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Serum SAA1 and APOE are novel indicators for human cytomegalovirus infection

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Human cytomegalovirus (HCMV) infection is a global concern and highly infectious. HCMV-infected individuals are often carriers with damaged immunity. However few diagnostic indicators block HCMV control and prevention. Thus, we measured 21 serum proteins related to HCMV infection using iTRAQ-labeling based quantitative proteomic approaches and SAA1 and APOE were confirmed as candidate serum indicators for identification of HCMV infection according to ROC curve analysis and that co-occurrence of SAA1 and APOE are better markers than individual proteins.

Human cytomegalovirus (HCMV) is also known as human herpesvirus-5 (HHV-5) and is within the Betaherpesvirinae subfamily which includes cytomegaloviruses from other mammals¹. Most HCMV-infected individuals are asymptomatic with latent infection. Once infected, individuals become long-term viral carriers. HCMV is identified in blood mononuclear cells, and some viruses are found in T cells². HCMV infection is typically unnoticed in healthy people, but can be lethal for immunocompromised persons, such as HIV-infected individuals, organ transplant recipients, or newborns. HCMV infection is global and highly infectious; most people acquire the infection during childhood. HCMV can infect NK cells individually and can mimic infectious mononucleosis or glandular fever³. Although the mechanisms of HCMV persistence remain unclear, frequent virus shedding into saliva and the genitourinary tract may account for most world-wide infection. The disease associated with HCMV infection is mostly attributed to lytic viral replication and organic damage either secondary to virus-induced cell death or from host immunological responses⁴. Virus-controlled phosphorylation accounts for ganciclovir-resistance and a recently discovered protein kinase homologue may be a potential therapy for HCMV infections⁵.

The chemokine, vCXCL-1s, is key to viral dissemination by binding to different chemokine receptors⁶. Another group reported that microRNA miR-21 can attenuate HCMV replication in neural cells by inhibiting expression of Cdc25a⁷. Studies also indicate that HCMV can evade the immune system by expressing class I MHC homologue on the infected host cell surface⁸.

Because no therapy exists, early diagnosis is necessary but few biomarkers are available. Here we report that SAA1 and APOE were differentially expressed in HCMV-infected patients and that both proteins were more robust for identifying infection than either protein alone.

Materials and Methods

Reagents. A Pierce C18 Spin Columns was purchased from Thermo Scientific (Rockford, IL). ProteoExtract kit was purchased from MERK (Kenilworth, NJ). The iTRAQ 8 Plex kit was purchased from SCIEX (Framingham, MA). The antibodies against GAPDH, Serum amyloid A-1 protein (SAA1) and apolipoprotein E (APOE) were purchased from Abcam (Cambridge, MA). The secondary antibodies and electro-generated chemiluminescence substrates were from Pierce (Rockford, IL). PhosphoSafe Extraction Reagent was purchased from MERCK (Darmstadt, Germany). Dithiothreitol (DTT) and iodoacetamide (IAA) were purchased from GE Healthcare (Pittsburgh, PA). Trichloroethylene, triethylammonium bicarbonate (TEAB), trifluoroacetic acid (TFA), trypsin, formic acid, acetonitrile, isopropyl alcohol, pyrrolidine, and ammonium hydroxide solution were from Sigma-Aldrich (Shanghai, China).

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Depletion of high abundance serum proteins and sample clean up. Human sera was collected according to approved methods of the Ethics Committee of Shenzhen Second People's Hospital. Samples (N = 10 each) from HCMV-infected, healthy controls and patients with hepatitis B virus infection were aliquoted and stored at -80°C before use. Albumin and IgG were removed from serum using a ProteoExtract kit. Sample cleanup was conducted using a TCA-acetone protein precipitation procedure. First, 100% TCA was added to each sample to a final volume of 33%, and samples were vortexed for 1 min and incubated on ice for 3 h. Samples were centrifuged at $10,000 \times g$, 4°C for 10 min. Supernatant was removed and ice cold 100% acetone was added. Samples were centrifuged at $10,000 \times g$, 4°C for 5 min, and this was repeated twice. Acetone was removed and protein was re-dissolved in extraction buffer (6 M urea, 10 mM DTT). Quantification of the total proteins was performed following the manufacturer's instructions for the 2-D Quant Kit (GE Healthcare, USA).

Protein digestion, iTRAQ labeling and sample desalting. Samples were pooled for each group and 200 μg protein was filtered using an Amicon Ultra 3 K filter device (Millipore, location) for desalting and buffer exchange. Extraction buffer was replaced with 10 mM DTT in 100 mM TEAB and samples were reduced at 50°C for 30 min. After the samples were cooled buffer was replaced with 20 mM IAA in 100 mM TEAB via 3 K filters. Samples were alkylated in the dark for 1 h and buffer was replaced with 100 mM TEAB via 3 K filters and 200 μg of trypsin was added to each sample and incubated overnight at 37°C .

Digestion products were lyophilized and re-suspended in 20 μL 500 mM TEAB. iTRAQ reagents (113, 114 were used to label two replicates of pooled HCMV infected samples, 115, 116 were used to label two replicates of pooled healthy control samples) were prepared according to the manufacturer instructions: iTRAQ reagents were mixed with respective samples within and between biological replicates and incubated at room temperature for 2 h. Reactions were quenched by adding 100 μL water to each sample and incubating them for 30 min at room temperature. Labeled samples were pooled and lyophilized again, then re-dissolved in sample buffer (5% acetonitrile, 0.1% TFA). Desalting of peptide samples using C18 spin columns was performed according to the manufacturer instructions, and samples were dried using a vacuum concentration system.

Peptide separation by liquid chromatography and data acquisition by mass spectrometry. Samples were re-dissolved in 2% acetonitrile, 0.1% formic acid, and loaded on a ChromXP C18 (3 μm , 120 \AA) nanoLC trap column. Online trapping and desalting was carried out at 2.0 $\mu\text{L}/\text{min}$ for 10 min with 100% solvent A. Solvents were composed of water/acetonitrile/formic acid (A, 98/2/0.1%; B, 2/98/0.1%). Then, an elution gradient of 4–55% acetonitrile (0.1% formic acid) in a 90 min gradient was used on an analytical column (3 μm , 100 \AA , 75 μm i.d. \times 15 cm, Acclaim PepMap100, C18, Dionex). MS/MS analysis was performed with a TripleTOF 5600 System (AB SCIEX, Concord, ON) fitted with a Nanospray III source (AB SCIEX). Data were acquired using an ion spray voltage of 2.3 kV, curtain gas of 30 PSI, nebulizer gas of 5.5 PSI, and an interface heater temperature of 150°C . The MS was operated with TOF-MS scans. For DIA, survey scans were acquired in 250 ms and as many as 30 product ion scans (80 ms) were collected if counts exceeded a threshold of 150 counts per second (counts/s) with a +2 to +5 charge-state. A rolling collision energy setting was applied to all precursor ions for collision-induced dissociation. Dynamic exclusion was set for $\frac{1}{2}$ of peak width (~ 8 s).

Protein identification, quantification and functional classification. MS/MS data were analyzed for protein identification and quantification using ProteinPilot Software v.4.5 (AB Sciex Inc.). The threshold of the local false discovery rate was estimated as 1.0% with the integrated PSPEP tool in the ProteinPilot Software, after searching against a decoy concatenated Uniprot human protein database (a total of 71434 entries) with MASCOT search engine. The search parameters were set as follows: iTRAQ quantification, cysteine modified with iodoacetamide, trypsin digestion, thorough searching mode and minimum protein threshold of 95% confidence (unused protein score > 1.3). All peptide intensities were normalized to protein intensities by calculating the medians and then the data were arcsine transformed to obtain the data fitting a normal distribution. A student's t-test was performed iteratively for each protein to test for statistical significances. Then, the data were transformed backwards to calculate the mean \pm SD and 95% confidence intervals. The differential serum proteins were profiled by functional classification through Pather (<http://www.pantherdb.org/>).

Protein validation using ELISA. Protein was quantified for each sample with a commercially available ELISA kit for human SAA1 and APOE (Abcam, Cambridge, MA) according to the manufacturer's instructions. Briefly, equal amounts of crude sera from 15 HCMV patients, 15 healthy controls and 8 hepatitis B virus infected patients were measured using ELISA. Standards and samples were assayed in triplicate. Protein was calculated and analyzed using R 3.1.2.

Protein validation with Western blot. Samples (N = 4 each) from HCMV-infected, healthy controls and hepatitis B virus infected patients were cleaned up using the TCA-acetone procedure. Samples were pooled with loading buffer and boiled at 96°C for 7 min. After electrophoresis, proteins were transferred onto PVDF membranes and antibodies against GAPDH, SAA1 and APOE were diluted at 1:1000 in TBST buffer and incubated for 1 h at room temperature. Secondary goat anti-mouse antibody was diluted at 1:3000 in TBST and incubated for 1 h at room temperature. Protein bands were imaged using ECL substrate and an ImageQuant RT ECL System (GE Healthcare). Relative protein quantification was performed using ImageQuant TL-1D Analysis Tool. A student's t-test was applied to test for statistical significance.

Results

Efficiency evaluation for IgG/albumin depletion. A challenge for measuring serum protein with proteomic analysis is the high abundance of proteins that suppress signals of low abundance proteins during LC-MS/MS analysis. IgG and albumin are the two most abundant serum proteins, comprising 10–20% and 50–70% of

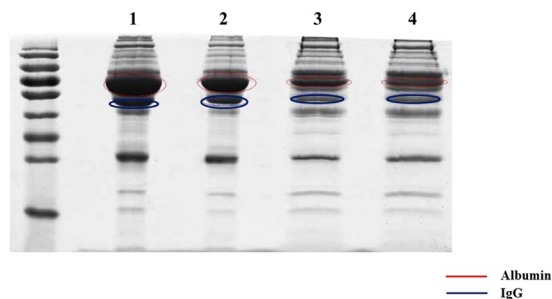


Figure 1. Removal of albumin and IgG from serum samples. 1 crude sera from healthy controls; 2 crude sera from HCMV-infected patients; 3 depleted sera from healthy control; 4 depleted sera from HCMV-infected patients.

| Accession # | Protein Name | Ratio | Pval |
|-----------------------|---|---------|--------|
| sp P0DJ18 SAA1_HUMAN | Serum amyloid A-1 protein | 10.7647 | 0.0161 |
| sp P02741 CRP_HUMAN | C-reactive protein | 6.0813 | 0.0154 |
| sp P01877 IGHA2_HUMAN | Ig alpha-2 chain C region | 5.0119 | 0.0292 |
| sp Q9UK55 ZPI_HUMAN | Protein Z-dependent protease inhibitor | 2.466 | 0.0551 |
| sp P04264 K2C1_HUMAN | Keratin, type II cytoskeletal 1 | 2.1478 | 0.0181 |
| sp P02748 CO9_HUMAN | Complement component C9 | 2.0701 | 0.0073 |
| sp P04196 HRG_HUMAN | Histidine-rich glycoprotein | 0.4742 | 0.0041 |
| sp P01009 A1AT_HUMAN | Alpha-1-antitrypsin | 0.4529 | 0.0133 |
| sp Q15582 BGH3_HUMAN | Transforming growth factor-beta-induced protein ig-h3 | 0.4406 | 0.0409 |
| sp P02749 APOH_HUMAN | Beta-2-glycoprotein 1 | 0.4325 | 0.0062 |
| sp P05155 IC1_HUMAN | Plasma protease C1 inhibitor | 0.4169 | 0.014 |
| sp P11226 MBL2_HUMAN | Mannose-binding protein C | 0.4093 | 0.0244 |
| sp P02656 APOC3_HUMAN | Apolipoprotein C-III | 0.4055 | 0.0073 |
| sp P01008 ANT3_HUMAN | Antithrombin-III | 0.3981 | 0.0197 |
| sp P10909 CLUS_HUMAN | Clusterin | 0.3499 | 0.0278 |
| sp P02649 APOE_HUMAN | Apolipoprotein E | 0.3281 | 0 |
| sp P01860 IGHG3_HUMAN | Ig gamma-3 chain C region | 0.3251 | 0.0352 |
| sp O43866 CD5L_HUMAN | CD5 antigen-like | 0.2884 | 0 |
| sp P01871 IGHM_HUMAN | Ig mu chain C region | 0.263 | 0.0111 |
| sp Q9BXR6 FHR5_HUMAN | Complement factor H-related protein 5 | 0.2559 | 0.0545 |
| sp P01861 IGHG4_HUMAN | Ig gamma-4 chain C region | 0.1406 | 0.0531 |
| sp P01880 IGHD_HUMAN | Ig delta chain C region | 0.1318 | 0 |
| sp P01857 IGHG1_HUMAN | Ig gamma-1 chain C region | 0.1148 | 0.0132 |
| sp P78417 GSTO1_HUMAN | Glutathione S-transferase omega-1 | 0.0112 | 0.0189 |

Table 1. Differential serum proteins in HCMV infected group.

total serum proteins, respectively. Thus to obtain data with high resolution and quality, a ProteoExtract kit was used to deplete serum albumin and IgG specifically. Densitometry analysis of stained protein bands showed that serum albumin and IgG were removed from the serum samples (Fig. 1).

Quantitative analysis and functional classification of HCMV related differentially expressed proteins. Before identification and quantification, peptides with less than 95% confidence or an unused protein score <1.3 were removed. Peptide intensities were transformed to protein signals by calculating medians and 538 proteins were identified in serum samples. Of these 21 were significantly ($P < 0.05$) altered in serum of HCMV-infected patients. Specifically, 5 proteins were up-regulated and 16 were down-regulated (Table 1). Differentially expressed serum proteins were classified by GO categories of biological processes and molecular functions (Fig. 2).

APOE and SAA1 are potential biomarkers for identification of HCMV. In all the differential serum proteins, SAA1 and APOE are not classic immune-related factors such as C-reactive protein, IgG, CD5 antigen-like protein *et al.* which may be altered in many other diseases. Moreover, SAA1 and APOE are with relatively high fold changes and low P values.. Western blot analysis indicated decreased APOE (Fig. 3AB) and

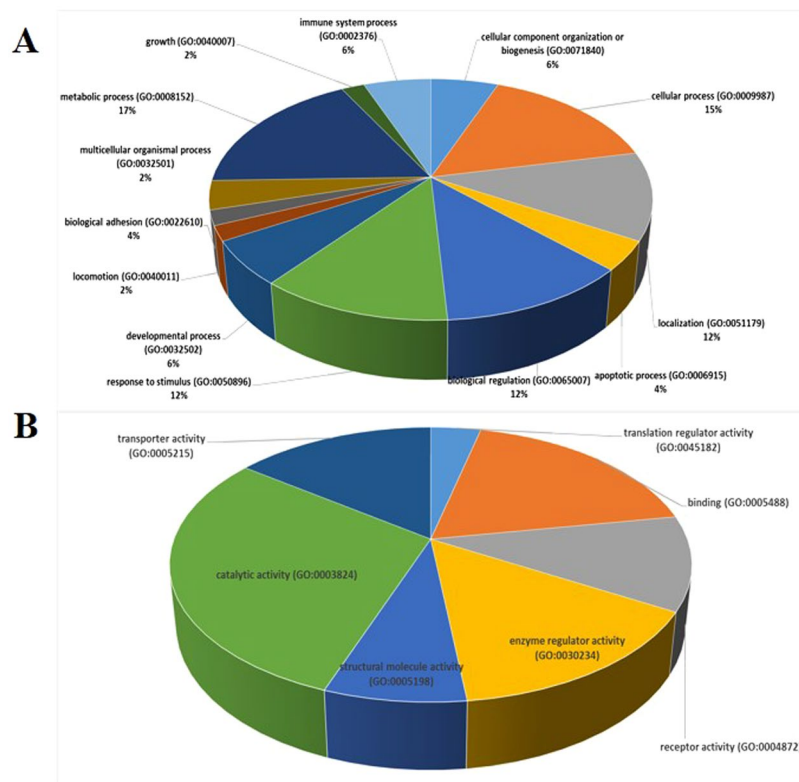


Figure 2. Functional protein classification using panther (<http://www.pantherdb.org>) with GO annotation data. Proteins were classified under the ontology of biological process and molecular function. (A) Protein classification based on biological processes. (B) Protein classification based on molecular function.

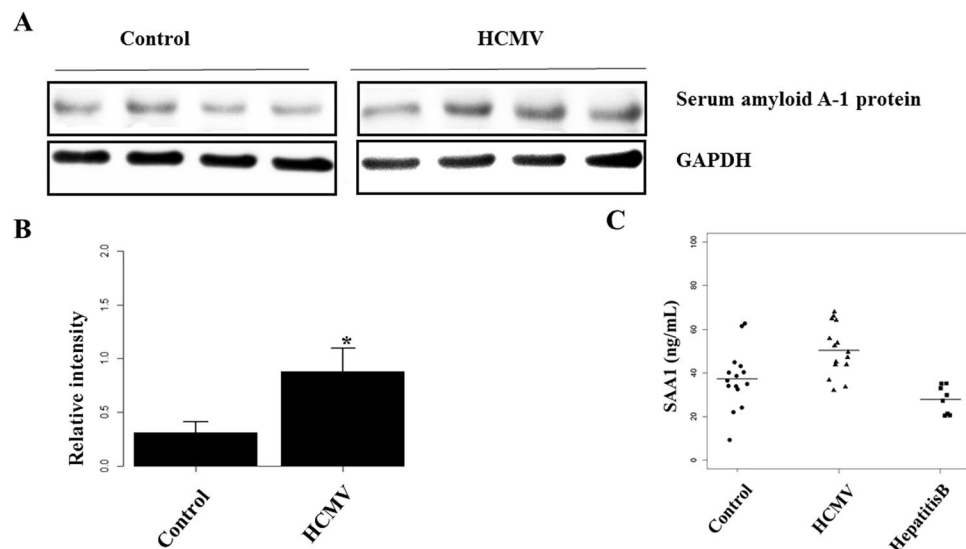


Figure 3. Validation data for potential serum markers for HCMV infection. Expression of proteins in crude sera of controls and infected patients validated by Western blot, and 15 more controls and patients by ELISA. An independent Student's t-test was used for statistical validation. *** $p < 0.001$; (A) Protein bands of SAA1 in serum sample; (B) abnormal expression of SAA1 in sera from individuals with HCMV infection according to Western blot; (C) abnormal expression of SAA1 in sera from individuals with HCMV infection according to ELISA.

increased SAA1 (Fig. 4AB) in HCMV-infected patients compared with healthy controls and hepatitis B virus infected patients respectively. ELISA confirmed down-regulation of APOE (Fig. 4C) and up-regulation of SAA1 (Fig. 3C) in HCMV-infected patients. To determine the potential ability of APOE and SAA1 to discriminate

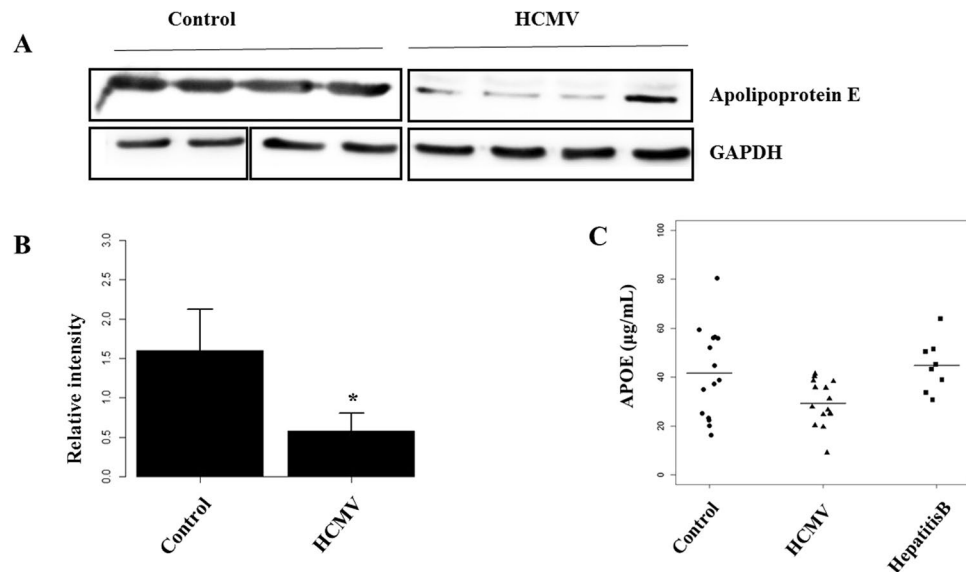


Figure 4. Validation data for potential serum markers for HCMV infection. Protein expression in crude sera of controls and patients enrolled in the proteomic analysis as validated by Western blot, and 15 more controls and patients as validated with ELISA. AN independent Student's t-test was used for statistical validation. *** $p < 0.001$; (A) Protein bands of APOE in serum sample; (B) abnormal expression of APOE in sera from individuals with HCMV infection according to Western blot; (C) abnormal expression of APOE in sera from individuals with HCMV infection according to ELISA.

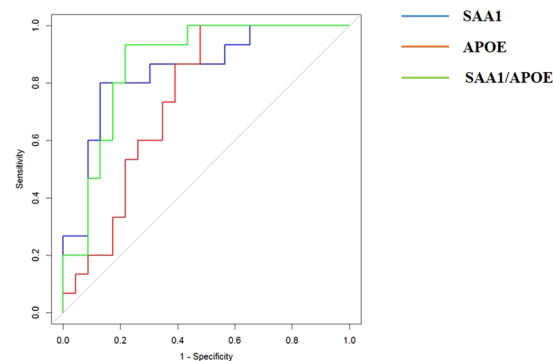


Figure 5. ELISA data based ROC curves for SAA1 and APOE in discriminating HCMV infection from healthy controls. AUC for SAA1 and APOE were 0.78 and 0.67 respectively.

HCMV infections, healthy controls and hepatitis B virus infections, receiver operating characteristic (ROC) curves were generated based on ELISA data. The data of hepatitis B virus infections were mixed with healthy controls and results showed that SAA1 (AUC = 84.3%, specificity = 87.0%, sensitivity = 80.0%) was a better biomarker than APOE (AUC = 74.5%, specificity = 52.2%, sensitivity = 100.0%) in discriminating HCMV infections from healthy controls and hepatitis B virus infections (Fig. 5). However the co-occurrence (ratio between SAA1 and APOE) of the two proteins was with best performance (AUC = 86.7%, specificity = 78.3%, sensitivity = 93.3%) identifying HCMV infections.

Discussion

Primary infection with HCMV is usually asymptomatic and can cause a lifelong latent infection of the host. Although in healthy carriers, no overt disease is observed, severe clinical symptoms are usually associated with HCMV reactivation in immunocompromised transplant patients and HIV sufferers. Transcription factors and histone proteins were found to influence HCMV gene expression profoundly and the virus-encountered cellular environment upon infection may have a critical role in determining a lytic or latent infection and subsequent reactivation from latency⁹. Research shows that the virus can interact with tumor suppressor p53 and induce excessive proliferation of smooth muscle cells which could eventually cause coronary restenosis after angioplasty¹⁰. HCMV infection is global and highly infectious. It is difficult to remove completely from the body, and the treatment is complicated and expensive. Thus, we need better early diagnostic methods for confirming HCMV infection.

To this end, we used a quantitative proteomic method to measure differential serum proteins and of the 538 proteins identified, 21 were differentially expressed. Among these differential proteins, 6 were up-regulated and 15 were down-regulated in HCMV-infected patients. Functional studies suggested enrichment of differential proteins mainly the in immune system and SAA1 and APOE were verified using Western blot and ELISA. ROC curve analysis was used to confirm protein accuracy as a biomarker.

SAA1 is a serum amyloid A family apolipoprotein and a major acute phase protein is highly expressed in response to inflammation and tissue injury. Different isoforms of SAA are expressed constitutively at different levels or in response to inflammatory stimuli. These proteins are produced predominantly by the liver. SAA1 was found to be protective for the development of amyloidosis in individuals with Familial Mediterranean fever¹¹. SAA1 increases dramatically and then rapidly decreases. However, binding to high-density-lipoprotein can prolong the plasma half-life of SAA1¹². SAA1 was reported to aggravate T-cell-mediated hepatitis by producing chemokine via activation of T-cells through the Toll-like receptor 2¹³.

APOE is a lipoprotein that combines with lipids to form complex molecules that are responsible for packaging cholesterol and other fats and carrying them through the bloodstream. Apolipoprotein E is a major component of low-density lipoproteins (VLDLs) which remove excess cholesterol from the blood and carry it to the liver for processing. Maintaining normal cholesterol is essential for prevention of disorders that affect the heart and blood vessels. IFN-gamma was found to increase lesion collagen content but reduce atherosclerotic lesion size, decrease lesion lipid accumulation and lesion cellularity in APOE knock-out mice¹⁴. Additional work indicated that deficiency of endogenous APOE could protect mice from angiotensin II-induced abdominal aortic aneurysm formation¹⁵. Our work not only gives insights into early diagnosis of HCMV but also provides a scientific basis for the future development of a rapid and specific serological diagnostic reagent.

Ethics section. The collection of human sera was approved by the Ethics Committee of Shenzhen Second People's Hospital.

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Author Contributions

Ni Xie wrote the manuscript. Jianhui Yuan designed the study, revised the manuscript and collected serum samples. Zhonghang Li removed the albumin and IgG then digested serum proteins. Suwen Qi labeled digestion products with iTRAQ reagent and profiled the differentially serum proteins. Ran Zuo verified SAA1 using ELISA. Ting Zhu verified APOE using ELISA. Litao Liu verified SAA1 and APOE using Western-blot analysis. Lili Wan performed the statistical analysis.

Additional Information

Competing Interests: The authors declare that they have no competing interests.

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