positive	8 (8.25%)	36 (17.82%)	0.036
ANA positive	1 (1.03%)	15 (7.43%)	0.021
ACL positive	0 (0%)	1 (0.50%)	1
≥ 1 autoantibody positive	9 (9.28%)	47 (23.27%)	<0.001

PRL: prolactin; IgG: immunoglobulin G; IgM: immunoglobulin M; C3: complement 3; C4: complement 4; RF: rheumatoid factor; ANA: anti-nuclear antibody; ACL: anticardiolipin

Data are presented as the means ± SDs. *P* values were determined by the two-tailed Mann–Whitney test for continuous data and by Fisher's exact test for count data

Immune indexes in women with HPRL

To further investigate the clinical index for autoimmune diseases, the levels of IgG, IgM, C3, C4, IL-4 and IL-6 were tested (Fig. 2a). Interestingly, the levels of IL-4 (OD: 0.155 ± 0.110 vs 0.056 ± 0.049, $p < 0.0001$) and IL-6 (OD:

Table 2 Distribution of autoantibodies in women with HPRL

No.	PRL (ng/ml)	ANA panel	RF
1	36.02	SSA+ Ro-52++	–
2	40.08	SSA++ SSB+ nRNP/Sm+	+
3	71.03	–	+
4	30.90	Ro-52++ SSA+	–
5	60.27	–	+
6	34.76	–	+
7	31.24	–	+
8	28.61	–	+
9	57.05	–	+
10	35.17	SSA+	–
11	32.29	Ro-52+++ SSA+++ SSB+	–
12	33.90	–	+
13	30.81	–	+
14	35.32	–	+
15	115.22	–	+
16	41.52	–	+
17	28.09	–	+
18	36.43	CENP-B+ SSB± Ro-52+++ SSA+++ nRNP/Sm+	–
19	113.03	–	+
20	41.31	–	+
21	40.35	AMA M2++ CENP-B+++ nRNP/Sm+	–
22	35.47	–	+
23	60.14	nRNP/Sm+ Histone± Nucleosome± dsDNA±	–
24	35.30	–	+
25	45.54	–	+
26	30.57	–	+
27	42.53	–	+
28	29.40	SSA++ nRNP/Sm+	–
29	28.90	SSA+++ SSB+Ro-52+++ nRNP/Sm±	–
30	46.94	SSA+	–
31	28.65	–	+
32	43.90	–	+
33	68.35	–	+
34	27.24	–	+
35	82.84	–	+
36	48.62	–	+
37	29.53	–	+
38	49.98	–	+
39	158.14	–	+
40	42.09	–	+
41	38.93	–	+
42	47.46	–	+
43	48.64	–s	+
44	42.02	AMA M2++ CENP-B+++	–
45	50.00	Ro-52+++ SSA+++ nRNP/Sm±	+
46	48.14	Histone + CENP-B+++	+
47	60.17	AMA M2+ rRNP++ Histone ++ Nucleosome++ dsDNA+++ Ro-52++ SSA++	+

ANA: anti-nuclear antibody; ACL: anticardiolipin; dsDNA: double-stranded DNA; AMA M2: mitochondrial M2; CENP-B: centromere B; nRNP/SM: ribonucleoprotein/Smith; RF: rheumatoid factor

0.171 ± 0.197 vs 0.053 ± 0.111, $p < 0.0001$) were significantly higher in the sera of women with HPRL than in healthy women. However, no significant differences in the

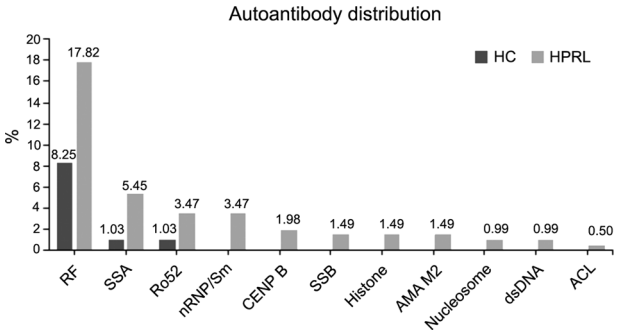

Fig. 1 Distribution of autoantibodies in women with HPRL and healthy women. The numbers represent the positivity rate of each autoantibody in each group. RF: rheumatoid factor; nRNP/SM: ribonucleoprotein/Smith; CENP-B: centromere B; AMA M2: mitochondrial M2; dsDNA: double-stranded DNA; ACL: anticardiolipin

levels of IgG, IgM, C3 and C4 were found between women with HPRL and healthy women. Furthermore, the correlations between IgG, IgM, C3, C4, IL-4 and IL-6 levels and the level of PRL were analysed. Interestingly, IgG, IgM, C3 and C4 did not show a correlation with the PRL level; however, significant positive correlations of IL-4 and IL-6 with PRL levels were observed (Fig. 2b).

B-cell subset distribution in women with HPRL

To verify whether the distribution of B-cell subsets was different between women with HPRL and healthy women, 22 additional women with HPRL and 19 healthy controls were recruited for flow cytometry analysis. The percentages of transitional ($CD19^+CD27^-IgD^+CD10^-$, Tr), naive IgD^+IgM^- ($CD19^+CD27^-IgD^+IgM^-$, B_{ND}), unswitched memory ($CD19^+CD27^+IgD^+$, UM), switched memory ($CD19^+CD27^+IgD^-$, SM) and antibody-secreting ($CD19^+IgD^-CD27^{++}CD38^{++}$, ASC) B cells among the total B cells were measured by flow cytometry. The gating strategy is shown in Fig. 3a. B_{ND} ($14.05 \pm 1.65\%$ vs $2.63 \pm 0.30\%$, $p < 0.0001$), ASC ($2.59 \pm 0.33\%$ vs $1.28 \pm 0.22\%$, $p = 0.007$) and UM ($16.83 \pm 1.48\%$ vs $10.15 \pm 1.00\%$, $p = 0.004$) B cells in HPRL women were significantly increased compared to those in healthy women (Fig. 3b).

Discussion

The immune and neuroendocrine systems are intimately linked and involved in bidirectional communication [15]. Furthermore, a complete regulatory loop between the immune and neuroendocrine systems has been postulated [15, 16]. To clarify the possible role of PRL in humoural immunity in HPRL women, we detected various autoantibodies, IgG, IgM, complements, cytokines and B-cell

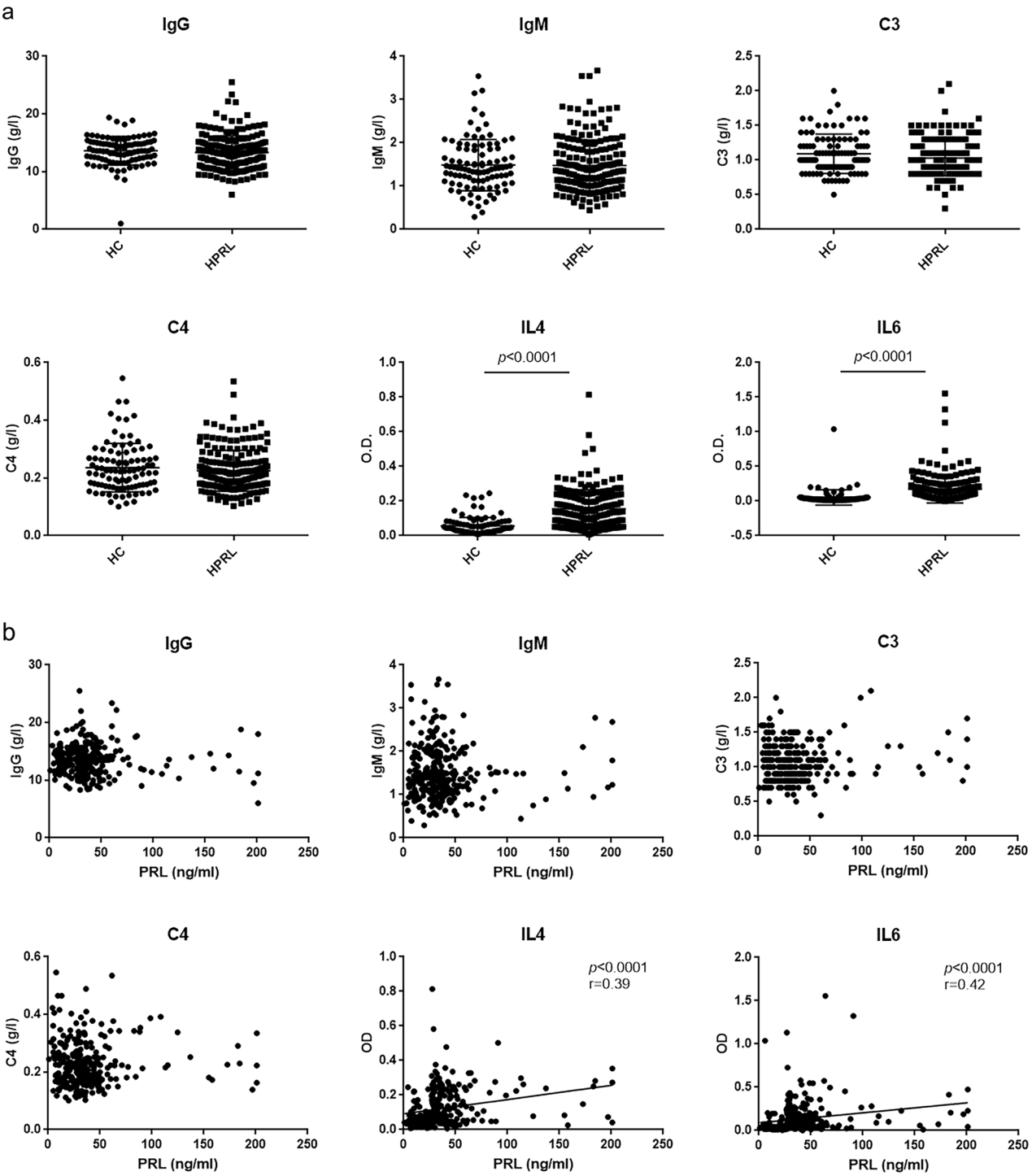

Fig. 2 Immune index assays in women with HPRL and healthy women. **a** Levels of immunoglobulin G (IgG), immunoglobulin M (IgM), complement 3 (C3), complement 4 (C4), IL-4 and IL-6 compared between women with HPRL ($n = 202$) and healthy women ($n = 97$). The data shown are the means ± SDs. *P* values were determined using the two-tailed Mann–Whitney test. **b** Correlation between concentration of prolactin and immune indexes (HPRL $n = 202$; HC $n = 97$). *P* values were calculated with Spearman's correlation

subset distributions in women with HPRL and healthy women.

Our results showed that 23.27% of women with HPRL present with at least one autoantibody, which was significantly higher than the percentage of healthy women. However, this prevalence of autoantibodies in HPRL is relatively lower than that reported in earlier published studies. Buskila reported that 25 of 33 (75.7%) HPRL women

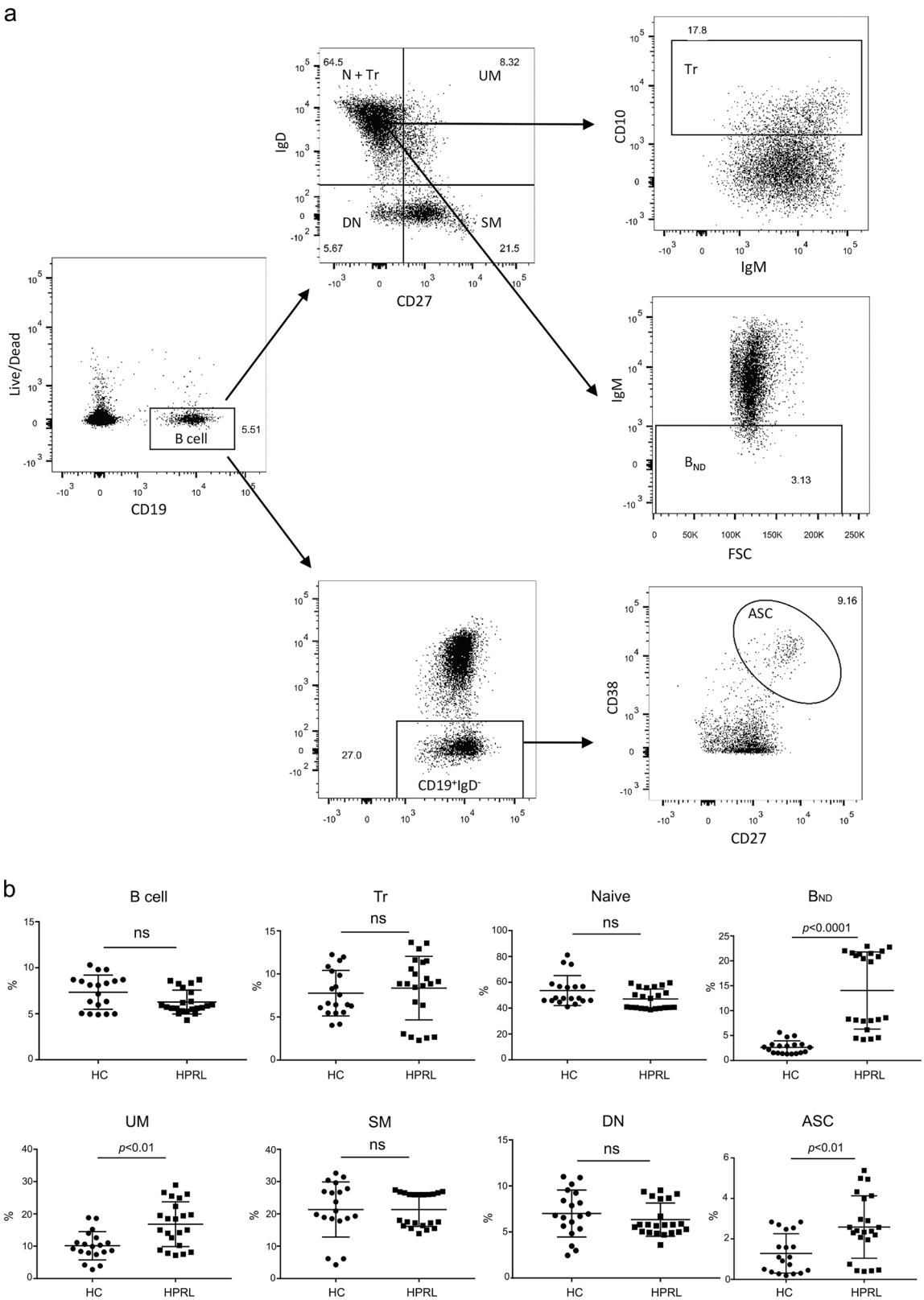

Fig. 3 B-cell subset distribution in women with HPRL and healthy women. **a** Gating strategy of B-cell subsets. N: naïve B cells; Tr: transitional B cells; UM: unswitched memory B cells; SM: switched memory B cells; DN: $CD27^-IgD^-$ B cells; B_{ND}: naive IgD^+IgM^- B cells; ASC: antibody-secreting cells. **b** Comparison of the percentage of different subsets between women with HPRL and healthy women. The data shown are the means ± SEMs. *P* values were determined using the two-tailed Mann–Whitney test

were found to have at least one autoantibody, while none of the 19 women with normal PRL had any [12]. Krause studied anti-endothelial cell antibodies in the sera of 25 HPRL women and found that 76% of the participants were positive for these autoantibodies [13]. One possible explanation for the discrepancy was that we did not detect thyroid autoantibodies and had ruled out patients with known hypothyroidism. Autoimmune thyroid disease (Hashimoto's thyroiditis) was the most common autoimmune disease associated with HPRL. Unlike most of the published studies focused on thyroid autoantibodies, our study aimed to reveal the association of HPRL with rheumatic disease related autoantibodies. In addition, genetic, methodological or other factors could also contribute to the disparity.

Knowing whether the autoantibody-positive rate could vary with different levels of PRL is of considerable interest. A previous study showed that in vitro, the physiological concentration of PRL (20 ng/ml) induced IgG production in PBMCs more effectively than PRL at 100 ng/ml. These results indicated a potential role for mild HPRL in influencing IgG production [17]. However, in our study, the IgG level in women with HPRL was not significantly different from that in healthy women. In addition, no significant difference in the autoantibody-positive rate was found between the PRL mild increase group and the PRL moderate-to-severe increase group. However, the factors affecting the production of antibodies were much more complex in vivo than in vitro.

Although a high prevalence of serum autoantibodies was found, none of the HPRL patients presented with clinical manifestations of autoimmunity. Some studies have shown that the responsiveness of the immune system to PRL is genetically determined. PRL was demonstrated to enhance the in vitro production of IgG in PBMCs from patients with SLE but not in those from healthy controls [17]. Treatment of transgenic female BALB/c mice with PRL for 4 weeks induced a lupus-like phenotype with elevated serum anti-DNA antibody titres but this finding was not observed in C57BL/6 mice [18]. These data could help explain why no correlation was found between PRL and IgG, IgM, C3 or C4. A recent meta-analysis demonstrated that the PRL −1149 G/T polymorphism was associated with susceptibility to RA, but not SLE, in Caucasians [19]. In line with these findings, our results showed that RF was the most prevalent autoantibody in HPRL women.

The association between PRL and cytokines has been suggested in animal studies. Furthermore, PRL has been reported to increase the release of IL-4 in mouse splenocytes and the mRNA expression of IL-6 in bovine thymic stromal cells and murine spleen CD11c-positive dendritic cells [20–22]. Moreover, increased levels of IL-4 and IL-6 were detected in the lungs of rats when HPRL was induced [23]. PRL-R knockdown Jurkat cells showed decreased secretion of IL-4 and IL-2, supporting a mechanism involving the expression of inflammatory cytokines by the autocrine pathway of PRL in lymphocytes [24]. A recent clinical study, which included 102 patients with HPRL, reported that the serum level of IL-4 was significantly higher in patients with HPRL than in healthy controls [14]. Consistent with this study, our results provided evidence for the association of HPRL and inflammatory cytokines (IL-4 and IL-6). Future studies are needed to clarify the possible mechanism of PRL in autoimmune conditions via regulation of specific cytokines.

The B-cell phenotype is considered an important autoimmune marker. However, few studies of this have been reported in HPRL human peripheral blood. In our study, the observation of a high prevalence of autoantibodies and increased cytokines in HPRL women indicated that their B-cell subset distribution could also be altered. Antibody-secreting cells are considered the main effector B cells in humoural immunity. The expansion of antibody-secreting cells has been described in autoimmune diseases such as lupus flare [25], and rheumatoid arthritis [26]. Previous studies of antibody-secreting cells identified anti-apoptotic genes associated with increased PRL [27]. Moreover, IL-6, a key cytokine in the differentiation of antibody-secreting cells [28] was significantly elevated in women with HPRL in our study. However, whether the increase in antibody-secreting cells in women with HPRL was associated with pathogenic autoantibodies remains unclear.

B-cell anergy represents a key mechanism of peripheral immune tolerance in autoreactive B cells [29]. Persistently elevated PRL was found to promote autoimmune responses by decreasing the threshold for activation of anergic B cells. In addition, in the presence of IL-4, the anergy of B cells could be reversed and contribute to autoimmune pathogenesis in patients with SLE [30]. A reduction in anergic B cells (B_{ND}) was recently reported in patients with Hashimoto disease [31] and type 1 diabetes [32]. However, in contrast to these results, we observed that the B_{ND} cells were increased in women with HPRL. Notably, although a high prevalence of serum autoantibodies was found in women with HPRL, none of the women presented with symptoms related to autoimmune disorders. The increase in anergic B cells may represent a compensatory response in effort to maintain immune homoeostasis. Similarly, a loss of unswitched memory B cells is characteristic of rheumatic diseases, as observed in SLE, pSS and RA [33]. However, our data showed that HPRL women have more unswitched memory B cells in their peripheral blood. The meaning of the increase in unswitched memory B cells in HPRL women remains to be formally explored.

To our knowledge, the detection of autoantibodies in women with HPRL in such a large cohort has not been performed previously. However, our study had limitations.

Long-term follow-up of HPRL patients with high autoantibody titres should be performed to clarify whether these patients are at high risk of developing symptomatic autoimmune diseases. In addition, an abnormal distribution of peripheral B-cell subsets was found in the relatively small sample of women with HPRL in our study. Mechanistic studies are needed to explore the possible role of PRL in B-cell differentiation in HPRL in the future. In addition, macroprolactin, which is actually PRL bound to IgG, can lead to a falsely elevated PRL results [34]. However, the gold standard test for diagnosing macroprolactin was not performed in this study. Although a recent study showed that macroprolactin may also have a lower biological activity, no significant difference in autoimmune factors was observed between macroprolactin and true HPRL [14]. Screening to identify macroprolactin is important to avoid misdiagnosis of HPRL.

Conclusion

Our cross-sectional case–control study identified a higher prevalence of autoantibodies (particularly, RF and ANA), increased serum levels of cytokines (IL-4 and IL-6) and elevated peripheral B-cell subset levels (B_{ND} cells, antibody-secreting cells and unswitched memory B cells) in women with HPRL compared with those in healthy women. Although no autoimmune disease symptoms or signs were presented in women with HPRL, the altered autoimmune markers, especially the autoantibodies, indicated the existence of an autoimmune response in these women. Considering that many autoantibodies may be present a few years before the onset of autoimmune disease, patients with HPRL may require long-term follow-up, especially for genetically predisposed individuals.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All the recruited subjects who participated in the study provided informed consent. The study was reviewed and approved by the Medical Ethics Committee of Shanghai Changzheng Hospital, Second Military Medical University.

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