Published in final edited form as:

Vaccine. 2014 February 7; 32(7): 881–887. doi:10.1016/j.vaccine.2013.12.009.

Leptin and Leptin-Related Gene Polymorphisms, Obesity, and Influenza A/H1N1 Vaccine–Induced Immune Responses in Older Individuals

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Abstract

Obesity is a risk factor for complicated influenza A/H1N1 disease and poor vaccine immunogenicity. Leptin, an adipocyte-derived hormone/cytokine, has many immune regulatory functions and therefore could explain susceptibility to infections and poor vaccine outcomes. We recruited 159 healthy adults (5074 years old) who were immunized with inactivated TIV influenza vaccine that contained A/California/7/2009/H1N1 virus. We found a strong correlation between leptin concentration and BMI ($r=0.55$, $p<0.0001$), but no association with hemagglutination antibody inhibition (HAI), B-cell, or granzyme B responses. We found a slight correlation between leptin concentration and an immunosenescence marker (TREC: T-cell receptor excision circles) level ($r=0.23$, $p=0.01$). We found eight SNPs in the *LEP/LEPR/GHRL* genes that were associated with leptin levels and four SNPs in the *PTPNI/LEPR/STAT3* genes associated with peripheral blood TREC levels ($p<0.05$). Heterozygosity of the synonymous variant rs2230604 in the *PTPNI* gene was associated with a significantly lower (531 vs. 259, $p = 0.005$) TREC level, as compared to the homozygous major variant. We also found eight SNPs in the *LEP/PPARG/CRP* genes associated with variations in influenza-specific HAI and B-cell responses ($p<0.05$). Our results suggest that specific allelic variations in the leptin-related genes may influence adaptive immune responses to influenza vaccine.

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Presented in part: Federation of Clinical Immunology Societies (FOCIS) Meeting, Boston, MA, June 27-30, 2013. Abstract F100.

Conflict of Interests

Dr. Poland is the chair of a Safety Evaluation Committee for novel investigational vaccine trials being conducted by Merck Research Laboratories. Dr. Poland offers consultative advice on vaccine development to Merck & Co. Inc., CSL Biotherapies, Avianax, Sanofi Pasteur, Dynavax, Novartis Vaccines and Therapeutics, PAXVAX Inc, and Emergent Biosolutions. Dr. Jacobson serves as a member on a safety review committee and on a data monitoring committee concerning several non-influenza vaccines in studies funded by Merck Research Laboratories. These activities have been reviewed by the Mayo Clinic Conflict of Interest Review Board and are conducted in compliance with Mayo Clinic Conflict of Interest policies. This research has been reviewed by the Mayo Clinic Conflict of Interest Review Board and was conducted in compliance with Mayo Clinic Conflict of Interest policies.

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Keywords

Influenza Vaccines; Influenza A Virus; H1N1 Subtype; Immunity; Polymorphism; Single Nucleotide; Receptors; Leptin; Obesity; Adipose Tissue; Overweight; Aging; Adult; Adipocytes; A/H1N1 virus; immune response; SNPs; obese; immunosenescence; BMI

1. Introduction

Influenza A represents a devastating pathogen to humans, causing significant yearly morbidity and mortality, and periodic deadly pandemics [1, 2]. Although 60–65% of people 65 years of age undergo annual influenza vaccination in the U.S., that age group comprises 90% of the total deaths from influenza and influenza-induced complications [3]. Signs of immunosenescence observed in adults 50 [4, 5] include altered cytokine secretion, diminished clonal expansion and function of T cells, decreased in diversity of T cell repertoire, depleted T lymphocytes function, decreased B cell production and function, decreased antibody affinity, and increased chronic inflammation [6–8]. The latter is recognized as a “chronic inflammatory state” known as “inflamm-aging.” Aging also decreases the generation of new memory T cells in response to novel antigenic stimulation, such as influenza infection or vaccination [9]. A better understanding of the direct and indirect effects altering immune function in older individuals (immunosenescence) is required to reformulate or design an effective influenza vaccine for this particular high-risk group.

The adipocyte-derived hormone leptin (a member of the IL-6 superfamily) has many regulatory immunologic functions [10–12]. Leptin directly acts on hematopoietic cells (i.e., CD4⁺ helper, CD8⁺ cytotoxic T and B cells) [13] through its interaction with the membrane-bound leptin receptor (LEPR) and subsequent signaling via the JAK2/STAT3 pathway [11, 14]. Leptin receptor is a type I (150 kDa) transmembrane glycoprotein and is expressed on the cell surface of T (Treg), B and natural killer (NK) cells, mast cells, macrophages, monocytes, and dendritic cells (DC) [15]. The level of circulating leptin is proportional to body fat mass, but with advancing age, leptin concentrations have been shown to increase disproportionately to total body fat mass, which leads to leptin resistance, and is linked to obesity [16].

Obesity is a risk factor for influenza A/H1N1 disease and poor influenza vaccine immunogenicity [17–19]. Impaired immune functions and viral-induced outcomes have been found in diet-induced mouse models infected with influenza A/H1N1 virus, as well as with human populations [17, 20, 21]. For instance, obese individuals demonstrated decreased activation of influenza-specific CD8⁺ T cells, including decreased production of IFN- γ and granzyme B from PBMC, compared to healthy-weight persons, suggesting that influenza vaccination may not be as effective in the obese population as in healthy-weight individuals [17]. Despite the reduced efficacy of the influenza A/H1N1 vaccine in older individuals, the U.S. federal Advisory Committee on Immunization Practices recommends universal influenza vaccination including all older adults (age $\geq$ 65) [22]. A recent study has demonstrated that the number of obese persons who were hospitalized with complications of the influenza A/H1N1 infection in 2009 was greater than the number of persons of healthy weight [19]. Moreover, obese individuals had a higher death rate and a greater risk of complications following influenza A infections including A/H1N1 virus infection [23–25].

Many aspects of immunosenescence have been studied with regard to influenza vaccine response [4, 26, 27]. Genetic polymorphisms (SNPs), as well as immunosenescence, may affect immune function, reducing the effectiveness of seasonal influenza vaccines in older

individuals. However, it is unknown how age-dependent changes in leptin affect immune function prior to and following influenza vaccine in older individuals and how such effects relate to immunosenescence. We hypothesized that leptin concentrations may explain poor immunogenicity vaccine outcomes. This study aimed to identify correlations between circulating leptin concentrations and influenza vaccine-induced humoral and cellular responses and immunosenescence, or the thymic output marker TREC, in older individuals. It also aimed to examine associations between leptin and leptin-related gene polymorphisms and influenza vaccine-induced immune responses.

2. Materials and methods

2.1. Study subjects

Details of this study's recruitment and study subjects have been provided elsewhere [28]. Briefly, we enrolled 159 subjects (50 to 74 years of age) as part of a larger influenza vaccine study in Rochester, MN. All subjects received one intramuscular dose of the 2010–2011 trivalent inactivated seasonal influenza vaccine (TIV, Fluarix, 0.5 ml), containing influenza A/H1N1/California/2009 virus. Venous blood samples were obtained prior to (day 0) and after (day 28) vaccination. Both height and weight were obtained to determine body mass index (BMI). Overweight and obesity subcategories were defined by the National Institutes of Health (NIH) guidelines as BMI of 25–29 and BMI of ≥ 30 , respectively. The Mayo Clinic Institutional Review Board approved the study, and we obtained written, informed consent from each subject.

2.2. Leptin assay

Non-fasting leptin concentrations were quantified in subjects' sera from the day 0 and day 28 sample time points using the Human Leptin Elisa (R&D Systems, Minneapolis, MN) following the manufacturer's protocol. The level of sensitivity for the leptin assay was 7.8 pg/ml.

2.3. Influenza hemagglutination antibody inhibition (HAI) assay

Details of the HAI assay have been previously described [29]. Briefly, the HAI assays were performed on day 0 and day 28 samples using a standard WHO protocol using 0.6% solution of turkey red blood cells by measuring HAI titers against the influenza vaccine strain A/California/7/2009/H1N1. We considered an HAI titer of at least 1:40 seroprotective.

2.4. B-cell Elispot assay

Influenza virus-specific IgG-secreting B cells were quantified in PBMCs on day 0 and day 28 using the Human IgG ELISpot^{PLUS} kit (Mabtech, Inc., Mariemont, OH). The Elispot assays were performed following the manufacturer's protocol after coating the plates with influenza A/California/7/2009/H1N1 virus (50,000 TCID₅₀ per well). Plates were analyzed on an ImmunoSpot[®] S4 Pro Analyzer (Cellular Technology Ltd., Cleveland, OH) using ImmunoSpot[®] version 4.0 software (Cellular Technology Ltd.). The results are presented in spot-forming counts (SFC) per 2×10^5 PBMC as the median of influenza-specific value (measured in four replicates), minus the median unstimulated value (one replicate). The 95% confidence interval for the intra-class correlation coefficients (ICC) for the baseline samples stimulated with influenza virus was 0.85–0.90.

2.5. Granzyme B assay

Our description of the granzyme B Elispot assay is similar to those we published elsewhere [28]. Influenza-specific granzyme B-positive cells were quantified in PBMC cultures on day 0 and day 28 using the BD[™] Human Granzyme B Elispot kit (BD Biosciences, San Jose,

CA) following the manufacturer's protocol. PBMCs were stimulated with influenza A/California/7/2009/H1N1 virus at a multiplicity of infection (MOI) of 0.5 for 24 hours or left unstimulated. The results are presented in SFC per 2×10^5 PBMC as median of influenza-specific value (in triplicate), minus the median unstimulated value (in triplicate). The 95% CI for the ICC for the baseline samples stimulated with influenza virus was 0.55–0.68.

2.6. TREC analysis

During the process of TCR recombination, by-products of DNA rearrangements are generated in the form of T cell receptor rearrangement excision circles (TRECs) and their quantification is considered a valuable tool to estimate thymic T cell production and immunosenescence. TRECs were examined by quantitative real-time (RT)-PCR on the baseline samples (day 0) for all subjects. Signal joint (sj) TREC content was assessed relative to CCR5 copies in genomic DNA (50 to 400 ng input, isolated from PBMC) using quantitative RT-PCR with specific primers, as described previously [30]. Standard curves with known copies of human sjTREC plasmid and human CCR5 plasmid were generated in order to calculate copies of TREC versus CCR5 for test samples. All reactions were run in quadruplicate and results are expressed as TREC copy numbers/ 10^6 cells.

2.7. SNP selection and genotyping

The genotyping methods have been previously described and are identical to what we have previously published [31]. In brief, 96 tagSNPs from 12 candidate genes (specifically: leptin (*LEP*); leptin receptor (*LEPR*); Janus kinase 2 (*JAK2*); signal transducer and activator of transcription 3 (*STAT3*); suppressor of cytokine signaling 3 (*SOC3*); protein tyrosine phosphatase N1 (*PTPN1*); C-reactive protein (*CRP*); peroxisome-proliferator-activated receptor γ (*PPARG*); adiponectin (*ADIPOQ*); adiponectin receptor 1 (*ADIPOR1*); adiponectin receptor 2 (*ADIPOR2*); and ghrelin/obestatin (*GHRL*)) were selected based on computational selection using source databases, such as the International Hapmap Phase II, Seattle SNPs, NIEHS SNPs and the 1000 Genomes Project. SNP selection was limited to 10kb upstream and downstream from the gene. The linkage disequilibrium (LD)-based ldSelect algorithm was used to identify tagSNPs with a pairwise LD threshold of $r^2 = 0.90$ for Caucasians [31]. The SNP-Picker program was used to post-process and refine the SNP list in order to accommodate a set of Illumina platform limitations. Custom 96-plex SNP panels were designed for the simultaneous detection of multiple SNPs in a DNA sample ($n=159$; 250 ng each) using the Illumina BeadXpress Reader with VeraCode digital microbead technology (Illumina Inc., San Diego, CA). Three SNPs failed QC and were excluded from analysis, leaving 93 SNPs.

2.8. Statistical methods

Results are presented as the median and 25th and 75th percentiles, both overall and by allelic category. Median values were used to summarize the assays that were conducted in triplicate. Correlation of the medium leptin concentration with age, BMI, and immune outcomes were calculated using Spearman's rank correlation. Additive models were used to assess the ability of the SNPs to explain the variability in the immune outcomes and leptin concentration. The linear models with immune outcome were adjusted for leptin and outcomes were log base 2 transformed to satisfy assumptions of normality. Furthermore, the models for B-cell and granzyme B Elispot responses included the unstimulated condition as a covariate. The linear models were then run adjusting for BMI.

3. Results

3.1. Characteristics of the study subjects and immune response

The demographic characteristics of the study subjects are displayed in Table 1. The median age of the study subjects was 59.5 years. The majority of the subjects were Caucasian (98.7%) with near-equal gender representation (females 61.6%). Of 159 enrolled subjects, 147 (92.5%) had BMI data recorded. The median BMI for these subjects was 28.2 with an inter-quartile range (IQR) from 24.5 to 33.2, which is reflective of the U.S. population. Leptin concentrations, which may be reflective of food intake, varied broadly (median 8,100 pg/ml; IQR 4,320; 15,875) and the median peripheral blood TREC level was within the normal range at 442.4 (IQR 174.5; 914.4) copies/ 10^6 PBMC.

A comparison of the HAI titers determined for serum samples, as well as B-cell and granzyme B Elispot responses from PBMC obtained on days 0 (baseline) and 28 (post-vaccination), is shown in Table 2. At baseline (day 0), the influenza A/H1N1-specific HAI (median titer 1:80; IQR 1:40; 1:320) responses indicated that approximately 75 percent of the subjects were already immune. Baseline measures otherwise also included B-cell Elispot (median 9 SFC/ 2×10^5 cells; IQR 3; 20) and granzyme B (median 4 SFC/ 2×10^5 cells; IQR 1; 13) measures. HAI and B-cell Elispot responses increased such that, by day 28, subjects demonstrated a median antibody titer of 1:320 (IQR 1:160; 1:640, $p < 0.001$) and median B-cell Elispot response of 35 SFC/ 2×10^5 cells (IQR 15; 58, $p < 0.001$). Granzyme B activity was low and did not differ significantly between day 0 to day 28 ($p = 0.39$).

We found a strong positive linear relationship between leptin concentration and BMI ($r=0.55$, $p<0.0001$) (Figure 1). There was no evidence of a correlation between leptin concentration and age or leptin concentration and HAI titer; however, there was a slight and direct correlation between leptin concentration and both TREC level ($r=0.23$, $p=0.01$) and B-cell Elispot response ($r=0.14$, $p=0.09$) (Figure 1).

3.2. Associations between SNPs in leptin and leptin-related genes and influenza vaccine-induced responses

In total, 23 SNPs revealed associations ($p \leq 0.05$) (in 8 of 12 candidate leptin and leptin-related genes) with leptin concentrations, influenza vaccine-induced humoral and cellular responses, and the immunosenescence marker TREC (Table 3). Pairwise LD analysis demonstrated rs11811946 to be connected to rs7554485 located in the *LEPR* gene ($r^2=1$), which was associated with an increase in leptin concentrations (6,907; 8489; and 10,832 pg/ml; $p=0.013$). The occurrence of homozygous genotype AA for an intronic SNP (rs2071045) located in a LD block with rs4731429 and rs12706832, $r^2 = 0.86$ of the *LEP* gene on chromosome 7 (7q31.3) was associated with more than a four-fold increase in median leptin levels (2,347; 6,510; and 9,772 pg/ml; $p=0.018$) as compared to the homozygous GG variants.

Four SNP associations in the *PPARG* and *CRP* genes were found with influenza-induced HAI titers. SNP rs17793951 in the intronic region of the *PPARG* gene was associated with an allele-dependent increase in HAI titers (1:240; 1:320; and 1:640; $p=0.022$). We found that the presence of genetic polymorphisms in the *PPARG* (rs1175540 and rs2972164, $r^2 = 0.33$), *LEP* (rs2071045), and *CRP* (rs876537) genes were associated with influenza-specific B-cell Elispot responses (Table 3). Associations were also found for the promoter SNP in the *LEPR* (rs10493377, $p=0.026$), and two intronic SNPs in the *GHRL* (rs35683, $p=0.026$), and *ADIPOR* (rs12813694, $p=0.047$) genes and variations in the granzyme B responses.

For the immunosenescence marker, TREC, heterozygosity of the synonymous variant (rs2230604 in exon 8) in the *PTPN1* gene was associated with significantly lower ($p=0.005$)

peripheral blood TREC levels, as compared to the homozygous major variant. As the copies of the minor allele of an intronic SNP (3762274, $p=0.029$) in the *LEPR* gene and SNPs (rs6503695, $p=0.007$, and rs12949918, $p=0.023$, $r^2 = 0.69$) in the *STAT3* gene increased, PBMC TREC levels decreased (Table 3).

4. Discussion

Obesity has been shown to be a predictor of impaired immunogenicity (decreased antibody response) to hepatitis B, tetanus toxoid, and influenza vaccines [17, 32–34]. As individuals age, circulating leptin levels have been reported to rise with a concomitant reduction in leptin signaling, resulting in leptin resistance [35], which is a finding most frequently associated with obesity [16, 36]. Leptin resistance has been shown to adversely affect the immune response in obese subjects, including response to influenza virus [20, 37]. Currently, there is no clinical measure of leptin resistance, so the most appropriate method of detection is circulating leptin concentrations and leptin-induced STAT3 phosphorylation. It is unknown if, and to what extent, leptin is correlated with variations in influenza vaccine-induced immune response. It is also unknown if genetic polymorphisms (SNPs) in leptin and leptin-related genes account for the inter-individual variations in immune function among older subjects, increasing or decreasing susceptibility to the development of leptin resistance. The primary objective of this study was to ascertain if genomic and proteomic correlations exist between leptin and immune function following influenza A/H1N1 vaccination among older individuals.

We did not observe correlations of non-fasting leptin concentration with age or with HAI titer; however, we found a strong positive correlation between leptin and BMI ($r=0.55$), and a slight direct correlation between leptin concentration and B-cell Elispot response ($r=0.14$). It is not surprising that leptin and BMI (a commonly used indicator of body fat) were correlated, since circulating leptin concentrations are produced from large fat cells and are reflected by adiposity [38]. In this regard, leptin signaling is known to regulate B cell homeostasis through activation of Bcl-2 and cyclin D1 [39]. We found that the leptin level was correlated with peripheral blood TREC levels ($r=0.23$). Since TRECs are generated during TCR V(D)J gene recombination as T cells undergo maturation, TREC assessment has been used as an immunosenescence marker in newly produced naïve T cells. Robust TCR diversity has been related to immune protection and may prevent the development of immune escape variants during infection by increasing T cell exposure to cross-reactive epitopes carried by multiple influenza strains [40]. A recent study has demonstrated that increased concentrations of leptin with age may be an important factor to help the maintenance of a naïve T cell pool in the elderly [41].

It is known that genetic polymorphisms, as well as immunosenescence, affect immune function, reducing the effectiveness of seasonal influenza vaccines in older individuals [27, 42]. We used a candidate gene study to examine genetic variation in the leptin and leptin-related genes and inter-individual differences in influenza vaccine-specific immune responses among an older cohort of 159 subjects. Identifying SNPs in the leptin-related genes and testing their relation to leptin and inter-individual variations of the influenza vaccine-induced immune responses is particularly important because this information, for example, can be applied to finding similarities in leptin-specific mechanisms of vaccine failure between age and morbid obesity (both groups are high-risk for influenza virus-related morbidity and mortality). In our study, *LEP* and/or *LEPR* variants demonstrated associations with leptin, B-cell Elispot and TREC levels. This is consistent with reports of the involvement of leptin in the innate and adaptive immune responses [43]. Findings from this study suggest that SNP rs2071045 in an intronic region of the leptin gene is associated with both leptin levels and influenza-specific B-cell Elispot response in an allele-dependent

manner. However, this association was less significant when adjusting for BMI. Upon interaction with its receptor, leptin can induce IL-10, IL-1 and IL-6 synthesis via T cell activation and B cell proliferation and subsequent antibody production [10, 43]. Evidence also shows that intronic *cis*-regulatory polymorphisms can modulate gene expression and may increase the genetic risk of obesity [44].

Our data provide evidence for associations of polymorphisms in the peroxisome proliferator-activated receptor- γ (*PPARG*) gene with both influenza HAI titer and B-cell Elispot responses. For example, a decrease in HAI titer from 1:320 to 1:160 and a decrease in B-cell response from 38 to 32 SFC/ 2×10^5 cells were observed with increased representation of the minor alleles for SNP rs1151999 and SNP rs2972164, respectively, in the *PPARG* gene. The precise role of this genetic locus in influenza-induced immunity is unclear, although *PPARG* is known to target adipocyte metabolism and has been implicated in obesity [45]. As recently shown in the literature, humoral factors, such as IL-4, have been reported to have functional influence on the *PPARG* expression in human macrophages and PBMC [46]. By identifying and replicating genomic changes that are associated with vaccine-induced immune responses, improvements in vaccine design can be made to reduce the number of people infected each year with influenza, which is currently the 5th leading cause of death (including pneumonia) in older people [47].

We identified four nucleotide polymorphisms in the *PTPN1* (rs2230604) synonymous and *LEPR* (rs3762274) and *STAT3* (rs6503695 and rs12949918) intronic regions that were significantly associated with variations in peripheral blood TREC levels. It has been demonstrated that leptin directly acts on CD4+ and CD8+ T cells through its interaction with the membrane-bound leptin receptor and subsequent signaling via the JAK2/STAT3 pathway [14, 48]. Protein tyrosine phosphatase 1B (PTPN1) has been implicated in leptin and insulin pathways and obesity [49]. As an example, genetic variant *LEPR* rs1045895 in exon 2 has been previously linked with change in BMI over time [50]. In our study, this genetic marker rs1045895 was only marginally associated with leptin concentrations ($p=0.2$); however, it may be linked to functional SNPs that play a role in obesity and its related traits. Of note, in our study, SNPs in the *LEP* (rs2071045, rs4731429, rs12706832, rs1349419), *LEPR* (rs3762274), and *PPARG* (rs2972164, rs17793951, rs1175540) genes were associated with variations in BMI (range of p -values 0.003–0.04) (data not shown). A replication study using the same methods is necessary to confirm these findings.

To our knowledge, this is the first study to examine correlations between leptin concentrations, influenza vaccine-induced immune responses, and an immunosenescence marker (TREC), and to examine associations between leptin-related gene polymorphisms and vaccine-induced immunity in older individuals. The strength of our study is its focus on older individuals, allowing us to better elucidate the immunologic/immunogenetic causes of immune alteration leading to a decreased response to influenza vaccination. This could possibly influence the development of new approaches that lead to better prevention and infection control in this age group where 95% of the influenza-related deaths occur. Our findings are subject to several limitations. A relatively small number of subjects with restricted racial diversity (98.7% Caucasian) was used in this study. Nevertheless, our sample size was carefully selected to identify biologically relevant effect sizes. TREC copy numbers were assessed from total PBMC only, and cells were not fractionated into CD8+ and CD4+ T cells. However, we have learned the relative importance of specific immune outcomes and genetic markers that may explain or predict influenza A/H1N1 vaccine immunity in older individuals.

In conclusion, this study confirms results of previous reports regarding a relationship between serum leptin levels and BMI [51, 52], but it does not show a relationship between

leptin levels and HAI response to influenza A/H1N1 or with age. We did find that leptin concentrations were positively correlated with TREC responses and influenza vaccine-induced B-cell Elispot responses among older individuals. Further, this study identified several SNPs present within the leptin and leptin-related genes that are associated with inter-individual serum leptin concentrations and influenza-specific HAI, B-cell and granzyme B Elispot responses, and the immunosenescence marker TREC in older individuals. More data, including fine-mapping and functional studies, are needed to confirm these findings. Examination of immune response signaling pathways in older persons, as well as other cohorts susceptible to contracting influenza, such as the obese, may help provide a common mechanism (or contributing factor) of immune system dysfunction and, hence, provide the knowledge to design a more immunogenic next-generation vaccine that will target multiple high-risk groups.

Acknowledgments

We thank the Mayo Clinic Vaccine Research Group and the subjects who participated in this study. We thank Michael D. Jensen, M.D., for his useful comments and suggestions. We thank Caroline L. Vitse for her editorial assistance.

Funding

This work was supported by the National Institutes of Health [grant number U01AI089859] and the Retirement Research Foundation. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health or the Retirement Research Foundation.

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Highlights

- Leptin could explain susceptibility to infections and poor vaccine outcomes.
- Our study found strong correlation between leptin concentration and BMI.
- Slight correlation between leptin and immunosenescence
- Allelic variations in leptin-related genes may influence adaptive immune response

Figure 1. Leptin correlations with BMI, Age, TREC levels, and influenza-specific HAI, B-cell and granzyme B Elispot responses

Correlations between leptin concentration and demographic and clinical variables were evaluated using Spearman's method.

Table 1

Demographic and clinical variables of the study cohort.

Variable	No. of Subjects	Level (median, IQR*)
Age, years	159	59.5 (55.3; 66.4)
Gender (n, %)	159	
Female		98 (61.6%)
Male		61 (38.4%)
Race (n, %)	159	
Caucasians		157 (98.7%)
Others		2 (1.3%)
BMI	147	28.2 (24.5; 33.2)
Leptin (pg/ml)	147	8,100 (4,320; 15,875)
TREC**	156	442.4 (174.5; 914.4)

IQR*, interquartile range; BMI, body mass index; **TREC, T cell receptor rearrangement excision circles; 10^6 PBMC at day 0—ratio of the number of copies for TREC relative to the number of copies for a control gene (CCR5).

Table 2

Immune measures summary for the study cohort.

Time Point	No. of Subjects	HAI Titer, median (IQR) ^a	B-cell Elispot (SFC, median, IQR) ^b	Granzyme B Elispot (SFC, median, IQR) ^c
Day 0	159	1:80 (1:40; 1:320)	9.0 (3.0; 20.0)	4.0 (1.0; 13.0)
Day 28	159	1:320 (1:160; 1:640)	35.0 (15.0; 58.5)	5.0 (1.0; 13.0)

^aHAI, hemagglutination inhibition assay (subjects with an HAI titer of 1:40 were considered seroprotective); SFC, spot-forming cells per 2×10^5 PBMC.

^bInfluenza-specific IgG producing B-cells per 2×10^5 PBMC; Median of influenza-specific response (measured in four replicates) minus the median unstimulated response (in one replicate).

^cInfluenza-specific cytolytic activity per 2×10^5 PBMC; Median of influenza-specific response (measured in triplicate) minus the median unstimulated response (in triplicate).

Table 3

Associations between SNPs in the leptin-related genes and leptin concentration, influenza vaccine-induced humoral responses, and immunosenescence markers.

Immune Measure	Gene	SNP ID	Location	Genotype ^d	N	Median (IQR) ^b	P value ^c	P value ^d
Leptin (pg/ml)								
	LEPR	rs11811946	Intron	GG AG AA	48 72 20	6907 (3960, 11733) 8489 (3656, 16083) 10833 (6718, 21858)	0.013	0.023
	LEPR	rs7554485	Intron	GG AG AA	48 71 20	6907 (3960, 11733) 8905 (3530, 16085) 10833 (6718, 21858)	0.013	0.022
	LEP	rs4731429	Intron	GG AG AA	35 70 35	6960 (4410, 12970) 7533 (3869, 14125) 10540 (5748, 21950)	0.049	0.294
	LEP	rs2071045	Intron	AA AG GG	80 57 3	9773 (4483, 18370) 6510 (4275, 11420) 2348 (1192, 16345)	0.018	0.189
	LEP	rs1349419	Intron	GG AG AA	36 69 35	6733 (3970, 12195) 7640 (3396, 14125) 10540 (5773, 21950)	0.014	0.203
	LEP	rs12706832	Intron	AA AG GG	36 70 34	10315 (5760, 20618) 7870 (3315, 15875) 6732 (4410, 11420)	0.019	0.206
	GHRL	rs35683	Intron	CC AC AA	41 72 27	10540 (4745, 21575) 7823 (4303, 13803) 7095 (3254, 15875)	0.031	0.030
	GHRL	rs27647	Intron	AA AG GG	50 65 25	11138 (4203, 18620) 7425 (4416, 14205) 5538 (2720, 11420)	0.037	0.009
HAI titer								
	PPARG	rs796313	Intron	AA AC CC	37 83 20	1:320 (1:320, 1:640) 1:320 (1:80, 1:640) 1:240 (1:80, 1:320)	0.013	0.011
	PPARG	rs1151999	Intron	AA AC CC	37 84 19	1:320 (1:320, 1:640) 1:320 (1:120, 1:640) 1:160 (1:80, 1:320)	0.011	0.010
	PPARG	rs17793951	Intron	AA AG GG	54 65 21	1:240 (1:80, 1:640) 1:320 (1:160, 1:640) 1:640 (1:160, 1:640)	0.022	0.010

Immune Measure	Gene	SNP ID	Location	Genotype ^a	N	Median (IQR) ^b	P value ^c	P value ^d
B-Cell Elispot (SFC 2×10^5 PBMC) ^e								
	CRP	rs1130864	3' UTR	GG AG AA	78 49 13	1:320 (1:160, 1:640) 1:320 (1:160, 1:640) 1:160 (1:80, 1:160)	0.033	0.029
	PPARG	rs1175540	Intron	CC AC AA	49 66 25	29 (13, 59) 35 (18, 53) 44 (20, 68)	0.040	0.082
	PPARG	rs2972164	Intron	AA AG GG	42 69 29	38 (16, 60) 36 (18, 60) 32 (9, 59)	0.035	0.069
	LEP	rs2071045	Intron	AA AG GG	80 57 3	32 (14, 49) 40 (17, 60) 60 (16, 68)	0.018	0.027
	CRP	rs876537	3'downstream	GG AG AA	57 60 23	37 (20, 60) 37 (16, 62) 18 (10, 44)	0.015	0.015
Granzyme B Elispot (SFC 2×10^5 PBMC) ^f								
	LEPR	rs10493377	5'upstream	AA AG GG	34 84 22	5 (2, 17) 6 (1, 13) 2 (0, 8)	0.026	0.023
	GHRL	rs35683	Intron	CC AC AA	41 72 27	8 (3, 16) 5 (1, 12) 3 (1, 12)	0.026	0.028
	ADIPOR2	rs12813694	Intron	GG AG AA	67 52 20	5 (1, 17) 5 (3, 10) 2 (0, 4)	0.047	0.039
TREC (Copy numbers per 10^6 PBMC)								
	PTPN1	rs2230604	Synonymous	GG AG AA	120 19 ---	532 (213, 1050) 259 (101, 501) --- (---, ---)	0.005	0.006
	LEPR	rs3762274	Intron	AA AG GG	49 75 13	665 (259, 1354) 421 (149, 901) 403 (209, 650)	0.029	0.037
	STAT3	rs6503695	Intron	AA AG GG	66 61 13	613 (249, 1227) 367 (141, 710) 282 (115, 500)	0.007	0.007
	STAT3	rs12949918	Intron	AA AG GG	54 62 24	650 (282, 1354) 397 (116, 701) 324 (188, 937)	0.023	0.017

--- no subject for that genotype, A-Adenine, C-Cytosine, G-Guanine, T-thymine, IQR-interquartile range, SNP-single nucleotide polymorphism, LD-linkage disequilibrium.

A total of 93 SNPs were examined; only those found to be statistically significant ($P \leq 0.05$) were included in the table.

Rs11811946 and rs7554485 are in LD ($r^2=1$), rs796313 and rs1151999 are in LD ($r^2=0.98$), rs1175540 and rs1151999 are in LD ($r^2=0.54$), rs12706832 and rs4731429 are in LD ($r^2=0.86$), and rs6503695 and rs12949918 are in LD ($r^2=0.69$).

^aValues are presented as homozygous major allele/heterozygous/homozygous minor allele.

^bIQR, interquartile range.

^cTest for trend p-value from the linear regression analysis. The p-values are adjusted for leptin concentration, except when the immune outcome is leptin concentration.

^dTest for trend p-value from the linear regression analysis. The p-values are adjusted for leptin concentration and BMI. Models with leptin concentration as the outcome are only adjusted for BMI.

^eValues presented are for the median stimulated values less the unstimulated value.

^fValues presented are for the difference of the median stimulated and median unstimulated values.