Isotypes of autoantibodies against novel differential 4-hydroxy-2-nonenal-modified peptide adducts in serum is associated with rheumatoid arthritis in Taiwanese women

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Abstract

Background: Rheumatoid arthritis (RA) is an autoimmune disorder with systemic inflammation and may be induced by oxidative stress that affects an inflamed joint. Our objectives were to examine isotypes of autoantibodies against 4-hydroxy-2-nonenal (HNE) modifications in RA and associate them with increased levels of autoantibodies in RA patients.

Methods: Serum samples from 155 female patients [60 with RA, 35 with osteoarthritis (OA), and 60 healthy controls (HCs)] were obtained. Four novel differential HNE-modified peptide adducts, complement factor H (CFAH)1211–1230, haptoglobin (HPT)78–108, immunoglobulin (Ig) kappa chain C region (IGKC)2–19, and prothrombin (THRB)328–345, were re-analyzed using tandem mass spectrometric (MS/MS) spectra (ProteomeXchange: PXD004546) from RA patients vs. HCs. Further, we determined serum protein levels of CFAH, HPT, IGKC and THRB, HNE-protein adducts, and autoantibodies against unmodified and HNE-modified peptides. Significant correlations and odds ratios (ORs) were calculated.

Results: Levels of HPT in RA patients were greatly higher than the levels in HCs. Levels of HNE-protein adducts and autoantibodies in RA patients were significantly greater than those of HCs. IgM anti-HPT78–108 HNE, IgM anti-IGKC2–19, and IgM anti-IGKC2–19 HNE may be considered as diagnostic biomarkers for RA. Importantly, elevated levels of IgM anti-HPT78–108 HNE, IgM anti-IGKC2–19, and IgG anti-THRB328–345 were positively correlated with the disease activity score in 28 joints for C-reactive protein (DAS28-CRP). Further, the ORs of RA development through IgM anti-HPT78–108 HNE (OR 5.235, \( p < 0.001 \)), IgM anti-IGKC2–19 (OR 12.655, \( p < 0.001 \)), and IgG anti-THRB328–345 (OR 5.761, \( p < 0.001 \)) showed an increased risk. Lastly, we incorporated three machine learning models to differentiate RA from HC and OA, and performed feature selection to determine discriminative features. Experimental results showed that our proposed
Background

Rheumatoid arthritis (RA) is an autoimmune disorder with systemic inflammation and may be induced by oxidative stress that affects inflamed joints [1]. Age, sex, antinuclear antibodies, rheumatoid factor (RF), environmental factors and smoking have been investigated as risk factors of the RA etiopathogenesis [2–4]. Several autoantibodies against neoeptopes, including peptide modified with glycation, citrullination, carbamylation, and oxidation and malondialdehyde (MDA), have been measured in RA patients. In clinical practice, Anti-cyclic citrullinated peptide (anti-CCP) antibodies and RF are clinical biomarkers in patients with RA [5, 6]. One of the lipid peroxidation-generated highly bioactive electrophilic oxidation products, 4-hydroxy 2-nonenal (HNE), is produced through oxidative stress-generated reactive oxygen species (ROS)-targeted n-6 polyunsaturated fatty acids that contribute to the pathogenesis of various diseases, including RA, diabetes mellitus (DM), systemic lupus erythematosus (SLE), alcoholic liver disease (ALD), aging, neurodegenerative diseases, chronic obstructive pulmonary disease (COPD), cardiovascular diseases (CDs), and inflammation-driven cancers [7–10].

HNE has two reactive electrophilic groups, an aldehyde group and an alkene bond, and can react with residues in amino acid. The C=C double bond in HNE can be targeted via Michael addition and has a mass addition at 156 Da in its non-reduced form [alanine (A), arginine (R), cysteine (C), glutamine (Q), histidine (H), lysine (K) and leucine (L)] or 158 Da in its reduced form (CHKRQ) [11–13]. The aldehyde group in HNE can react by forming Schiff base adducts and increase mass of 138 Da in the non-reduced form (CHKAL) or 140 Da in the reduced form (CHKR) [11–13]. The non-reduced form of the Schiff base adducts (CHKR) further spontaneously rearranges to form a pyrrole adduct with a mass increase of 120 Da [14, 15].

Oxidation-specific epitopes (OSEs) include MDA, HNE, 2-(ω-carboxyethyl) pyrrole, oxidized phosphatidylserine, oxidized cardiolipin, oxidized phosphatidylethanolamine, and phosphocholine-oxidized phospholipids, which react with lipids or free amino groups in side chains of protein [5]. Eggleton et al. indicated that HNE-protein adducts present OSEs and are excellent immunogens to induce autoantibodies [8, 16]. Binder et al. revealed that chronic inflammation can be triggered by accumulation of OSEs [5]. Chou et al. suggested that many anti-OSEs are immunoglobulin M (IgM)-natural autoantibodies (IgM-NAAs) [17], and Gronwall et al. proposed that IgM-NAAs provide protection against pathogenesis of autoimmunity [18].

Levels of HNE-protein adducts in diseased states are higher than those in healthy controls (HCs), including Alzheimer’s disease (AD), CDs, Menkes kinky hair disease (MKHD), hyperthyroidism, preeclampsia, mild cognitive impairment (MCI), RA, SLE, and breast cancer [7, 8, 19–25]. Luczaj et al. reported that amounts of HNE-protein adducts were significantly 1.21-fold greater in the plasma of patients with RA than levels in HCs [24]. Autoantibodies against HNE-derived epitopes are present in a variety of diseases comprising SLE, ALD, and AD [26–28]. However, to date, only few studies have reported autoantibodies against HNE-modified human serum albumin in RA [29].

In the present report, four differential novel HNE-modified peptides were re-analyzed via acquired tandem mass spectrometry (MS/MS) data (ProteomeXchange: PXD004546) using PEAKS 7 software (Bioinformatics Solutions, Waterloo, Canada) [6]. Acquired MS/MS spectra were obtained through concanavalin (Con) A affinity chromatography, one-dimensional (1-D) sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), in-gel digestion, and nano-liquid chromatography tandem mass spectrometry (nano-LC–MS/MS) in patients with RA versus HCs [6]. We further validated HNE modifications of proteins and examined proteins level in serum and HNE-protein adducts. Moreover, we evaluated the performance of novel diagnostic autoantibodies against unmodified and HNE-modified peptides, which can possibly be used as diagnostic biomarkers for patients with RA, osteoarthritis (OA), and HCs. Herein, we aimed to determine correlations of IgM and IgG autoantibody titers against unmodified and HNE-modified peptide adducts with disease activity and clinical variables in RA patients. Further, the association between higher levels of serum autoantibodies in RA patients with a risk for RA development was assessed compared
to HCs. Lastly, to thoroughly evaluate the potential of serum autoantibodies for biomarker development, we incorporated three machine learning algorithms and performed feature selection with WEKA (version 3.8.3) to further classify our subjects.

**Methods**

**Patient samples**

Serum samples from 155 female patients [60 with RA (54.8 ± 10.47 years old), 35 with OA (56.2 ± 11.44 years old), and 60 HCs (54.3 ± 8.70 years old)] were obtained from the Division of Allergy, Immunology, and Rheumatology, Department of Internal Medicine and the Department of Laboratory Medicine, Shuang-Ho Hospital (New Taipei City, Taiwan). Patients with RA had received a diagnosis from a rheumatologist and followed the appropriate criteria for classification—either the 2010 American College of Rheumatology (ACR)/European League Against Rheumatism classification criteria [30] or 1987 ACR classification criteria [31]. Further, patients with RA received a disease activity score in 28 joints for C-reactive protein (DAS28-CRP) (4.4 ± 6.7) assessment when they were diagnosed as RA. RA patients included in this study had suffered from this disease for a duration of 5.4 ± 6.41 years (Additional file 1: Table S1). OA patients had been diagnosed according to clinical symptoms with assistance from OA criteria by the ACR [32, 33]. Therapies were given to patients with OA (65% non-steroidal anti-inflammatory drugs (NSAIDs), 11.4% disease-modifying anti-rheumatic drugs (DMARDs)) and RA (42.9% NSAIDs, 99.3% DMARDs) by clinicians (Additional file 1: Table S1). The institutional review board of the study hospital approved this study, and informed consent was provided by all volunteers before participating. Four novel differential HNE-modified peptide adducts were re-identified using PEAKS 7 software (Bioinformatics Solutions) from previous MS/MS data (ProteomeXchange with the identifier PXD004546) [6]. S-Pyrolylation (C)/+ 105.057849 Da was set as the fixed modification, whereas oxidation (M)/+ 15.994915 Da and the following HNE modifications were specified as variables: CHKQRQ/ + 156.11504 Da, CHKQRQ/ + 158.13068 Da, CHKQRQ/ + 138.10446 Da, CHKQRQ/ + 140.12012 Da, and CHKQRQ/ + 120.1916 Da in Additional file 2: Figure S1B. All of the modified MS spectra were identified manually, and fragmented ions were labeled as y, b, y-NH₂, and b-H₂O ions. Details are provided in the "Additional file 3: Supplementary Information" section.

**Con A affinity chromatography and IP-Western blotting**

Serum-derived Con A-captured serum proteins were purified using the protocol of Uen et al. [34]. Protein concentrations were examined using a Pierce™ Coomassie Plus (Bradford) Assay Kit (Thermo Scientific, Waltham, MA, USA) following to the protocol from manufacturer. Con A-captured proteins were used in IP. IP-Western blotting of pooled Con A-captured proteins was used to confirmed modifications of HNE-modified proteins. We used antibodies in IP including complement factor H (CFAH), haptoglobin (HPT), the Ig kappa chain C region (IGIK), and prothrombin (THRB), following to the protocol of Liao et al. [35]. HNE modifications of proteins were detected using a goat polyclonal anti-HNE antibody. Details of experiments are provided in the "Additional file 3: Supplementary Information" section.

**Detection of proteins and HNE-protein adducts**

Levels of CFAH, HPT, IGKC, and THRB were detected using Western blotting. HNE-protein adducts were quantified using an enzyme-linked immunosorbent assay (ELISA) [36]. All samples were detected in duplicate. Details of the protocol are provided in the "Additional file 3: Supplementary Information" section.

**Measurement of autoantibodies against unmodified and HNE-modified peptides**

Polypeptides were synthesized and used in the ELISA [35]. Unmodified peptides are presented as...
CFAH\textsuperscript{1211−1230}, HPT\textsuperscript{78−108, 19}, IGKC\textsuperscript{83−19}, and THRB\textsuperscript{328−345}, HNE-modified peptides, marked as CFAH\textsuperscript{1211−1230} HNE, HPT\textsuperscript{78−108} HNE, IGKC\textsuperscript{2−19} HNE, and THRB\textsuperscript{328−345} HNE, were prepared using HNE (CAS 75899−68-2, Millipore, Darmstadt, Germany) \cite{1}. Then, CFAH\textsuperscript{1211−1230} HNE and HPT\textsuperscript{78−108} HNE were reductively stabilized using NaBH\textsubscript{4} \cite{11}. In total, 155 serum samples were evaluated for the presence of IgG and IgM isotypes of anti-unmodified and anti-HNE-modified peptide autoantibodies. All of the samples were detected in duplicate. The protocol details are provided in the “Additional file 3: Supplementary Information” section.

Results

Identification of differential HNE-modified peptide adducts

MS/MS spectra of the four HNE-modified peptides of patients were RA-specific as shown in Additional file 2: Figure S1 and Additional file 1: Table S1. HNE modifications were identified through manual examination. Among HNE-modified peptides, 1211−SHTLRTTCWDGKLEYPTCAK\textsuperscript{1230} (CFAH\textsuperscript{1211−1230}, Additional file 2: Figure S1C), 78−AVGDKLPCEADDGCPKPEIAHGYVEH SVR\textsuperscript{108} (HPT\textsuperscript{78−108}, Additional file 2: Figure S1D), 2−TVAAPSVFIFPPSDEQLK\textsuperscript{19} (IGKC\textsuperscript{2−19}, Additional file 2: Figure S1E, upper panel), and 328−TFGSGEADCGLRPLFEKK\textsuperscript{345} (THR\textsuperscript{328−345}, Additional file 2: Figure S1F) were identified. The HNE reduced form of Michael adducts of CFAH (at K1230) and HPT (at C92) corresponded to a mass increase of 158.13068 Da. The HNE Michael adducts of IGKC (at A5) and THRB (at K344) had a 156.11504 Da shift in the two residues. Moreover, four HNE-modified peptides that were HC-specific were shown in Additional file 2: Figure S1 and Additional file 1: Table S1: 284−ERTGDEITYQCRNGFYPATRGNTA\textsuperscript{308} (CFAH\textsuperscript{284−308}, Additional file 2: Figure S1G), 162−ILGGLHDL\textsuperscript{170} (HPT\textsuperscript{162−170}, Additional file 2: Figure S1H), 83−VYACEVTHQGLSSPVTKSF\textsuperscript{103} (IGKC\textsuperscript{83−103}, Additional file 2: Figure S1I), and 328−TFGSGEADCGLRPLFEK\textsuperscript{344} (THR\textsuperscript{328−344}, Additional file 2: Figure S1J) were identified. The HNE Schiff base adducts of CFAH (at K308) corresponded to a mass increase of 138.10446 Da. The HNE Michael adducts of HPT (at A169) and THRB (at L341) caused a 156.11504 Da shift in the two residues, and the HNE-reduced form of Michael adducts of THRB (at C336) corresponded to a mass increase of 140.12012 Da. The HNE Michael adducts of IGKC (at Q91) corresponded to a mass increase of 158.13068 Da. In particular, 2−TVAAPSVFIFPPSDEQLK\textsuperscript{19} (IGKC\textsuperscript{2−19}, Additional file 2: Figure S1E, bottom panel) was identified from RA and HC, respectively. The HNE Michael adducts of IGKC (at A4) corresponded to a mass increase of 156.11504 Da.

Statistical analysis

The significance of blot densitometric differences, and levels of serum proteins and HNE-protein adducts were determined using Student’s t-test. A one-way analysis of variance (ANOVA) was used to examine levels of autoantibody isotypes against unmodified and HNE-modified peptides between RA and OA patients and HCs. Scheffe’s post-hoc test was applied to evaluate the difference of mean between any two groups, as well as a post-hoc test using the Bonferroni method with a 0.0167 adjusted significance level. We used GraphPad Prism (vers. 5.0; GraphPad Software, San Diego, CA, USA) to evaluate differences in Student’s t-test between groups, correlations between measurements, and generated receiver operating characteristic (ROC) curves to evaluate the diagnostic performance of autoantibodies. Pearson’s or Spearman’s rank correlation coefficients were used to assess correlations among different parameters. To estimate multivariate-adjusted odds ratios (ORs) and their 95% confidence intervals (CIs) for RA risk, Logistic regression models were performed in this study. The positivity of autoantibody isotypes and HNE-protein adducts was decided by ROC curves. The cut-off value for an ROC curve was determined by Youden index, which represents the sum of sensitivity and 1-specificity, and the maximum value of Youden index is the suitable cut-off point for that curve. Pair-wise comparisons of ROC curves were assessed using MedCalc Statistical Software (vers. 15.4; MedCalc Software, Ostend, Belgium). One-way ANOVA and power were determined using SAS (vers. 9.3; SAS Institute, Cary, NC, USA), and power estimations were calculated according to the ROC analysis. The area under the ROC curve (AUC), sensitivity, and specificity were calculated at a 95% confidence level. The significance level of all statistical tests was set to \( p < 0.05 \). For feature selection, we first used ‘Information Gain’ as the attribute evaluator with ‘Ranker’ as the search method in WEKA (version 3.8) \cite{38} to select discriminative features in identify RA patients. Next, we incorporated ten-fold cross-validation to evaluate our model based on decision trees (DT) \cite{39}, random forests \cite{40}, and support vector machines (SVM) \cite{41} in scikit-learn (version 0.21.3) \cite{42}. Parameter tuning was performed for each training and validation. During model selection, a forward selection algorithm was used to select the most effective combination of features for classification. In forward selection, a feature was selected in to the optimal feature set if adding the feature into the prediction model improved the AUC. To evaluate the predictive performance, we applied a confusion matrix to calculate the accuracy, precision, sensitivity, specificity, and AUC for assessments.
Validation of HNE modifications on HNE-modified peptide adducts
HNE modifications in four differential HNE-modified peptide adducts were validated by IP-Western blotting with two pooled Con A-captured serum samples from RA and HCs, which detected signals of approximately 184, 43, 25, and 80 kDa, respectively, indicating CFAH, HPT, IGKC, and THRB (Fig. 1).

Detection of protein levels and HNE-protein adduct levels
Protein levels of CFAH, IGKC, and THRB from 32 randomly paired individual serum samples from RA patients and HCs showed no significant differences (Fig. 2a, c, d). However, levels of HPT in patients with RA were greatly higher than the levels in HCs (1.24-fold, \( p < 0.041 \), Fig. 2b). Serum levels of HNE-protein adducts in RA patients were significantly higher than the levels in OA patients (1.16-fold, \( p = 0.0311 \)) and HCs (1.20-fold, \( p = 0.0062 \), Additional file 1: Table S1).

Measuring autoantibodies against unmodified and HNE-modified peptides
The AUC, sensitivity, and specificity were used to assess clinical performances of IgG and IgM that against unmodified and HNE-modified peptides. The ANOVA analysis indicated that differences in all autoantibodies against unmodified and HNE-modified peptides were significant among patients with RA, OA, and HCs (Fig. 3, Table 1).

Levels of IgG against CFAH\(^{1211-1230}\) in patients with RA were greatly higher than those in patients with OA (1.36-fold, \( p = 0.0002 \)) and HCs (1.25-fold, \( p = 0.0007 \)) (Fig. 3a, left panel), and AUC values were 0.66 (with 63.3% sensitivity and 51.7% specificity), and 0.58 (with...
60.0% sensitivity and 60.0% specificity) for detecting patients with RA and OA (Table 2). Levels of IgG against CFAH\(^{1211-1230}\) HNE in RA patients were greatly higher than the levels in OA patients by 1.32-fold (\(p=0.0006\)), and HCs by 1.32-fold higher (\(p=0.0001\)) (Fig. 3b, left panel), and AUC values were 0.71 (with 76.7% sensitivity and 55.0% specificity) and 0.52 (with 51.4% sensitivity and 50.0% specificity) for detecting patients with RA and OA (Table 2). HC-RA versus HC-OA showed a statistically significant difference (\(p=0.0135\), Fig. 3b, right panel, Table 2) in pair-wise comparisons of ROC curves. Further, levels of IgM against CFAH\(^{1211-1230}\) HNE in RA patients were greatly higher than the levels in HCs by 1.40-fold (\(p=0.0002\), Fig. 3d, left panel), and AUC values were 0.70 (with 85.0% sensitivity and 41.7% specificity) and 0.59 (with 80.0% sensitivity and 36.7% specificity) for detecting patients with OA and RA (Table 2). Further, HC-OA versus HC-RA showed a significant difference (\(p=0.0421\), Fig. 3d, right panel, Table 2) in pair-wise comparisons of ROC curves.

Levels of IgG against HPT\(^{78-108}\) were significantly 1.32-fold higher (\(p=0.0008\) and 1.26-fold (\(p=0.0003\) greater in patients with RA than the levels in OA patients and HCs (Fig. 3e, left panel), and AUC values were 0.67 (with 71.7% sensitivity and 55.0% specificity) and 0.53 (with 51.4% sensitivity and with 46.7% specificity) for detecting patients with RA and OA (Table 2). Levels of anti-HPT\(^{78-108}\) IgG in RA patients were greatly higher than the levels in OA patients and HCs by 1.46- (\(p<0.0001\)) and 1.35-fold (\(p=0.0002\)), respectively (Fig. 3f, left panel), and AUC values were 0.66 (with 71.7% sensitivity and 48.3% specificity) and 0.58 (with 62.9% sensitivity and 55.0% specificity) for detecting patients with RA and OA (Table 2). Levels of IgM against HPT\(^{78-108}\) were
Fig. 3 Dot plots and receiver operating characteristics (ROC) curves of serum concentrations (absorbance units at 450 nm) of autoantibody isotypes: IgG anti-complement factor H (CFAH)\textsuperscript{1211–1230} (a), IgG anti-CFAH\textsuperscript{1211–1230} 4-hydroxy-2-nonenal (HNE) (b), IgM anti-CFAH\textsuperscript{1211–1230} (c), IgM anti-CFAH\textsuperscript{1211–1230} HNE (d), IgG anti-haptoglobin (HPT)\textsuperscript{78–108} (e), IgG anti-HPT\textsuperscript{78–108} HNE (f), IgM anti-HPT\textsuperscript{78–108} (g), IgM anti-HPT\textsuperscript{78–108} HNE (h), IgG anti-immunoglobulin kappa chain C region (IGKC)\textsuperscript{2–19} (i), IgG anti-IGKC\textsuperscript{2–19} HNE (j), IgM anti-IGKC\textsuperscript{2–19} (k), IgM anti-IGKC\textsuperscript{2–19} HNE (l), IgG anti-prothrombin (THRB)\textsuperscript{328–345} (m), IgG anti-THRB\textsuperscript{328–345} HNE (n), IgM anti-THRB\textsuperscript{328–345} (o), and IgM anti-THRB\textsuperscript{328–345} HNE (p) in healthy controls (HCs), osteoarthritis (OA) patients, and rheumatoid arthritis (RA) patients using an ELISA. OD\textsubscript{450}, optical density at 450 nm. ANOVA was used to test levels of autoantibody isotypes among patients with RA and OA, and HCs. Scheffe’s post-hoc test was applied to compare the mean difference between any two groups. Values of mean ± 1 standard deviation are also indicated in the figure as horizontal lines.
1.35-fold ($p = 0.0013$) greatly higher in RA patients than HCs (Fig. 3g, left panel), and AUC values were 0.67 (with 71.7% sensitivity and 56.7% specificity) for detecting RA and 0.53 (with 51.4% sensitivity and 50.0% specificity) for detecting OA (Table 2). Levels of anti-HPT$^{78-108}$ HNE IgM in RA patients were greatly higher than the levels in OA patients by 3.45-fold ($p < 0.0001$), while RA patients versus HCs was 3.83-fold higher ($p < 0.0001$) (Fig. 3h, left panel).
Table 1 Differential 4-hydroxy-2-nonenal (HNE)-modified peptide adducts in rheumatoid arthritis (RA) patients compared to healthy controls (HCs)

| Protein* | Modified peptideb | Positionc | Obs. d | Calc. e | dMf | dMg | Sequence found with modifications |
|----------|-------------------|-----------|--------|---------|------|------|---------------------------------|
| CFAH     | SHTLRTTCWDGKLEYPTCAK(+158) | 1211−1230 | 858.439 | 858.435 | 0.0041 | 5.4 | − | + |
| HPT      | HRTGDEITYQCRNGFFPATRGNTAK(+138) | 284−308 | 749.370 | 749.372 | −0.0018 | −1.8 | + | − |
| IGKC     | AVGDKLPEEADDGCL(+158)PKPPEIAGYVEHVR | 78−108 | 869.925 | 869.927 | −0.0017 | −1.3 | − | + |
|          | ILGGHILDA(+156)K | 162−170 | 540.329 | 540.327 | 0.0028 | 6.1 | + | − |
|          | TVAA(+156)PSVIFPSDEQLK | 2−19 | 701.384 | 701.386 | −0.0018 | −1.7 | − | + |
|          | TVA(+156)APSVIFPSDEQLK | 2−19 | 1051.582 | 1051.575 | 0.0070 | 7.1 | + | − |
|          | VYACETTHQ(+158)GLSSVTKSFNR | 83−103 | 862.780 | 862.785 | −0.0044 | −4.5 | + | − |
| THRB     | TFGSGEADCGLPFLFEK(+156)K | 328−345 | 1056.036 | 1056.046 | −0.0107 | −9.6 | − | + |
|          | TFGSGEADC(+140)GLPFL(+156)FEK | 328−344 | 708.376 | 708.375 | 0.0009 | 2.1 | + | − |

* CFAH, Complement factor H; HPT, Haptoglobin; IGKC, Ig kappa chain C region; THRB, Prothrombin
b Site of modified peptide
c Amino acid positions of the first and the last residues in peptide
d Obs: observed m/z of the modified peptides
e Calc: calculated m/z of the modified peptides
f dM (Obs.-Calc.): mass accuracy
g Modified peptide mass accuracy (ppm)

Levels of IgM against IGKC2−19 were greatly higher in RA patients than in OA patients and HCs, respectively (Fig. 3i, left panel), and AUC values were 0.86 (with 83.3% sensitivity and 66.7% specificity) for detecting RA and 0.58 (with 68.6% sensitivity and 51.7% specificity) for detecting OA (Table 2). Further, HC-RA versus HC-OA was statistically significant (p < 0.0001, Fig. 3j, right panel, Table 2) in pair-wise comparisons of ROC curves. Levels of IgM against IGKC2−19 in RA patients were greatly higher than in OA patients and HCs (1.29-fold, p = 0.0157) and HCs (1.47-fold, p = 0.0003) (Fig. 3i, left panel), and AUC values were 0.72 (with 80.0% sensitivity and 60.0% specificity) for detecting RA and 0.59 (with 65.7% sensitivity and 53.3% specificity) for detecting OA (Table 2).

Levels of IgG against IGKC2−19 were greatly higher in RA patients than OA patients (1.20-fold, p = 0.0017) and HCs (1.47-fold, p = 0.0003) (Fig. 3i, left panel), and AUC values were 0.72 (with 80.0% sensitivity and 60.0% specificity) for detecting RA and 0.59 (with 65.7% sensitivity and 53.3% specificity) for detecting OA (Table 2). Further, HC-RA versus HC-OA were statistically significant (p < 0.0001, Fig. 3k, right panel, Table 2) in pair-wise comparisons of ROC curves. Levels of IgG against IGKC2−19 were greatly higher in RA patients than in OA patients and HCs, respectively (Fig. 3j, left panel), and AUC values were 0.86 (with 83.3% sensitivity and 66.7% specificity) for detecting RA and 0.58 (with 68.6% sensitivity and 51.7% specificity) for detecting OA (Table 2). Further, HC-RA versus HC-OA were statistically significant (p < 0.0001, Fig. 3l, right panel, Table 2) in pair-wise comparisons of ROC curves. Levels of IgG against IGKC2−19 were greater in RA patients than in OA patients and HCs (1.29-fold, p = 0.0157) and HCs (1.47-fold, p = 0.0003) (Fig. 3i, left panel), and AUC values were 0.72 (with 80.0% sensitivity and 60.0% specificity) for detecting RA and 0.59 (with 65.7% sensitivity and 53.3% specificity) for detecting OA (Table 2).

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Table 2 Comparisons of the area under the receiver operating characteristic curve (AUC), sensitivity, and specificity of autoantibody isotypes against unmodified and 4-hydroxy-2-nonenal (HNE)-modified peptides in rheumatoid arthritis (RA) patients and osteoarthritis (OA) patients compared to healthy controls (HCs)

| Peptides | RA vs. HC | OA versus HC | p value a |
|----------|-----------|--------------|-----------|
|          | AUC (95%CI) | Sensitivity (95%CI) | Specificity (95%CI) | AUC (95%CI) | Sensitivity (95%CI) | Specificity (95%CI) |
| IgG anti-CFAH1121-1230 | 0.66 (0.57–0.76) | 63.3% (49.9–75.4) | 51.7% (38.4–64.8) | 0.58 (0.46–0.70) | 60.0% (42.1–76.1) | 60.0% (46.5–72.4) |
| Cutoff (OD450) > 0.24 | | | |<0.21| | |
| IgG anti-CFAH1121-1230 HNE | 0.71 (0.62–0.80) | 76.7% (64.0–86.6) | 55.0% (41.6–67.9) | 0.52 (0.40–0.64) | 51.4% (34.0–68.6) | 50.0% (36.8–63.2) |
| Cutoff (OD450) > 0.21 | | | |<0.20| | |
| IgM anti-CFAH1121-1230 | 0.68 (0.59–0.78) | 81.7% (69.6–90.5) | 53.3% (40.0–66.3) | 0.68 (0.58–0.79) | 77.1% (59.9–89.6) | 60.0% (46.5–72.4) |
| Cutoff (OD450) > 0.58 | | | |>0.63| | |
| IgM anti-CFAH1121-1230 HNE | 0.70 (0.61–0.79) | 85.0% (73.4–92.9) | 41.7% (29.1–55.1) | 0.59 (0.47–0.71) | 80.0% (63.1–91.6) | 36.7% (24.6–50.1) |
| Cutoff (OD450) > 0.60 | | | |<0.0001| | |
| IgG anti-HPT78-108 | 0.67 (0.58–0.77) | 71.7% (58.6–82.6) | 55.0% (41.6–67.9) | 0.53 (0.41–0.65) | 51.4% (34.0–68.6) | 46.7% (33.9–68.6) |
| Cutoff (OD450) > 0.64 | | | |<0.64| | |
| IgG anti-HPT78-108 HNE | 0.66 (0.57–0.76) | 71.7% (58.6–82.6) | 48.3% (35.2–61.6) | 0.58 (0.46–0.70) | 62.9% (44.9–78.5) | 55.0% (41.6–67.9) |
| Cutoff (OD450) > 0.25 | | | |<0.25| | |
| IgM anti-HPT78-108 | 0.67 (0.57–0.77) | 71.7% (58.6–82.6) | 56.7% (43.2–69.4) | 0.53 (0.40–0.66) | 51.4% (33.9–68.6) | 50.0% (36.8–63.2) |
| Cutoff (OD450) > 0.30 | | | |<0.29| | |
| IgM anti-HPT78-108 HNE | 0.89 (0.80–0.93) | 86.7% (75.4–94.1) | 66.7% (53.3–78.3) | 0.58 (0.46–0.69) | 65.7% (47.8–80.9) | 55.0% (41.6–67.9) |
| Cutoff (OD450) > 0.24 | | | |<0.0001| | |
| IgG anti-IGKC2-19 | 0.72 (0.62–0.81) | 80.0% (67.7–89.2) | 60.0% (46.5–72.4) | 0.59 (0.47–0.70) | 65.7% (47.8–80.9) | 53.3% (40.0–66.3) |
| Cutoff (OD450) > 0.64 | | | |<0.41| | |
| IgG anti-IGKC2-19 HNE | 0.70 (0.61–0.79) | 78.3% (65.8–87.9) | 56.7% (43.2–69.4) | 0.55 (0.43–0.67) | 65.7% (47.9–80.9) | 50.0% (36.8–63.2) |
| Cutoff (OD450) > 0.25 | | | |<0.37| | |
| IgG anti-THRB328-345 | 0.70 (0.61–0.79) | 66.7% (53.3–78.3) | 53.3% (40.0–66.3) | 0.53 (0.42–0.65) | 62.9% (44.9–78.5) | 48.3% (35.2–61.6) |
| Cutoff (OD450) > 0.20 | | | |<0.16| | |
| IgG anti-THRB328-345 HNE | 0.75 (0.67–0.84) | 80.0% (67.7–89.2) | 53.3% (40.0–66.3) | 0.60 (0.48–0.71) | 68.6% (50.7–83.2) | 50.0% (36.8–63.2) |
| Cutoff (OD450) > 0.45 | | | |<0.39| | |
| IgM anti-IGKC2-19 | 0.83 (0.75–0.90) | 81.7% (69.6–90.5) | 60.0% (46.5–72.4) | 0.56 (0.44–0.68) | 57.1% (39.4–73.7) | 50.0% (36.8–63.2) |
| Cutoff (OD450) > 0.61 | | | |<0.37| | |
| IgM anti-THRB328-345 | 0.86 (0.79–0.93) | 83.3% (71.5–91.7) | 66.7% (53.3–78.3) | 0.58 (0.46–0.70) | 68.6% (50.7–83.2) | 51.7% (38.4–64.8) |
| Cutoff (OD450) > 0.27 | | | |<0.0001| | |
| IgG anti-THRB328-345 | 0.70 (0.61–0.79) | 66.7% (53.3–78.3) | 53.3% (40.0–66.3) | 0.53 (0.42–0.65) | 62.9% (44.9–78.5) | 48.3% (35.2–61.6) |
| Cutoff (OD450) > 0.20 | | | |<0.19| | |
| IgM anti-THRB328-345 HNE | 0.75 (0.67–0.84) | 80.0% (67.7–89.2) | 53.3% (40.0–66.3) | 0.60 (0.48–0.71) | 68.6% (50.7–83.2) | 50.0% (36.8–63.2) |
| Cutoff (OD450) > 0.32 | | | |<0.19| | |
| IgM anti-THRB328-345 | 0.63 (0.53–0.73) | 63.3% (50.0–75.4) | 53.3% (40.0–66.3) | 0.56 (0.44–0.68) | 60.0% (42.1–76.1) | 53.3% (40.0–66.3) |
| Cutoff (OD450) > 0.61 | | | |<0.0001| | |
| IgM anti-THRB328-345 HNE | 0.78 (0.69–0.86) | 76.7% (64.0–86.6) | 71.7% (58.6–82.6) | 0.56 (0.44–0.68) | 65.7% (47.8–80.9) | 46.7% (33.7–60.0) |
| Cutoff (OD450) > 0.64 | | | |<0.52| | |

*Pairwise comparison of predictive performance, HC-RA versus HC-OA, was performed by Student’s t-test.
sensitivity and 53.3% specificity) and 0.56 (with 60.0% sensitivity and 53.3% specificity) for detecting RA and OA (Table 2). Levels of IgM against anti-THRB328–345 HNE were 1.33- (p = 0.0008) and 1.52-fold (p < 0.0001) greater in RA patients than in OA patients and HCs, respectively (Fig. 3p, left panel), and AUC values were 0.78 (with 76.7% sensitivity and 71.7% specificity) and 0.56 (with 65.7% sensitivity and 46.7% specificity) for detecting RA and OA (Table 2). HC-RA versus HC-OA was statistically significant (p < 0.0001, Fig. 3p, right panel, Table 2) in pair-wise comparisons of ROC curves.

Correlations of serum anti-unmodified and anti-HNE-modified peptide autoantibodies with clinical variables in RA patients

A correlation analysis was conducted of autoantibody reactivities against unmodified and HNE-modified peptides with DAS28-CRP measurements and serum clinical variables (RF, anti-CCP, CRP, ESR, and the HNE-protein adduct) in patients with RA. In Table 3, there were significant positive correlations between DAS28-CRP scores and autoantibodies, including IgM anti-HPT78–108 HNE (r = 0.2703, p = 0.0367), IgM anti-IGKC2–19 (r = 0.2816, p = 0.0293), and IgG anti-THRB328–345 (r = 0.2703, p = 0.0367). RF vs. autoantibodies exhibited significant positive correlations, including IgM anti-HPT78–108 HNE (r = 0.6140, p < 0.0001), IgM anti-IGKC2–19 (r = 0.5674, p < 0.0001), IgM anti-IGKC2–19 HNE (r = 0.5404, p < 0.0001), IgG anti-THRB328–345 (r = 0.614, p < 0.0001), IgG anti-THRB328–345 HNE (r = 0.3072, p = 0.019), and IgM anti-THRB328–345 HNE (r = 0.2845, p = 0.0304). Anti-CCP versus autoantibodies exhibited significant positive correlations, including IgG anti-HPT78–108 HNE (r = 0.2782, p = 0.0314) and IgG anti-THRB328–345 HNE (r = 0.2549, p = 0.0494). ESR vs. autoantibodies exhibited significant positive correlations, including IgM anti-IGKC2–19 (r = 0.2692, p = 0.0376), IgM anti-IGKC2–19 HNE (r = 0.2985, p = 0.0205), and IgM anti-THRB328–345 HNE (r = 0.2597, p = 0.0451). Moreover, HNE-protein adduct vs. autoantibodies exhibited significant positive correlations, including IgM anti-IGKC2–19 (r = 0.2667, p = 0.0394) and IgM anti-IGKC2–19 HNE (r = 0.2709, p = 0.0363). However, autoantibodies exhibited significant negative correlations between DAS28-CRP scores, including IgG anti-IGKC2–19 (r = -0.3538, p = 0.0056) and IgG anti-IGKC2–19 HNE (r = -0.3432, p = 0.0073). HNE-protein adducts vs. IgM anti-THRB328–345 (r = -0.2796, p = 0.0305) had a significantly negative correlation. Otherwise, there were no significant correlations between CRP and the other autoantibodies (Table 3).

Associations of serum anti-unmodified and anti-HNE-modified peptide autoantibodies with RA patients compared to HCs

As shown in Table 4, an age adjusted logistic regression analysis demonstrated that ORs of RA development were significantly associated with levels of autoantibodies against unmodified and HNE-modified peptides in patients with RA compared to HCs: IgG anti-CFAH1121–1230 (OR 3.293, p = 0.013, power = 0.874), IgG anti-CFAH1121–1230 HNE (OR 2.808, p < 0.005, power = 0.870), IgM anti-CFAH1121–1230 (OR 5.204, p < 0.001, power = 0.979), IgM anti-CFAH1121–1230 HNE (OR 2.700, p = 0.004, power = 0.838), IgM anti-HPT78–108 (OR = 2.695, p = 0.005, power = 0.841), IgM anti-HPT78–108 HNE (OR 5.235, p < 0.001, power = 0.985), IgG anti-IGKC2–19 (OR 4.679, p < 0.001, power = 0.974), IgM anti-IGKC2–19 (OR 3.206, p < 0.001, power = 0.905), IgM anti-IGKC2–19 (OR 12.655, p < 0.001, power > 0.999), IgM anti-IGKC2–19 HNE (OR 8.095, p < 0.001, power > 0.999), IgG anti-THRB328–345 (OR 5.761, p < 0.001, power = 0.951), IgG anti-THRB328–345 HNE (OR 9.524, p < 0.001, power = 0.962), and IgM anti-THRB328–345 HNE (OR 5.043, p < 0.001, power = 0.992). IgM anti-IGKC2–19 carried the highest risk of RA (Table 4). The OR results were not considered because of power values less than 0.7, including IgG anti-HPT78–108, IgG anti-HPT78–108 HNE, and IgM anti-THRB328–345. Further, HNE-protein adducts (OR 2.413, p = 0.014, power = 0.743) also demonstrated a high risk of RA development (Table 4).

Using serum anti-unmodified and anti-HNE-modified peptide autoantibodies to identify RA patients from HCs and OA patients

Experimental results from feature selection indicated that IgM anti-HPT78–108 HNE (HC vs. RA 0.3496, OA vs. RA 0.3496), IgM anti-IGKC2–19 HNE (HC vs. RA 0.2967), and IgM anti-IGKC2–19 HNE (HC vs. RA 0.2921) showed discriminative power in identifying RA patients from HC and OA patients (Table 5). Predictive performance of decision trees, random forests, and support vector machines based on all 16 autoantibodies and only forward-selected autoantibodies were summarized in Tables 6 and 7, respectively. For both groups (HC vs. RA and OA vs. RA), we observed that using only forward-selected autoantibodies consistently performed better than all autoantibodies, which supports our assumption that feature selection is effective to identify RA. For HC versus RA, the decision tree achieved an AUC of 0.86, random forest achieved AUC of 0.92, and support vector machine achieved an AUC of 0.82. For OA versus RA, decision tree achieved an AUC of 0.84, random forest
|                            | DAS28 | RF   | Anti-CCP | CRP  | ESR  | HNE adduct |
|-----------------------------|-------|------|----------|------|------|------------|
|                            | r     | p value | r     | p value | r     | p value | r     | p value | r     | p value |
| IgG anti-CFAH1121-1230      | 0.1580| 0.2279 | 0.0968 | 0.4696 | 0.0108| 0.9347 | 0.0229| 0.8659 | 0.2076| 0.1115 |
| IgG anti-CFAH1121-1230 HNE |       | -0.0441| 0.7379 | -0.0035| 0.9794| -0.0039| 0.7852| -0.1446| 0.2831| 0.1359| 0.3006 |
| IgM anti-CFAH1121-1230      | 0.0424| 0.7477 | 0.0626 | 0.6406| 0.2100| 0.1073 | -0.0164| 0.9036| 0.0756| 0.5658 |
| IgM anti-CFAH1121-1230 HNE  | 0.0377| 0.7748 | 0.0435| 0.746 | 0.2192| 0.0924 | -0.0821| 0.5435| 0.1343| 0.3063 |
| IgG anti-HPT78-108          | 0.0929| 0.4804 | 0.1386| 0.2996| 0.0659| 0.6169 | 0.1138| 0.3994| 0.1695| 0.1954 |
| IgG anti-HPT78-108 HNE      |       | -0.0266| 0.8400| 0.0195| 0.8848| 0.2782| 0.0314| 0.1081| 0.4236| 0.1346| 0.3052 |
| IgM anti-HPT78-108          |       | -0.0171| 0.8969| 0.1711| 0.1992| 0.0877| 0.5051| -0.0591| 0.6623| 0.0074| 0.9555 |
| IgM anti-HPT78-108 HNE      | 0.2703| 0.0367| 0.6140| < 0.0001| 0.0294| 0.8238| 0.1236| 0.3596| 0.2180| 0.0943 | 0.1836| 0.1603 |
| IgG anti-IGKC2-19           | -0.3538| 0.0056| -0.1144| 0.3926| -0.0023| 0.9862| -0.1061| 0.4320| -0.123| 0.3597| 0.2097| 0.1078 |
| IgG anti-IGKC2-19 HNE       | 0.6023| -0.3432| 0.0073| -0.1997| 0.1328| 0.1253| 0.3399| -0.0524| 0.6986| -0.0328| 0.8035| 0.0686 |
| IgM anti-IGKC2-19           | 0.2816| 0.0293| 0.5674| < 0.0001| -0.0588| 0.6535| 0.0511| 0.7059| 0.2692| 0.0376 | 0.2667| 0.0394 |
| IgM anti-IGKC2-19 HNE       | 0.2487| 0.0553| 0.5404| < 0.0001| 0.2439| 0.0604| 0.0429| 0.7512| 0.2985| 0.0205 | 0.2709| 0.0363 |
| IgG anti-THRB328-345        | 0.2703| 0.0367| 0.6140| < 0.0001| 0.2439| 0.0604| 0.1236| 0.3596| 0.218| 0.0943  | 0.1836| 0.1603 |
| IgG anti-THRB328-345 HNE    | 0.1274| 0.3320| 0.3072| 0.019 | 0.2549| 0.0494| -0.0575| 0.6712| 0.1341| 0.307 | 0.0170| 0.8972 |
| IgM anti-THRB328-345        | -0.0637| 0.6290| 0.2207| 0.096 | -0.0365| 0.7821| -0.0292| 0.8293| -0.1513| 0.2473| -0.2796| 0.0305 |
| IgM anti-THRB328-345 HNE    | 0.0818| 0.5345| 0.2845| 0.0304| -0.0438| 0.7397| 0.081 | 0.549 | 0.2597| 0.0451 | 0.1036| 0.4310 |
achieved an AUC of 0.92, and support vector machine achieved an AUC of 0.88. The ROC plots of these algorithms were presented in Additional file 4: Figure S2. Our results demonstrated that random forest performed better than the other algorithms for predicting RA from HC or OA.

### Discussion

To the best of our knowledge, this is the first study to investigate autoantibodies isotypes against unmodified and HNE-modified peptides, its correlation with activity of disease in Taiwanese women with RA, and associations of risks for RA compared to HCs. However, a critical limitation should be noted due to our samples used in this study were not strictly selected during the disease progression of RA. Therefore, the efficacy of this test may be affected. Levels of HNE-protein adducts in RA patients

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### Table 4 Associations of 4-hydroxy-2-nonenal (HNE)-protein adducts and autoantibody isotypes against unmodified and HNE-modified peptides in rheumatoid arthritis (RA) patients vs. healthy controls (HCs)

| Risk factor                  | Cutoff | HC     | RA     | Age-adjusted logistic regression model<sup>a</sup> | p value   | Power |
|------------------------------|--------|--------|--------|---------------------------------------------------|-----------|-------|
|                              |        | (n)    | (n)    | OR (95% CI)                                       |           |       |
| HNE-protein adduct           | <      | 2.386  | 26     | 1.0 (Ref)                                         | 0.014     | 0.743 |
|                              | >      | 2.386  | 34     | 2.413 (1.193, 4.880)                              | 0.013     | 0.874 |
| IgG anti-CFAH<sup>121−1230</sup> | <      | 0.175  | 14     | 1.0 (Ref)                                         | 0.005     | 0.870 |
|                              | >      | 0.175  | 46     | 3.293 (1.286, 8.431)                              |           |       |
| IgG anti-CFAH<sup>121−1230</sup> HNE | <      | 0.263  | 46     | 1.0 (Ref)                                         | <0.001    | 0.979 |
|                              | >      | 0.263  | 14     | 2.808 (1.363, 5.785)                              |           |       |
| IgM anti-CFAH<sup>121−1230</sup> | <      | 0.609  | 35     | 1.0 (Ref)                                         | <0.001    | 0.997 |
|                              | >      | 0.609  | 25     | 5.204 (2.550, 10.617)                             |           |       |
| IgM anti-CFAH<sup>121−1230</sup> HNE | <      | 0.333  | 39     | 1.0 (Ref)                                         | 0.004     | 0.838 |
|                              | >      | 0.333  | 21     | 2.700 (1.378, 5.289)                              |           |       |
| IgG anti-HPT<sup>78−108</sup> | <      | 0.662  | 35     | 1.0 (Ref)                                         | 0.024     | 0.645 |
|                              | >      | 0.662  | 25     | 2.156 (1.108, 4.193)                              |           |       |
| IgG anti-HPT<sup>78−108</sup> HNE | <      | 0.307  | 41     | 1.0 (Ref)                                         | 0.094     | 0.455 |
|                              | >      | 0.307  | 19     | 1.821 (0.904, 3.672)                              |           |       |
| IgM anti-HPT<sup>78−108</sup> | <      | 0.326  | 40     | 1.0 (Ref)                                         | 0.005     | 0.841 |
|                              | >      | 0.326  | 20     | 2.695 (1.356, 5.354)                              |           |       |
| IgM anti-HPT<sup>78−108</sup> HNE | <      | 0.187  | 39     | 1.0 (Ref)                                         | <0.001    | 0.985 |
|                              | >      | 0.187  | 21     | 5.235 (2.594, 10.656)                             |           |       |
| IgG anti-IGKC<sup>2−19</sup> | <      | 0.392  | 35     | 1.0 (Ref)                                         | <0.001    | 0.974 |
|                              | >      | 0.392  | 25     | 4.679 (2.317, 9.449)                              |           |       |
| IgG anti-IGKC<sup>2−19</sup> HNE | <      | 0.427  | 33     | 1.0 (Ref)                                         | <0.001    | 0.905 |
|                              | >      | 0.427  | 27     | 3.206 (1.621, 6.340)                              |           |       |
| IgM anti-IGKC<sup>2−19</sup> | <      | 0.743  | 56     | 1.0 (Ref)                                         | <0.001    | >0.999 |
|                              | >      | 0.743  | 4      | 12.655 (4.244, 37.734)                             |           |       |
| IgM anti-IGKC<sup>2−19</sup> HNE | <      | 0.738  | 53     | 1.0 (Ref)                                         | <0.001    | >0.999 |
|                              | >      | 0.738  | 7      | 8.095 (3.312, 19.780)                              |           |       |
| IgG anti-THRB<sup>328−345</sup> | <      | 0.154  | 19     | 1.0 (Ref)                                         | <0.001    | 0.951 |
|                              | >      | 0.154  | 41     | 5.761 (2.228, 14.895)                              |           |       |
| IgG anti-THRB<sup>328−345</sup> HNE | <      | 0.281  | 26     | 1.0 (Ref)                                         | <0.001    | 0.962 |
|                              | >      | 0.281  | 34     | 9.524 (3.777, 24.012)                              |           |       |
| IgM anti-THRB<sup>328−345</sup> | <      | 0.791  | 46     | 1.0 (Ref)                                         | 0.029     | 0.697 |
|                              | >      | 0.791  | 14     | 2.249 (1.086, 4.659)                              |           |       |
| IgM anti-THRB<sup>328−345</sup> HNE | <      | 0.682  | 48     | 1.0 (Ref)                                         | <0.001    | 0.992 |
|                              | >      | 0.682  | 12     | 5.043 (2.377, 10.696)                              |           |       |

<sup>a</sup> OR, odds ratio; Ref, reference value
were greater than the levels in HC (Additional file 1: Table S1), which is consistent with results from a previous study [24]. Barrera et al. suggested that HNE-protein adducts also featured a pathogenic contribution of oxidative stress [43]. HNE-protein adducts, OSEs, are recognized as danger signals by innate immune receptors, such as the lectin-like oxidized LDL receptor 1 (LOX1) [44, 45]. HNE-protein adducts (OR 2.413) showed a risk of the alternative complement pathway, is to inhibit the inflammatory response through oxidative stress and to protect host tissues from complement-mediated damage [55–57]. Okroj et al. reported that complement activation would act as damage-associated molecular patterns (DAMPs) that trigger sterile inflammation [46]. Pattern recognition receptors (PRRs) can recognize DAMPs and activate the innate immune response to trigger sterile inflammation [47]. LOX1, a cellular PRR, can recognize and bind to HNE to mediate its uptake and inflammatory effect in atherosclerosis [48]. Macrophages can take up the IgM-NAA-HNE complex by C1q-calreticulin-C91-dependent or mannose-binding lectin (MBL) and MBL receptor-dependent mechanisms in chronic inflammatory diseases and atherosclerosis [49]. Siloşi et al. reported that B-1 cells produce NAAs (IgM > IgG > IgA) and pathogenic autoantibodies (IgG > IgM > IgA) [50]. However, the boundary line between natural immunity and pathogenic autoimmunity is unclear [50]. Further, IgM-NAAs can control IgG-NAA activity and regulate expression of natural IgG autoreactive repertoire by F(ab′)2 fragments of IgG-NAAs in human and mice serum [51, 52]. Chen et al. proved that inhibition of Toll-like receptor (TLR) and IgG-immune complex-mediated inflammatory responses mediate anti-inflammatory features of IgM-NAAs [53]. Moreover, IgG-NAAs may be involved in autoimmune disease pathogenesis, including SLE, Sjögren’s syndrome, and Graves’ disease [50, 54], and anti-OSE NAAs themselves may have a protective effect. Further, we deduced that elevated levels of anti-OSE NAAs may be a risk index of RA development based on protein function and disease activity when oxidative stress occurs over a sustained period in patients with RA (Additional file 1: Table S1, Table 4). In this study, four different novel HNE-modified peptide adducts were identified: CFAH[121–1230], HPT[78–108], IGKC[2–19], and THRB[328–345] (Table 1, Fig. 1).

The biological function of CFAH, a soluble inhibitor of the alternative complement pathway, is to inhibit the inflammatory response through oxidative stress and to protect host tissues from complement-mediated damage [55–57]. Okroj et al. reported that complement activation

Table 5 Feature importance ranking of 4-hydroxy-2-nonalen (HNE)-protein adducts and autoantibody isotypes against unmodified and HNE-modified peptides in rheumatoid arthritis (RA) patients versus healthy controls (HCs) and osteoarthritis (OA) patients

| Score | Feature                      | Score | Feature                      |
|-------|------------------------------|-------|------------------------------|
| 0.3496 | IgM anti-HPT[78–108] HNE     | 0.352 | IgM anti-HPT[78–108] HNE     |
| 0.2967 | IgM anti-IGKC[2–19]          | 0.245 | IgM anti-IGKC[2–19]          |
| 0.2921 | IgM anti-IGKC[2–19] HNE      | 0.223 | IgM anti-IGKC[2–19] HNE      |
| 0.1689 | IgG anti-THRB                | 0.186 | IgG anti-HPT[78–108] HNE     |
| 0.1395 | IgG anti-THRB[328–345] HNE   | 0.172 | IgG anti-CFAH[121–1230]      |
| 0.1382 | IgG anti-THRB[328–345] HNE   | 0.139 | HNE adducts                  |
| 0.1312 | IgG anti-THRB[78–108] HNE    | 0.134 | IgG anti-THRB[328–345] HNE   |
| 0.1186 | IgG anti-IGKC[2–19]          | 0.128 | IgG anti-CFAH[121–1230] HNE  |
| 0.1123 | IgG anti-CFAH[121–1230]      | 0.126 | IgG anti-HPT[78–108]         |
| 0.1108 | IgG anti-CFAH[121–1230]      | 0.114 | IgG anti-THRB[328–345]       |
| 0.1007 | IgG anti-HPT[78–108]         | 0.0994| HNE adducts                  |
| 0.0980 | IgG anti-CFAH[121–1230]      |       | HNE adducts                  |

Table 6 Predictive performance using all 16 features in decision trees, random forests, and support vector machines for healthy controls (HCs) versus rheumatoid arthritis (RA) patients and osteoarthritis (OA) versus RA patients

| Algorithms* | Accuracy | Precision | F1 score | Sensitivity | Specificity | AUC  |
|-------------|----------|-----------|----------|-------------|-------------|------|
| HC v.s. RA  |          |           |          |             |             |      |
| DT          | 0.75 (0.70–0.79) | 0.77 (0.69–0.86) | 0.69 (0.63–0.75) | 0.62 (0.56–0.69) | 0.85 (0.81–0.90) | 0.74 (0.69–0.78) |
| RF          | 0.80 (0.79–0.82) | 0.83 (0.81–0.85) | 0.79 (0.77–0.80) | 0.76 (0.74–0.79) | 0.84 (0.82–0.87) | 0.88 (0.87–0.89) |
| SVM         | 0.71 (0.64–0.78) | 0.90 (0.78–1.00) | 0.62 (0.51–0.73) | 0.49 (0.37–0.62) | 0.94 (0.89–1.00) | 0.86 (0.78–0.93) |
| OA v.s. RA  |          |           |          |             |             |      |
| DT          | 0.78 (0.73–0.83) | 0.90 (0.85–0.95) | 0.77 (0.70–0.84) | 0.68 (0.58–0.78) | 0.89 (0.82–0.96) | 0.79 (0.74–0.83) |
| RF          | 0.80 (0.75–0.85) | 0.84 (0.78–0.90) | 0.84 (0.80–0.88) | 0.86 (0.79–0.92) | 0.68 (0.52–0.85) | 0.87 (0.82–0.92) |
| SVM         | 0.66 (0.56–0.70) | 0.66 (0.56–0.70) | 0.79 (0.72–0.82) | 0.47 (0.33–0.60) | 0.93 (0.88–0.99) | 0.82 (0.80–0.85) |

*DT, decision trees; RF, random forests; SVM, support vector machines
Table 7 Improved predictive performance using only selected features in decision trees, random forests, and support vector machines for healthy controls (HCs) versus rheumatoid arthritis (RA) patients and osteoarthritis (OA) versus RA patients

| Algorithm* | Accuracy | Precision | F1 score | Sensitivity | Specificity | AUC | Selected feature |
|------------|----------|-----------|----------|-------------|-------------|-----|------------------|
| **HC v.s. RA** |          |           |          |             |             |     |                  |
| DT         | 0.84 (0.80–0.87) | 0.92 (0.88–0.95) | 0.80 (0.76–0.85) | 0.73 (0.67–0.80) | 0.93 (0.89–0.97) | 0.86 (0.82–0.89) | IgM anti-HPT78–108 HNE IgG anti-THRB128–345 |
| RF         | 0.87 (0.85–0.90) | 0.91 (0.87–0.95) | 0.85 (0.82–0.88) | 0.81 (0.76–0.87) | 0.93 (0.91–0.96) | 0.92 (0.90–0.95) | IgM anti-HPT78–108 HNE IgM anti-HPT78–108 IgG anti-HPT78–108 IgG anti-THRB128–345 HNE adducts |
| SVM        | 0.71 (0.62–0.80) | 0.97 (0.91–1.00) | 0.56 (0.42–0.70) | 0.42 (0.27–0.58) | 0.98 (0.95–1.00) | 0.82 (0.75–0.89) | IgM anti-HPT78–108 HNE IgG anti-HPT78–108 HNE IgM anti-IGKC2–19 IgM anti-CFAH1121–1230 HNE |
| **OA v.s. RA** |          |           |          |             |             |     |                  |
| DT         | 0.81 (0.75–0.87) | 0.94 (0.89–0.98) | 0.82 (0.76–0.89) | 0.74 (0.62–0.83) | 0.93 (0.88–0.98) | 0.84 (0.79–0.89) | IgM anti-HPT78–108 HNE IgG anti-THRB128–345 IgG anti-IGKC2–19 |
| RF         | 0.85 (0.81–0.89) | 0.87 (0.82–0.91) | 0.87 (0.82–0.92) | 0.88 (0.79–0.97) | 0.79 (0.71–0.87) | 0.92 (0.88–0.96) | IgM anti-HPT78–108 HNE IgG anti-HPT78–108 HNE IgG anti-IGKC2–19 IgM anti-CFAH1121–1230 HNE |
| SVM        | 0.68 (0.61–0.76) | 0.68 (0.61–0.76) | 0.81 (0.75–0.86) | 0.53 (0.31–0.66) | 0.94 (0.89–0.98) | 0.88 (0.83–0.93) | IgM anti-HPT78–108 HNE IgG anti-THRB128–345 |

*DT, decision trees; RF, random forests; SVM, support vector machines

contributes to the pathological process of RA [58]. The complement system is a central innate immune system that participates in eliminating pathogens and promotes inflammatory responses [55]. Weismann et al. indicated that CFAH is able to bind MDA, and as an MDA-binding protein, it blocks the proinflammatory effects that induced by MDA in vivo in mice [59]. Moreover, it was reported that the HNE modification was unbound by CFAH [59]. However, we found that CFAH was modified with HNE at K1230 in RA patients (Table 1, Additional file 2: Figure S1C). Trojná et al. identified three linear epitopes on serum CFAH (CFAH1137–1171, CFAH1177–1191, and CFAH1207–1226) in atypical hemolytic uremic syndrome (aHUS) [60]. Interestingly, CFAH1211–1230 is also an autoantigen in RA (Fig. 3a, c). The HNE-modified CFAH1211–1230 peptide can enhance autoantibody levels in patients with RA compared to patients with OA and HCs (Fig. 3b, d). Several studies demonstrated that aHUS was associated with the presence of autoantibodies against CFAH [56, 57]. Autoantibodies against CFAH are also present in significant ratios in RA [57]. Insufficient inhibition of CFAH activity may be caused by pathology-associated autoantibodies [56, 58]. Thus, high autoantibody titers against CFAH are not specific to RA, but may be important for pathologic processes in RA. However, IgG anti-CFAH1121–1230, IgG anti-CFAH1121–1230 HNE, IgM anti-CFAH1121–1230, and IgM anti-CFAH1121–1230 HNE were not significantly correlated with DAS28-CRP, RF, CRP, ESR, or HNE-protein adducts, respectively (Table 3); but, IgG anti-CFAH1121–1230 (OR 3.293), IgG anti-CFAH1121–1230 HNE (OR 2.808), IgM anti-CFAH1121–1230 (OR 5.204), and IgM anti-CFAH1121–1230 HNE (OR 2.700) showed risks for RA development (Table 4).

HPT is a hemoglobin-binding protein that can prevent oxidative damage to organs and participates in activating innate and adaptive immune responses [61]. Increased synovial fluid (SF) and serum HPT levels found in RA patients were associated with inflammation and tissue destruction [62]. Yildirim et al. indicated that serum HPT was an acute-phase protein and significantly correlated with disease activity in patients with RA [63]. In this study, we identified one novel HNE modification at C92 on HPT78–108 in RA patients (Table 1, Additional file 2: Figure S1D). Korngold indicated that the HPT-anti-HPT reaction can block HPT-hemoglobin-binding action [64]. Muta et al. reported that the level of the anti-HPT antibody in serum increased and the level of HPT decreased after febrile non-hemolytic transfusion reactions (FNHTRs) [3]. In this study, higher levels of HPT in serum were greatly 1.24-fold higher (p = 0.041) in RA patients than in HCs (Fig. 2b). IgG and IgM against HPT78–108 and HPT78–108 HNE were greatly higher in RA patients than in HCs (Fig. 3e–h). Thus, we inferred that high levels of autoantibodies against HPT may inhibit HPT’s function and play a role in the risk of developing...
of thrombin activity in SF via strong mitogenic activity toward synovial fibroblast-like cells play a significant role in the RA pathogenesis [71]. Yang et al. reported that anti-THRB autoantibodies can display prothrombinase activity and contribute to thrombosis in anti-phospholipid syndrome (APS) and SLE [72]. In this study, novel HNE modifications at K344 on THRB 328–345 in RA patients were identified (Table 1, Additional file 2: Figure S1F). Next, levels of IgG and IgM against THRB 328–345 and THRB 328–345 HNE were greatly higher in patients with RA than in HCs (Fig. 3m–p). Thus, high levels of anti-THRB autoantibodies may be considered as a risk factor for RA. Interestingly, IgG anti-THRB 328–345 was greatly positively correlated with DAS28-CRP (r = 0.2703) and RF (r = 0.6140), IgG anti-THRB 328–345 HNE was significantly positively correlated with RF (r = 0.3072) and anti-CCP (r = 0.2549), IgM anti-THRB 328–345 HNE was greatly positively correlated with RF (r = 0.2845) and ESR (r = 0.2597), but IgM anti-THRB 328–345 was significantly negatively correlated with the HNE-protein adduct (r = −0.2796) as shown in Table 3. Further, IgG anti-THRB 328–345 (OR 5.761), IgG anti-THRB 328–345 HNE (OR 9.542), and IgM anti-THRB 328–345 HNE (OR 5.043) exhibited risks for RA development (Table 4).

Several previous studies showed the feasibility in early diagnosis of autoimmune diseases using a machine learning application for RA. Rodrigo Torres et al. suggested that feature selection can be a powerful tool in biomarker discovery [73]. Therefore, we believe that with appropriate proteomic data and machine learning algorithms, the biomarker candidates we developed can be optimized into a set of highly accurate features. In our experiment, we incorporated feature ranking and a forward selection method to identify IgM anti-HPT 78–108 and HNE-protein adducts that can identify RA from HC combined with random forest algorithm. We then compared our results with the accuracy of anti-CCP which was considered a well performance biomarker in RA. Other studies reported that the sensitivity and specificity of anti-CCP were 63% ~ 91.4% and 69.7% ~ 97.6%, respectively [74–77]. However, we found only 50% of positive anti-CCP in RA samples. Moreover, consistency was observed between statistical analyses of odds ratio and prediction results of classification. Our results from statistical models, feature selection, and machine learning classifiers supported that IgM anti-HPT 78–108 HNE, IgM anti-IGKC 2–19, and IgM anti-IGKC 2–19 HNE showed potential to be developed as biomarkers for RA.
Conclusions

In the present study, we found that some IgG- and IgM-NAAs and anti-HNE IgM-NAAs may be correlated with activity of disease and inflammation in RA. We concluded that increased levels of IgM anti-HPT78–108 HNE, IgM anti-IGKC2–19, and IgM anti-IGKC2–19 HNE in serum can be used as diagnostic biomarkers of RA, and high levels of IgM anti-HPT78–108 HNE, IgM anti-IGKC2–19, and IgG anti-THRB328–345 are related to increased disease activity during RA development.

Supplementary information

The online version contains supplementary material available at https://doi.org/10.1186/s12191-020-01380-y.

Additional file 1: Table S1. Demographic and clinical characteristics of individual subjects contributing to serum for healthy controls (HCs), and patients with osteoarthritis (OA) and rheumatoid arthritis (RA).

Additional file 2: Fig. S1. Differential 4-hydroxy-2-nonal (HNE)-modified peptide adducts were re-analyzed through PEAKS 7 using previous MS/MS spectra (ProteomeXchange: PXD004546). Acquired MS/MS spectra were obtained through pooled concanavalin (Con) A-captured serum proteins (nine rheumatoid arthritis (RA) and nine healthy control (HC) pooled samples), 1-D SDS-PAGE, in-gel digestion, and nano-LC-MS/MS (A). HNE reacts with amino acid residues of proteins to form HNE-protein adducts by Michael addition and Schiff base adducts, respectively (B). Representative MS/MS spectrum of the 1211-SHTLRTTCWDGKLEYPTCAK-1230 peptide sequence and the modified peptide bearing an HNE modification at the K1230 residue in RA patients (C). A representative MS/MS spectrum of the peptide sequence 78-AVGDQKPEEADEDCGPCKPPEAHDGTVREH SVR-108 and the modified peptide bearing the HNE modification at the C92 residue in RA patients (D). The MS/MS spectrum 2-TVAAPSFVFPSPDSEQK-19 and the modified peptide bearing the HNE modification at the AS residue in RA patients (E, upper panel); 2-TVAAPSFVFPSPDSEQK-19 and the modified peptide bearing the HNE modification at the A4 residues in RA patients and HCs (E, bottom panel). Representative MS/MS spectrum of 328-TFGSGEADGCLRPFLFKK-345 and the modified peptide bearing the HNE modification at the K345 residue in RA patients (F). The MS/MS spectrum 284-HRTGDEITYQCRNGFYPATRGNTAK-308 and the modified peptide bearing the HNE modification at the K308 residue in HCs (G). A representative MS/MS spectrum of the peptide sequence 162-ILGGLHD1K-170 and the modified peptide bearing the HNE modification at the A169 residues in HCs (H). Representative MS/MS spectrum of 83-VYACEVTHQGLSSPVTKSFKR-93 and the modified peptide bearing the HNE modification at the C336 and L341 residues in HCs (J).

Additional file 3. Supplementary methods.

Additional file 4: Fig. S2. Comparison of receiver operating characteristic (ROC) curves from unselected features and selected features in (A) decision tree, (B) random forest classifier, and (C) support vector machine classifier.

Additional file 5: Table S2. Sequences of unmodified and 4-hydroxy-2-nonal (HNE)-modified peptides.

Abbreviations

1-D SDS-PAGE: One-dimensional sodium dodecylsulfate polyacrylamide gel electrophoresis; ACR: American College of Rheumatology; AD: Alzheimer’s disease; aHUS: Atypical hemolytic uremic syndrome; ALD: Alcoholic liver disease; AUC: Area under the receiver operating characteristic curve; CBB: Coomassie brilliant blue; CD: Cardiovascular disease; CFH: Complement factor H; Con A: Concanavalin A; COPD: Chronic obstructive pulmonary disease; CRP: C-reactive protein; DAMP: Damage-associated molecular pattern; DAS28-CRP: Disease activity score in 28 joints; DM: Diabetes mellitus; DMARD: Disease-modifying anti-rheumatic drug; ELISA: Enzyme-linked immunosorbent assay; ESR: Erythrocyte sedimentation rate; FDR: False detection rate; HC: Healthy control; HNE: 4-Hydroxy-2-nonal; IgM: Immunoglobulin M; IGKC: Immunoglobulin kappa chain; IA: Immuno precipitation; IMB: Mannose-binding lectin; MCI: Mild cognitive impairment; MKHD: Menisk kinked hair disease; MDA: Malondialdehyde; NAA: Natural autoantibody; nLC-MS/MS: Nano-liquid chromatography tandem mass spectrometry; NSAID: Non-steroidal anti-inflammatory drug; OSE: Oxidation-specific epitope; PRR: Pattern recognition receptor; PTM: Post-translational modification; RF: Rheumatoid factor; Anti-CCP: Anti-cyclic citrullinated peptide; ROC: Receiver operating characteristic; SF: Synovial fluid; SLE: System lupus erythematosus; THRB: Prothrombin.

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Authors’ contributions

CYL, CCC, YSC, KLT, SHL, and CYL conceived and designed the experiments; 2-TVAAPSFVFPSPDSEQK-19 and the modified peptide bearing the HNE modification at the A4 residues in RA patients; 2-TVAAPSFVFPSPDSEQK-19 and the modified peptide bearing the HNE modification at the A4 residues in RA patients; 2-TVAAPSFVFPSPDSEQK-19 and the modified peptide bearing the HNE modification at the A4 residues in RA patients (E, upper panel); 2-TVAAPSFVFPSPDSEQK-19 and the modified peptide bearing the HNE modification at the A4 residues in RA patients (E, bottom panel). Representative MS/MS spectrum of 83-VYACEVTHQGLSSPVTKSFKR-93 and the modified peptide bearing the HNE modification at the C336 and L341 residues in HCs (J).
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