ンスリン抵抗性，5以上の場合は高度のインスリン抵抗性と考えられる．脂肪肝の程度が進行するほどHOMA-IRが高値となり，血清ALT値はBMI，HOMA-IRと有意に正の相関を示し，ALTが40U/l以上の例の大半がBMIが25以上，あるいはHOMA-IRが2以上である[5]．また，NASHの肝線維化の進行はインスリン抵抗性と関連があるといわれている[3]．このようなことから，インスリン抵抗性の存在はNASHを疑う所見の一つである．

2. 高感度CRP（high-sensitivity C-reactive protein: hsCRP）

高感度CRP測定法の開発により，従来のCRPでは検出されない慢性的で微小な炎症を評価できるようになった．hsCRPレベルの上昇は，心血管疾患，2型糖尿病，メタボリックシンドローム発症の予測因子であることが証明されている[6]．また，メタボリックシンドロームの一表現形といわれるNAFLDではhsCRPの上昇が脂肪肝に伴う炎症を反映していると考えられている．さらにNASHにおいてhsCRPの上昇と肝線維化の程度には相関があるといわれ[1]，メタボリックシンドロームを有しないNAFLDの血清ALT高値群と正常群の比較では，有意にALT高値群のhsCRPが高値であるという報告[5]もあり，今後NASHの新しいマーカーとなることが期待される（図5-2）．

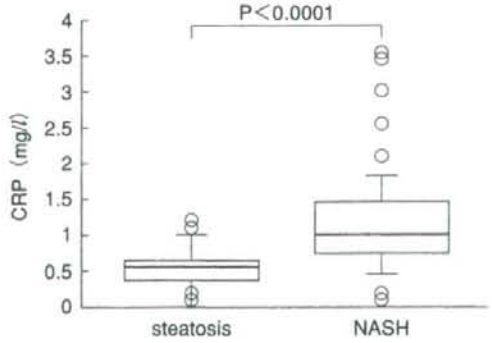

図5-2　NASHと単純性脂肪肝におけるhsCRP濃度（文献1を改変）

3. 酸化ストレスマーカー

ミトコンドリアの呼吸鎖複合体はROSを産生し，通常は速やかに消去されるが，NASHではミトコンドリア異常によりROS過剰になると考えられている．また，肝細胞の過剰な脂肪酸はミトコンドリアのβ酸化を亢進させ，CYP2E1の過剰発現を誘導する．さらに，NASH患者の肝組織中の鉄沈着レベルは単純性脂肪肝より高値である．このような病態はNASHにおいて酸化ストレスを誘導すると考えられる．

酸化ストレスマーカーには，活性酸素による修飾化合物と活性酸素消去系酵素や抗酸化物質がある[7]．heat shock protein（HSP）やチオレドキシン thioredoxin（TRX）などは酸化ストレスによって誘導される蛋白質であり，チオレドキシンは抗酸化物質としても知られている．NAFLDでは健常者と比較して，肝組織中のmalondialdehyde（MDA）など酸化ストレスマーカーは発現が高く，NAFLDの中でも単純性脂肪肝よりNASHのほうが，肝組織中MDAや血

清中チオレドキシンが高い（図5-3）[8]．このように，NASHの病態形成に酸化ストレスが強く関与すると考えられ，酸化ストレスマーカーはNASHの診断や病態進展予測に役立つ．特に，チオレドキシンは血清中の濃度を測定できることから，診断マーカーとしての有用性が期待できる．

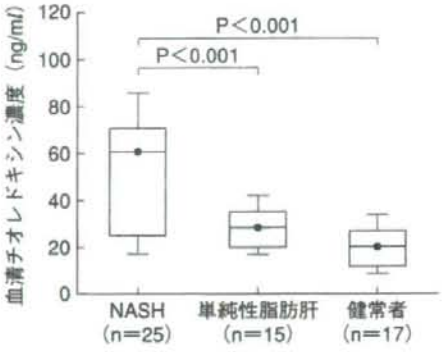

図5-3　**NASH，単純性脂肪肝と健常者における血清チオレドキシン濃度**（文献8を改変）

4. アディポサイトカイン

脂肪組織は単なる余剰エネルギーの蓄積臓器であるだけでなく，非常に多くの生理活性物質を分泌する組織である．このような脂肪組織由来生理活性物質は総称してアディポサイトカインとよばれる．代表的なアディポサイトカインとして，TNF-α，レプチン，アディポネクチン，PAI-1（plasminogen activator inhibitor type1），レジスチンなどがあげられるが，NAFLDの中でも特にNASHではこれらの分泌異常があるといわれる．

TNF-αは炎症性サイトカインの一つであるが，脂肪組織においても発現している．肥満により血中TNF-αは上昇し，脂肪組織におけるTNF-αの発現は肥満度と相関し，NASHでは血中TNF-αの上昇や肝でのTNF-α受容体の増加が認められる[9,10]．

アディポネクチンはメタボリックシンドローム発症に抑制的に働き，肥満によりその血中濃度は低下し，内臓脂肪量と強い逆相関を示す．骨格筋や肝臓においてAMP kinaseやPPARαを活性化し，インスリン抵抗性やメタボリックシンドロームを改善する作用をもつ．NAFLD患者では血中アディポネクチン濃度は低下している[11]．また，インスリン抵抗性改善薬ピオグリタゾンは，PPARγを介して脂肪細胞を小型化し，アディポネクチン産生低下を回復することで

MEMO

プロテオミクス

プロテオーム（proteome＝protein＋ome）とは1個の生物がもつすべての遺伝情報であるゲノム（gene＋ome）に相応した用語であり，その生物や細胞に発現しているすべての蛋白質の集合体のことである．また，網羅的に蛋白質の発現や性質を研究することを，プロテオーム解析もしくはプロテオミクス proteomicsと表現する．プロテオーム解析は(1)蛋白質の分離，(2)蛋白質の同定の2つの作業からなる．最も広く行われているのは，二次元電気泳動によって蛋白質を分離し，質量分析で目的の蛋白質を同定する方法である．質量分析には液体クロマトグラフィーと一体となったLC-MSと蛋白質の質量と飛行時間の関係を利用したTOF-MSがある．

NASH 患者に有効であると考えられている．このようなことから，アディポネクチンは診断だけでなく，アディポネクチンを用いた治療やピオグリタゾンなどの治療薬の効果のモニタリングにも応用できる可能性がある．

レプチンは中枢神経系に作用して強力な食欲抑制作用を示すほか，インスリン効果増強作用，エネルギー消費亢進，血圧上昇作用などさまざまな生理作用を示す．体脂肪量が増加するとレプチン濃度が上昇し，食欲を低下させるとともにエネルギーの消費を高めるが，肥満患者ではレプチン抵抗性の状態と考えられ，その機構が破綻した状況となっている．また，レプチンは肝線維化と関連する肝星細胞の活性化を促進することが報告されている[9,12]．このように，血中レプチン濃度は NASH では上昇し，診断に有用である可能性がある．

D. 血液検査の next approach

NAFLD や NASH のスクリーニングのための血液検査は簡便に測定できる．しかし，NASH を疑う根拠となるような検査は保険外診療で測定しなければならない検査項目も多く，必ずしも NAFLD もしくは NASH に特異的なものではなく，NASH の確定診断には肝生検が必要である．すなわち，簡便に測定でき，臨床の場で有用な NASH の特異的な血清診断マーカーはない．

血清 cytokeratin-18（CK-18）断片濃度は NAFLD における肝細胞のアポトーシスと関連して増加し[13]，男性ホルモンの中間代謝産物であるデヒドロエピアンドロステロンサルフェート（DHEA-S）は NAFLD の肝線維化の進行とともに低下する[14]．さらに，いくつかのマーカーを組み合わせた早期 NASH の診断法も報告されるようになってきている（表 5-3）[15]．また，プロ

表 5-3　**アディポネクチン，HOMA-IR，Ⅳ型コラーゲン 7S，およびマーカーの組み合わせを用いた線維化軽度（Stage1-2）NASH の診断率**

parameter	cut-off Value	AUC	感度	特異度
adiponectin	≦4.0（μg/ml）	0.765	68	79
HOMA-IR	≧3.0	0.757	51	95
Type Ⅳ Col.7S	≦5.0（ng/ml）	0.758	41	95
combination of markers			94	74

（文献 15 を改変）

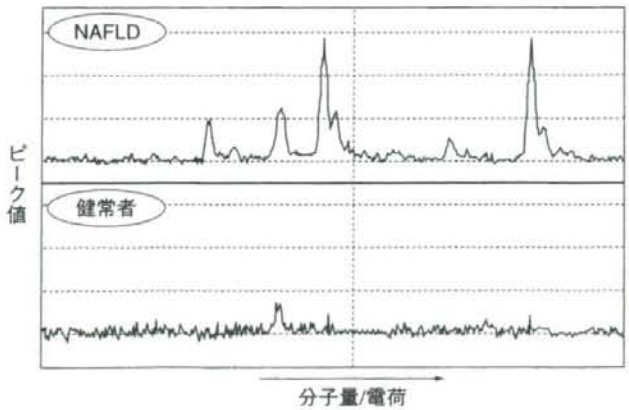

図 5-4　**SELDI TOF/MS プロテインチップシステムを用いた NAFLD 患者血清の蛋白発現パターン**

テオミクスなどを用いたバイオマーカー探索が悪性疾患を中心に広く行われるようになってきているが，NAFLD や NASH にも応用可能あると考えられ[16, 17]，今後新しいバイオマーカーの発見が期待されている．我々も，プロテオミクスを用いて，健常者に比較して NAFLD で高値を示し，NAFLD のバイオマーカーとなる可能性がある蛋白ピークをみいだしており（図 5-4），今後の研究の発展が期待できる．

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NASH/NAFLDとメタボリックシンドローム

NASH and NAFLD in Metabolic Syndrome

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Key Words:
NAFLD, NASH, metabolic syndrome, Life-style related Disease

Abstract

食習慣の欧米化や運動不足から，生活習慣病とその複合病態であるメタボリックシンドロームの増加が危惧されている。非アルコール性脂肪性肝疾患（NAFLD）は生活習慣病の肝病変と考えられ，肝機能異常や脂肪肝を指摘される健診受診者も増加していることから，NAFLDが注目されている。NAFLDは単純性脂肪肝と非アルコール性脂肪肝炎（NASH）に大別され，後者は肝硬変や肝癌のリスクがある。NASHを予測する簡便なバイオマーカーはなく，その診断には肝の組織診断が必須であるが，脂肪肝の頻度が高いこと，肝生検が侵襲的であることから，簡便かつ迅速に測定できる診断マーカーの同定が急務である。NASHはインスリン抵抗性を基盤とし，アディポネクチン低下やTNF-αの上昇がみられ，NASHの病態進展には内臓脂肪型肥満や生活習慣病が密接に関係している。NASHの治療薬としてインスリン抵抗性改善薬などが試みられており，NAFLD/NASHの治療には生活習慣病の改善が極めて肝要である。

■はじめに

非アルコール性脂肪性肝疾患(nonalcoholic fatty liver disease: NAFLD)とは，飲酒量が1日エタノール換算で20g以下で脂肪肝を呈するものであり，アルコール性脂肪肝や各種ウイルス性肝炎及び自己免疫性肝疾患は除外される。NAFLDは単純性脂肪肝(simple steatosis)と非アルコール性脂肪肝炎(nonalcoholic steatohepatitis: NASH)の2つに分けられが，臨床的に重要な疾患は肝硬変や肝癌に進行するNASHである。しかし，単純性脂肪肝はNASHに進展する可能性もあり，単純性脂肪肝でも定期的な経過観察が必要である。臨床的には生化学検査や画像所見でNAFLDと診断されることが多いが，NAFLDやNASHの確定診断には肝組織所見が不可欠である。また，進行したNASHでは画像所見や組織所見で典型的な脂肪肝を認めないこともあり，注意が必要である。

NASHの発症機序としてDayらが提唱したtwo hit theoryが広く知られている。すなわち，遺伝，肥満，高脂血症，糖尿病などの原因(first hit)で脂肪肝となり，インスリン抵抗性や酸化ストレス，エンドトキシン等の原因(second hit)が重なりNASHとなる。このtwo hit theoryで提唱されている原因の多くはメタボリックシンドロームに大きく関わっていることからNASH/NAFLDはメタボリックシンドロームの肝病変と考えられている。本稿ではNASH/NAFLDとメタボリックシンドロームの関連について最近の知見を紹介する。

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表1　肥満の診断基準

BMI値	日本肥満学会基準	WHO基準
BMI<18.5	低体重	Underweight
18.5≦BMI<25.0	普通体重	Normal range
25.0≦BMI<30.0	肥満（1度）	Preobese
30.0≦BMI<35.0	肥満（2度）	Obese I
35.0≦BMI<40.0	肥満（3度）	Obese II
40.0≦BMI	肥満（4度）	Obese III

（文献1から引用改変）

表2　肥満に起因ないしは関連して発症する健康障害のうち脂肪細胞の質的異常による疾患

1．耐糖能障害・2型糖尿病
2．脂質代謝異常
　高コレステロール血症
　低HDLコレステロール血症
　高トリグリセリド血症
3．高血圧
4．高尿酸血症・痛風
5．脂肪肝
　非アルコール性脂肪性肝疾患（NAFLD）
　（非アルコール性脂肪肝炎；NASHを含む
6．冠動脈疾患
　心筋梗塞
　狭心症
7．脳梗塞
　脳血栓症
　一過性脳虚血発作

（文献1から引用改変）

■肥満とNASH/NAFLD

肥満の評価はBMI〔(体重kg)/(身長m)^2〕が用いられており，BMIが22で有病率が最小になることから，これを基準に理想体重が算出される。日本肥満学会基準(肥満症治療ガイドライン2006)では18.5以上25未満が標準，25以上30未満が肥満(1度)，30以上が肥満2～4度となる（表1）[1]。日本成人15万人を対象とした研究ではBMIが25～28の1度の肥満でも耐糖能異常，2型糖尿病，高血圧，高中性脂肪血症，高コレステロール血症，低HDLコレステロール血症などを発症する危険率が標準体重群の2倍に増加すると報告されている。また，肥満は内臓脂肪型肥満と皮下脂肪型肥満の2種類に分けられるが，腹腔内に存在する脂肪の蓄積が皮下脂肪の蓄積よりも動脈硬化性疾患のリスクとなり，日本人の肥満は前者が多いことが指摘されている。

NASHを含む脂肪肝は肥満に起因ないし関連して発症する健康障害の一つである（表2）[1]。平成17年国民健康・栄養調査では成人の肥満人口(BMI≧25)の比率は，男性では40歳代，女性では閉経後の60歳代が最も高く，それぞれ34.1%，29.0%であった。また，非アルコール性かつ非ウイルス性肝障害の約80%に肥満が合併している。さらに，脂肪肝は，肥満1度では50%，肥満2度以上であれば75%にみられる。NASHの頻度はNAFLDの約10%，成人の0.5～1%程度と言われているが，今後肥満の増加とともに，NAFLDやNASHの頻度もさらに増加すると考えられる[2]。

■インスリン抵抗性とNASH/NAFLD

NASH/NAFLDは内臓脂肪蓄積に伴うインスリン抵抗性を基盤とする病態である。内臓脂肪からはTNF-α，アディポネクチン，レプチン，アンジオテンシノーゲンなどのアディポサイトカインと総称される生理活性物質が分泌され，門脈血流を介してインスリン抵抗性に影響する。アディポネクチンは内臓脂肪増加と逆相関して血中濃度が低下し，肝臓および骨格筋のインスリン感受性を低下させ，TNF-α，レプチン，アンジオテンシノーゲンはインスリン抵抗性を亢進させる。NASH症例の77%がアディポネクチン 10μg/ml以下，かつインスリン抵抗性を表わすHOMA-IRが3以上と高値であるのに対し，単純性脂肪肝症例では33%のみであることから，アディポネクチン減少はNASHの指標となる[3]。内臓脂肪量もNASHの予測因子とされ，TNF-αは内臓脂肪量に相関し，脂肪組織に浸潤した炎症細胞からも分泌され，TNF-αはNASHの繊維化を促進させる因子の一つである[4]。このようにアディポサイトカインはNASHの発症や進展に関与し，それに伴い誘導されるインスリン抵抗性はNASH/NAFLDの基本病態と考えられる。さらに，PPAR(peroxisome proliferators-activated

receptor) γ agonistであるチアゾリジン誘導体やビグアナイド薬はインスリン抵抗性を改善させることでNASHの肝障害を改善させる。

■脂質代謝異常とNASH/NAFLD

血清中性脂肪が200mg/dl以上の高値もしくはHDLコレステロールが35mg/dl未満の低値であれば，NAFLDの危険性が上昇することが報告されている。内臓脂肪蓄積に伴いインスリン抵抗性が惹起された結果，中性脂肪分泌が亢進する。脂肪細胞で中性脂肪が分解され，遊離脂肪酸が増加し肝臓へ流入する。また高インスリン血症に伴う肝での脂肪酸合成の促進，あるいは肝臓内でのβ酸化の低下などにより肝に脂肪が蓄積し脂肪肝が進行していく[5]。

フィブラート系薬剤はPPAR-α agonistであり，脂肪酸の燃焼を促し組織内の中性脂肪量を減少させることで脂肪肝を改善させる可能性がある[2]。また，スタチン系薬剤はHMG-CoA還元酵素阻害によるコレステロール合成抑制作用の他，レニン・アンギオテンシン系抑制作用，TNF-αなどのサイトカイン発現抑制作用などを介してNASHにおける肝機能改善及び肝脂肪化改善作用が期待されている。

■高血圧とNASH/NAFLD

NASH/NAFLDの病態の基盤であるインスリン抵抗性は，高血圧の増悪因子でもある。インスリン抵抗性が血圧上昇に関与する機序として，腎におけるNa貯留作用，交感神経亢進作用，レニン・アンギオテンシン系活性亢進作用，血管抵抗増加作用などが考えられる。また，NASH/NAFLDの病態形成には酸化ストレスが関与しているが，酸化ストレス亢進により血管内皮細胞から産生される一酸化窒素(NO)が減少し，NOが血管収縮あるいは交感神経刺激を誘導することで血圧を上昇させる[6]。一方，高血圧患者とくに肥満合併例ではレニン・アンギオテンシン系が亢進し，その中でもアンギオテンシンⅡはインスリン抵抗性を惹起し，鉄の過剰沈着，酸化ストレス増強などを引き起こす。このようにNASH/NAFLDと高血圧は密接に関連している。

さらに，降圧剤であるアンギオテンシン変換酵素阻害剤やアンギオテンシンⅡ 1型受容体拮抗薬は肝星細胞の活性化を抑制し，NASHの肝線維化を改善する可能性がある。このように，NASH/NAFLDに対しては降圧効果を介した心・血管イベントの抑制に加えて，直接もしくは間接的にNASHの進行も抑制することが期待出来る。

表3　日本の8学会合同の診断基準準備委員会によるメタボリックシンドロームの診断基準（文献[7]を引用）

腹腔内脂肪蓄積 ・ウエスト周囲径	（内臓脂肪面積　男女とも≧100cm²に相当） 男性≧85cm　女性≧90cm
上記に加え以下のうち2項目以上	
1）高トリグリセライド血症 かつ/または 低HDLコレステロール血症	≧150mg/dl <40mg/dl
2）収縮期血圧 かつ/または 拡張期血圧	≧130mm/Hg ≧85mm/Hg
3）空腹時高血糖	≧110mg/dl

・CTスキャンなどで内臓脂肪量測定を行うことが望ましい。
・ウエスト径は立位、軽呼気時、臍レベルで測定する。脂肪蓄積が著名で、臍が下方に変異している場合は肋骨下縁と前腸骨きょくの中点の高さで測定する。
・メタボリックシンドロームと診断された場合、糖負荷試験が薦められるが診断には必須ではない。
・高TG血症、低HDL-C血症、高血圧、糖尿病に対する薬物療法を受けている場合は、それぞれの項目に含める。
・糖尿病、高コレステロール血症の存在はメタボリックシンドロームの診断から除外されない。

■NASH/NAFLDとメタボリックシンドローム

過食，運動不足を背景に心筋梗塞や脳卒中等の動脈硬化性疾患の罹患数が増加傾向であったことから，シンドロームX，死の四重奏，内臓脂肪症候群などの様々な症候群が報告され，1999年WHOよりメタボリックシンドロームの概念が提唱された。さらに2005年4月，8学会合同で本邦でのメタボリックシンドローム診断基準が示された（表3）[7]。メタボリックシンドロームは，内臓脂肪型肥満を共通の要因とした高血糖，脂質異常，高血圧を呈する病態であり，動脈硬化を促進させる重要な複合生活習慣病である。CTスキャンなどで内臓脂肪量を定量することが勧められているが，診断基準

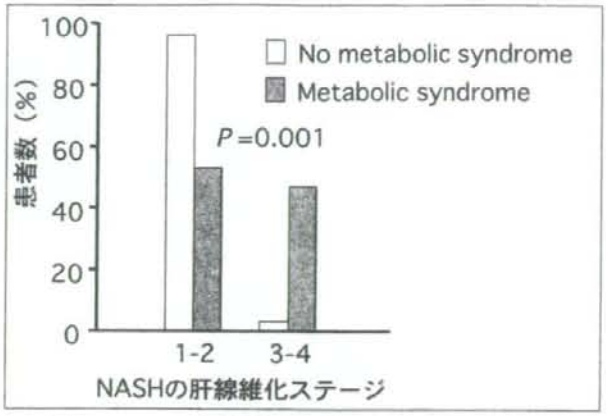

図　NASHの肝線維化の程度とメタボリックシンドロームの有無（文献[10]を引用改変）

では内臓脂肪評価の項目として計測が簡単な腹囲を用いている点が特徴である。また脂質異常の項目では，動脈硬化の指標として用いられるLDLコレステロールではなくトリグリセライドとHDLコレステロールを用いている点も特徴として挙げられる。

本邦のメタボリックシンドロームの頻度は男性ではおよそ8%～25%，女性は2%～22%程度で，対象年齢と調査時期により頻度は異なる。平成17年国民健康・栄養調査の報告では，40歳～74歳人口のうち，メタボリックシンドロームの該当者数は約920万人，予備軍数は約980万人，合わせて約1900万人と推計している。男性の2人に1人，女性の5人に1人はメタボリックシンドロームが強く疑われる者もしくはその予備軍と考えられており，メタボリックシンドロームは非常に頻度が高い。

日本成人4401人の調査では，男性25%，女性10%がNAFLDで，そのうち男性40%，女性26%がメタボリックシンドロームの診断基準を満たしている[8]。

さらに，平均414日後に再度腹部超音波検査を施行され，男性14%，女性5%が新たにNAFLDと診断され，メタボリックシンドロームの存在がNAFLDの発症に強く関与していると報告されている。また，NAFLDとNASHにおけるメタボリックシンドロームの頻度はそれぞれ34～79%，24～88%と報告されており[9]，NASH/NAFLDとメタボリックシンドロームの関連性は極めて高く，メタボリックシンドロームの存在はNASHの肝線維化に促進的に作用すると考えられる（図）[10]。NASH/NAFLDに対する治療法は確立していないが，NASH/NAFLDの治療の基本は生活習慣病をコントロールしていくことである。また単純性脂肪肝であっても将来NASHとなる可能性があり定期的な経過観察が必要である。

■おわりに

NAFLD/NASHと生活習慣病およびその複合病態であるメタボリックシンドロームとの関連について概略を説明した。NAFLD/NASHの病態は十分解明されておらず，NAFLD/NASHと内臓脂肪蓄積，インスリン抵抗性やアディポサイトカインなどとの相互関係を解明していくことが，NASHの病態解明や治療につながる。さらに，メタボリックシンドロームを形成する各病態を総合的に治療していくことは，NASHにおける肝硬変・肝癌への進展を抑制でき，NASHの生命予後の改善につながると考えられる。

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Original Article

Association between changes in body composition and the increasing prevalence of fatty liver in Japanese men

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Aim: Prevalence of fatty liver is increasing. In this study, to elucidate the factor that contributes most to recent increases in prevalence of fatty liver, we determined the independent predictors for the onset of fatty liver and compared these predictors between 2000 and 2005.

Methods: Japanese persons, aged 30–74 years, who participated in regular health checks at Kagoshima Kouseiren Medical Health Care Center (10 336 persons in 2000 and 11 011 persons in 2005) were enrolled in the study. Diagnosis of fatty liver was performed by ultrasonography. Body fat percentage (BFP) was determined using a bipedal bioimpedance instrument.

Results: The prevalence of fatty liver has increased between 2000 and 2005 in men (33.3 vs 38.5% in 2000 vs 2005, respectively, $P < 0.0001$), but not in women (21.3 vs 21.0%, $P = 0.8101$). Logistic regression analysis revealed that both body mass index (BMI) and BFP are independent predictors of fatty liver in both men and women. BMI did not change in either men (23.4 ± 2.9 vs 23.8 ± 3.0 kg/m^2, $P = 0.0528$) or women (22.8 ± 3.1 vs 22.8 ± 3.3 kg/m^2, $P = 0.9862$) during the survey period. In contrast, BFP increased in men (20.6 ± 4.7 vs 22.3 ± 5.0 kg/m^2, $P = 0.0003$), but not in women (27.4 ± 5.5 vs 28.4 ± 5.9 kg/m^2, $P = 0.3993$). There was no significant change in triglycerides and glucose levels.

Conclusion: These results suggest that altered body composition, particularly increased BFP without an increase in BMI, has developed in men and is strongly associated with the increasing prevalence of fatty live amongst Japanese men.

Key words: fatty liver, body fat percentage, body mass index, body composition, life-style, metabolic syndrome

1. INTRODUCTION

FATTY LIVER HAS become a significant problem on a worldwide scale, including in Japan, because the prevalence of fatty liver is increasing.[1–3] Although body mass index (BMI) is considered to be a major risk factor for fatty liver, BMI is only slightly increased amongst Japanese men, and is slightly decreased in women according to national surveys.[4] Although the increase in the prevalence of fatty liver cannot be explained simply by the increase in the prevalence of obesity, the underlying factors have yet to be fully clarified.

In this study, to elucidate the factors that contribute to the recent increase in the prevalence of fatty liver, we determined the predictors of fatty liver in participants who underwent health checks and compared these predictors between the participants in 2000 and 2005.

2. METHODS AND MATERIALS

THE SUBJECTS IN this study were Japanese persons aged 30–74 years, who participated in regular health checks at Kagoshima Kouseiren Medical Health Care Center: 10336 persons (6484 men, 3852 women) from April 2000 to March 2001 (2000 group) and 11011

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Table 1 Comparison of variables between participants with and without fatty liver

Fatty liver	Men			Women		
	With	Without	*P* value	With	Without	*P* value
Age (years)	51 ± 9	54 ± 10	<0.0001	56 ± 9	54 ± 10	0.0015
BMI (kg/m^2)	25.6 ± 2.8	22.6 ± 2.5	<0.0001*	25.9 ± 3.4	21.9 ± 2.7	<0.0001*
BFP (%)	25.4 ± 4.4	20.3 ± 4.3	<0.0001*	33.9 ± 5.5	26.9 ± 5.0	<0.0001*
ALT (IU/L)	38 ± 24	24 ± 29	<0.0001*	28 ± 19	18 ± 8	<0.0001*
γ-GTP (IU/L)	58 ± 60	40 ± 49	0.0012*	24 ± 21	16 ± 14	<0.0001*
TC (mg/dL)	218 ± 36	206 ± 33	<0.0001*	224 ± 35	215 ± 33	<0.0001*
TG (mg/dL)	179 ± 148	118 ± 106	<0.0001*	221 ± 62	81 ± 41	<0.0001*
HDL-C (mg/dL)	51 ± 12	59 ± 15	<0.0001*	57 ± 12	67 ± 14	<0.0001*
BG (mg/dL)	113 ± 26	105 ± 19	0.0002*	108 ± 27	97 ± 11	0.0004*
S-BP (mmHg)	125 ± 17	121 ± 17	0.0003*	122 ± 17	114 ± 18	<0.0001*
D-BP (mmHg)	82 ± 11	77 ± 11	<0.0001*	77 ± 10	72 ± 10	<0.0001*

Data are expressed as mean ± SD. ALT, alanine aminotransferase; BFP, body fat percentage; BG, blood glucose; BMI, body mass index; D-BP, diastolic blood pressure; HDL-C, high density lipoprotein-cholesterol; S-BP, systolic blood pressure; TC, total cholesterol; TG, triglycerides; γ-GTP, γ-glutamyl transpeptidase. *P value after adjusting for age (ANCOVA).

persons (6829 men and 4182 women) from April 2005 to March 2006 (2005 group).

Diagnosis of fatty liver was carried out using ultrasonography (SSA-250A and SSA-750A, Toshiba, Japan; Logic 400, GE Yokogawa, Japan), which was based on the findings of bright liver (increased echogenity) with liver-kidney contrast (increased echo level of the liver compared with the right kidney). BMI was calculated as follows: body weight (kg)/height^2 (m^2). Body fat percentage (BFP) was determined using a bipedal bioimpedance instrument (Model TBF-220; Tanita, Japan). Venous blood samples were taken from all subjects before 09.00 hours after overnight fasting and analyzed immediately. Alanine aminotransferase, γ-glutamyl transpeptidase, total cholesterol, triglycerides and glucose were measured by standard laboratory procedures. High-density lipoprotein (HDL) cholesterol level was determined by direct homogeneous assay of the serum using detergents (Daiichi Chemicals, Japan). History of alcohol intake was determined by questionnaire in which subjects reported a rough approximation of their daily intake.

Differences between groups were examined for statistical significance using the χ^2 test test, unpaired t-test and analysis of covariance (ANCOVA). Multivariate analysis was performed using logistic regression analysis. Correlations were examined by linear regression analysis using the coefficient of correlation. All data analyses were performed using Statview software version J-5.0 (Abacus Concepts, CA, USA). A P-value less than 0.05 was considered significant.

3. RESULTS

3.1. Independent predictors of fatty liver in the 2005 group

TO IDENTIFY FACTORS that associated with the pathogenesis of fatty liver, we compared the clinical and laboratory features between persons with and without fatty liver (Table 1) and performed logistic regression analysis (Table 2).

In both men and women, there was a significant difference in age between the fatty liver and non-fatty liver groups. In men, the age of the fatty liver group was lower than that of the non-fatty liver group; in contrast, the age of women in the fatty liver group was higher. This may reflect the gender difference in incidence of fatty liver, which has been reported elsewhere.[3] Only in men was age found to be an independent predictor.

Markers of obesity, BMI and BFP, were significantly higher in the fatty liver group. In addition, both BMI and BFP were found to be independent predictors of fatty liver in both men and women.

Triglycerides, HDL-cholesterol, glucose and ALT were also independent predictors in both sexes. Total cholesterol and diastolic blood pressure were independent predictors only in men.

As for alcohol, we compared the proportion of persons who drink more than 20 g/day between those with and without fatty liver, and found no difference between the groups (37.4 vs. 39.8%, $P = 0.0569$ in men; 1.1 vs. 1.8%, $P = 0.1463$ in women).

Table 2 Independent predictors of fatty liver by logistic regression analysis

	Regression Coefficient	Standard	Error	*P* value	Odds ratio	95% CI
Men	Age	−0.011	0.003	<0.0001	0.989	0.983–0.995
	BMI	0.207	0.019	<0.0001	1.227	1.183–1.273
	BFP	0.126	0.011	<0.0001	1.135	1.110–1.160
	ALT	0.017	0.002	<0.0001	1.017	1.017–1.021
	TC	0.006	0.001	<0.0001	1.006	1.004–1.008
	TG	0.001	0.000	0.0035	1.001	1.000–1.008
	HDL-C	−0.026	0.003	<0.0001	0.974	0.968–0.999
	FBS	0.011	0.001	<0.0001	1.011	1.008–1.014
	D-BP	0.009	0.003	0.0015	1.009	1.003–1.015
Women	BMI	0.161	0.034	<0.0001	1.175	1.098–1.256
	BFP	0.136	0.020	<0.0001	1.146	1.102–1.191
	ALT	0.049	0.005	<0.0001	1.051	1.041–1.061
	TG	0.009	0.001	<0.0001	1.009	1.007–1.011
	HDL-C	−0.022	0.004	<0.0001	0.978	0.971–0.987
	FBS	0.025	0.003	<0.0001	1.025	1.019–1.031

ALT, alanine aminotransferase; BFP, body fat percentage; BG, blood glucose; BMI, body mass index; CI, confidence interval; D-BP, diastolic blood pressure; HDL-C, high density lipoprotein-cholesterol; TC, total cholesterol; TG, triglycerides.

3.2. Comparison between 2000 and 2005 groups (Table 3)

The prevalence of fatty liver increased between 2000 and 2005 in men (from 33.3 to 38.5%), but not in women (from 21.3 to 21.0%).

Age was significantly higher in the male 2005 group. Age might not be involved in the higher prevalence of fatty liver in 2005 group, because the age of men was lower in the fatty liver group (Table 1).

There was no significant difference in BMI of both sexes. BFP increased significantly in men, but not in women.

Total cholesterol level was significantly elevated in both men and women. HDL-cholesterol levels decreased significantly in men. There was no significant difference in triglycerides and glucose levels. There was a significant difference in men's diastolic blood pressure.

4. DISCUSSION

FATTY LIVER IS an increasingly recognized condition, linked to the metabolic syndrome associated with obesity and insulin resistance. BMI has been considered to be the most important risk factor for fatty liver.[3,5]

Table 3 Comparison between the 2000 and 2005 groups

	Men			Women		
	2000	2005	*P* value	2000	2005	*P* value
Fatty liver (%)	33.3	38.5	<0.0001*	21.3	21.0	NS*
Age (years)	52 ± 10	53 ± 10	<0.0001**	54 ± 10	55 ± 10	0.0243**
BMI (kg/m^2)	23.4 ± 2.9	23.8 ± 3.0	NS***	22.8 ± 3.1	22.8 ± 3.3	NS***
BFP (%)	20.6 ± 4.7	22.3 ± 5.0	0.0003***	27.4 ± 5.5	28.4 ± 5.9	NS***
ALT (IU/L)	28 ± 28	29 ± 28	NS***	19 ± 13	20 ± 12	NS***
TC (mg/dl)	204 ± 33	211 ± 35	0.0058***	209 ± 34	217 ± 34	<0.0001***
TG (mg/dl)	151 ± 126	142 ± 127	NS***	100 ± 57	89 ± 42	NS***
HDL-C (mg/dl)	60 ± 16	56 ± 14	0.0230***	69 ± 16	65 ± 14	NS***
BG (mg/dl)	109 ± 21	108 ± 23	NS***	102 ± 16	97 ± 17	NS***
D-BP (mmHg)	78 ± 11	79 ± 23	<0.0001***	73 ± 16	73 ± 16	NS***

Data except fatty liver prevalence were expressed as mean ± SD. ALT, alanine aminotransferase; BFP, body fat percentage; BG, blood glucose; BMI, body mass index; D-BP, diastolic blood pressure; HDL-C, high density lipoprotein-cholesterol; TC, total cholesterol; TG, triglycerides. *χ^2 test; **unpaired *t*-test; ***analysis of covariance (ANCOVA, adjusted for age).

However, the recent increase in prevalence has not necessarily involved increasing BMI;[3] factors other than an increase in BMI are concerned. In the present study, as shown in Table 3, the increasing prevalence of fatty liver amongst men was associated with changes in BFP, and total cholesterol and HDL-cholesterol levels. It is significant that the prevalence of fatty liver increased with increasing BFP, even without an increase in BMI.

The increase in BFP without a corresponding increase in BMI may indicate changes in body composition; that is, an increase in body fat deposits and corresponding decrease in the fat-free component. As for fat deposits, Eguchi *et al.* report that the severity of fatty liver is positively correlated with visceral fat accumulation regardless of BMI.[6] It is possible that the increase in BFP corresponds to an increased accumulation of visceral fat. On the other hand, Caprosto *et al.* report that the resting metabolic rate (RMR) is lower in patients with non-alcoholic steatohepatitis than in controls.[7] Because of the correlation between RMR and muscle mass, lower RMR possibly reflects a decrease in muscle mass. Thus, the decrease in the fat-free component may correspond to a decrease in muscle mass. The literature suggests that Asian populations have a high level of abdominal fat at a lower BMI relative to Caucasians.[8,9] Therefore, Asians are at a higher risk for obesity-associated disorders even without obesity, and this is the rationale behind the World Health Organization's Regional Office for the Western Pacific criteria (overweight at risk) for Asians.[9] It is considered that the characteristic body composition of the Asian population has been further impacted upon by the present-day lifestyle in Japan, resulting in an increased prevalence of fatty liver without an increase in BMI. Since visceral fat obesity strongly associates with the metabolic syndrome, our study underscores the importance of monitoring visceral fat accumulation using representative indicators such as waist circumference or waist/hip ratio, or monitoring visceral fat volume by CT scan, during regular health checks. Table 3 shows that the prevalence of fatty liver has increased with increases in total cholesterol and decreases in HDL-cholesterol, possibly suggesting an association with insulin resistance.[10] Additional studies are required to further clarify these associations.

The changes in body composition in the present study should be distinguished from obesity. Obesity may bring about increases in both BFP and BMI. We consider that inadequate dieting (and rebound), irregular eating habits (e.g. fasting at breakfast) and a lack of exercise are the probable causes of the reported changes in body composition. As described above, it seems difficult to prevent the increasing prevalence of fatty liver only by weight (BMI) control. Thus, we must emphasize the need for a new strategy to reduce risk of fatty liver, in which relevant nutritional support and exercise are employed to reduce body fat deposits and develop muscle mass.

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Human Neutrophil Peptides 1–3 Are Useful Biomarkers in Patients with Active Ulcerative Colitis

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Background: A specific useful biomarker for diagnosing ulcerative colitis (UC) has not yet been described. This study employed proteomics to identify serum protein biomarkers for UC.

Methods: Ninety-four blood samples were isolated from patients and controls (including 48 UC, 22 Crohn's disease [CD], 5 colorectal cancer, and 6 infectious colitis patients and 13 healthy subjects). Serum samples were analyzed using the SELDI-TOF/MS ProteinChip system. After applying the samples to ProteinChip arrays, we assessed differences in the proteomes using Ciphergen ProteinChip software and identified candidate proteins, which were then characterized in immunoassays.

Results: Preliminary analysis using the ProteinChip system revealed significant peak-intensity differences for 27 serum proteins between 11 patients with UC and 7 healthy subjects. Among these proteins, 3 proteins (with mass/charge ratios of approximately 3400) were identified as human neutrophil peptides 1–3 (HNP 1–3). The presence of HNP 1–3 in the patient sera was confirmed using immunoassays. Enzyme-linked immunosorbent assays demonstrated that the mean plasma concentration of HNP 1–3 was significantly higher in patients with active UC ($n = 28$) than in patients whose UC was in remission ($n = 20$) or patients with CD ($n = 22$), infectious colitis, or healthy subjects, and tended to be higher than in patients with colon cancer. In addition, the plasma concentration of HNP 1–3 in patients that responded to corticosteroids-based therapy decreased after treatment, whereas it was not changed in nonresponders.

Conclusions: HNP 1–3 is a novel biomarker that may be useful for diagnosing patients with active UC and predicting treatment outcomes.

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Key Words: biomarkers, inflammatory bowel disease, ulcerative colitis, human neutrophil peptides 1–3, SELDI-TOF/MS, proteomics

Genetic and environmental factors contribute to the disease process of inflammatory bowel disease (IBD), including ulcerative colitis (UC).[1,2] The presence of active inflammation of the gut in patients with UC is associated with an acute-phase reaction and the migration of leukocytes to the gut. This, in turn, promotes the production of a large number of proteins.[3] Determination of inflammatory activity is important for the comprehensive assessment of patients with UC and for the tailoring of therapy.[4] Many clinical activity indices are used to stratify patients with UC. For example, the UC Disease Activity Index (UCDAI)[5] is a widely used measure of clinical parameters of disease activity. These indices, however, only provide indirect assessments of disease activity. Whereas albumin, hemoglobin, the erythrocyte sedimentation rate (ESR), and acute-phase protein levels are commonly used biological parameters for assessing UC, there are no accurate markers to assess the inflammatory activity observed with histopathologic or endoscopic analyses.[6]

Proteomic array technology, in which a ProteinChip system is coupled with surface-enhanced laser desorption ionization/time-of-flight/mass spectrometry (SELDI-TOF/MS) for the profiling of serum or plasma, is a powerful tool that allows the identification of new biomarkers for malignant tumors and autoimmune diseases.[7,8] This technology is a rapid and sensitive technique, in which the detected peak intensities for some proteins correlate with concentrations determined using enzyme-linked immunosorbent assay (ELISA). Novel blood biomarkers which are identified by this proteomics, may provide clinicians with more accurate parameters to assess inflammatory activity in UC.

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Host defense processes, which rely on both innate and adaptive immune mechanisms, are critical for the development of IBD.[1,2] Innate immunity participates in the activation of antigen-specific adaptive immune responses, including the production of antimicrobial peptides/proteins. In mammals, defensins, a class of antimicrobial peptides, can be divided into 2 major groups: α-defensins and β-defensins.[9] Six types of α-defensins have been identified, 4 of which are produced predominantly by neutrophils and phagocytes and stored in the granules of these cell types (denoted human neutrophil peptides 1–4; HNP 1–4). The remaining 2 α-defensins are localized in Paneth cell granules (denoted human α-defensins 5 and 6; HD 5 and 6). Although the amino-acid sequences of HNP 1, HNP 2, and HNP 3 are very similar, the sequence of HNP 4 is different than those of HNP 1–3. HD 5 is expressed by metaplastic Paneth cells in the colons of patients with UC or CD. The expression levels of HD 5 in blood, however, have not been examined; there are currently no data evaluating HNP 1–3 expression in patients with IBD.

In this study we clearly demonstrate serum profiling with increased levels of HNP 1–3 in the sera of patients with UC using a proteomics-based approach. We also compared the protein levels of HNP 1–3 in plasma samples from patients with UC and Crohn's disease (CD), before and after treatment for UC, and in patients in which treatment was effective or not effective. These analyses will contribute to our understanding of the pathogenesis of UC and aid in the discovery novel biomarkers to assess disease activity and therapeutic responses.

MATERIALS AND METHODS

Patients

After obtaining written informed consent, we analyzed a total of 94 blood samples from patients with IBD, colorectal cancer (CRC), infectious colitis, and control subjects. Forty-eight patients were diagnosed with UC (20 females and 28 males; median age, 39 years; age range, 12–72 years) and 22 with CD (11 females and 11 males; 29 years; 16–57 years). The control group contained 13 healthy subjects (5 females and 8 males; median age 30 years; age range, 24–34 years) and 5 with CRC (1 female and 4 males; median age 62 years; age range, 52–80 years) and 6 with infectious colitis (3 females and 3 males; median age 42 years; age range, 17–77 years). The study protocol was approved by the Ethics Committee of the Kagoshima University Graduate School of Medical and Dental Sciences (Kagoshima, Japan) and the Faculty of Medicine at the University of Miyazaki (Miyazaki, Japan). All IBD patients were diagnosed using established endoscopic, radiological, histological, and clinical criteria. The inactive or remission phase of UC was defined as a UCDAI score less than or equal to 2, whereas the active phase was defined as a UCDAI score greater than or equal to 3.[5] Twenty and 28 patients with UC were identified as inactive-phase and active-phase patients, respectively. All of the patients with active-phase UC were treated with oral corticosteroids, whereas 23 received leukocytapheresis therapy (LCAP) (Table 1). Furthermore, 4 of the active UC patients did not respond to treatment and eventually underwent a total colectomy. Fourteen patients with CD had high disease activities based on an International Organization for the Study of Inflammatory Bowel Disease (IOIBD) score of 2 or greater[10] and were regarded as active-phase patients. Eight patients that had lower IOIBD scores (0 or 1) were defined as inactive-phase patients. All 5 CRC patients were diagnosed with Duke's A group cancers by endoscopic, radiological, and histological examinations. All 6 patients with infectious colitis had diarrhea and fever, and were diagnosed based on clinical findings.

SELDI-TOF/MS

We used chips with cationic surfaces for analysis (CM10; Bio-Rad Laboratories, Hercules, CA). Serum samples were denatured in urea buffer (7 M urea, 2 M thiourea, 4% CHAPS, 1% dithiothreitol, and 2% ampholites), and then diluted 1:9 in binding/washing buffer (50 mM sodium acetate, pH 4). After washing the chip twice in binding/washing buffer, we applied 100 μL of diluted serum to each chip spot. Samples were incubated for 40 minutes and washed 3 times. After rinsing the chips once in water, 0.5 μL CHCA (α-cyano-4-hydoroxycinnamic acid; Nacalai Tesque, Kyoto, Japan) was applied twice to each spot and allowed to air-dry. Arrays were analyzed using a ProteinChip Reader (ProteinChip Biology System II; Bio-Rad Laboratories). TOF spectra were generated with laser shots collected in positive mode. The laser intensity ranged from 190 to 195 with a detector sensitivity of 6. On average, 65 laser shots per spectrum were used. A mixture of standard mass calibrant proteins (All-in-one Peptide Standard; Bio-Rad Laboratories) in 500 nL was used to calibrate the system for mass accuracy. The standards were applied to a single spot of the normal phase chip array (NP20; Bio-Rad Laboratories), after which two 1.0-μL samples of saturated CHCA were applied. TOF values were compared to the molecular masses of the standard proteins; calibration was performed according to the manufacturer's instructions.[7]

Immunodepletion Assay

Initially, 6 μL of anti-HNP 1–3 antibody solution (120 ng; Hycult Biotechnology, Netherlands) was bound to 30 μL of Protein A–agarose (Sigma Chemical, St. Louis, MO) for 15 minutes on ice. The postcentrifugation supernatant was discarded and the pellet was washed twice in buffer containing 20 mM HEPES (pH 7.8), 25 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, and 0.05% NP40. Then 15 μL of sera from each patient with UC was incubated with a pellet for 45 minutes on ice. As a negative control, samples were incubated with

TABLE 1. Characteristics of Patients with UC or CD

	UC		CD	
Disease activity[a]	Active	Inactive	Active	Inactive
Number	28	20	14	8
Gender (M/F)	19/9	9/11	10/4	6/2
Age (range), yr	41 ± 16 (14–68)	31 ± 16 (12–72)	32 ± 13 (16–57)	28 ± 7 (18–40)
Disease duration (range), yr	5.6 ± 4.8 (1–19)	5.2 ± 4.3 (1–18)	9.4 ± 7.4 (3–22)	6.0 ± 3.8 (1–13)
Treatment[b]				
5-aminosalicylic acid	28	19	14	8
Corticosteroid	28	7	10	2
Leukocytapheresis	23	0	0	0
Type of UC				
Left-side colitis	4	8	—	—
Pancolitis	24	12	—	—
Type of CD				
Ileal	—	—	4	2
Ileocolonic	—	—	9	5
Colonic	—	—	1	1

UC, ulcerative colitis; CD, Crohn's disease. Data are shown as the means ± SD or range.
[a]Active UC is defined as an Ulcerative Colitis Disease Activity Index score equal to or greater than 3, and active CD is defined as an International Organization for the Study of Inflammatory Bowel Disease score equal to or greater than 2.
[b]Includes the overlap treatment.

Protein A–agarose in the absence of a specific antibody. After incubation, samples were cleared by centrifugation; 3 μL of each supernatant was analyzed on NP20 ProteinChip arrays using a PBS II reader.[11]

ELISA

We determined the HNP 1–3 (P59665, P59666) concentrations in plasma using a human HNP 1–3 ELISA kit (Hycult Biotechnology) according to the manufacturer's instructions. Samples were analyzed in duplicate using a plate reader (Bio-Rad Laboratories) at 450 nm. The concentration of each protein in the plasma was calculated according to a standard curve.

Immunohistochemical Studies

HNP 1–3 expression in colon tissue was evaluated using immunohistochemistry. Abnormal colon tissues were obtained by total colectomy in patients with UC, whereas normal colon tissues were isolated in surgical resections for colon cancer by taking surrounding normal tissue without malignant cells. Colon tissues were fixed in 10% formalin and embedded in paraffin. For histological examination, 5-μm slices were stained with hematoxylin and eosin (HE). The anti-HNP 1–3 monoclonal antibodies (BMA Biochemicals, Augst, Switzerland) was diluted to a final concentration of 0.5% (w/v) in phosphate-buffered saline (PBS) supplemented with 1% fetal bovine serum (FBS). Immunohistochemical analysis of paraffin-embedded sections using antibodies against HNP 1–3 was performed as described.[12] EnVision plus horseradish peroxidase (Dako, Carpinteria, CA) was applied to samples; chromatin 3′,3′-diaminobenzidine was used to detect bound antibody.

Statistical Analysis

Values shown are the means ± SD. Statistical significance, including that for differences in laboratory data and individual peaks in SELDI-TOF/MS, was determined using Mann–Whitney *U*- and paired *t*-tests. *P*-values < 0.05 were considered to be statistically significant. The discriminatory power for each putative marker was described via the area under the curve (AUC) from receiver operating characteristic (ROC) analysis. The statistical analyses were performed using StatView 4.5 software (Abacus Concepts, Berkeley, CA), SPSS software (SPSS, Chicago, IL), and Ciphergen ProteinChip Software (Fremont, CA) v. 3.0.2.

RESULTS

Profiling Serum Proteins in Patients with UC

We performed differential profiling of serum proteins in 11 patients with UC and 7 normal healthy controls using the SELDI ProteinChip system. Peaks were automatically detected using Ciphergen ProteinChip Software 3.0.2.[7,13] Twenty-seven serum peaks in the 3000–10,000 m/z range

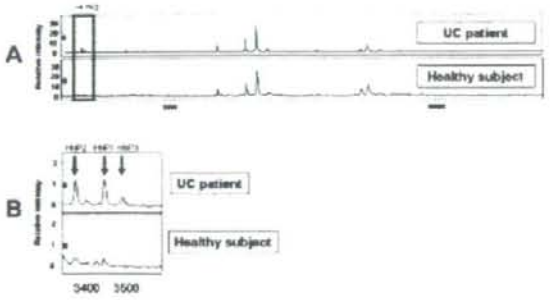

Figure 1. Serum proteomics of UC patients and healthy controls using SELDI-TOF/MS. (A) Spectra representing the serum proteins of a patient with UC and a healthy volunteer. The horizontal axis shows a range from 3000 to 10,000 m/z. Significant differences in peak intensities between patients with UC and healthy volunteers were found for 27 peaks. (B) The intensities of the protein peaks are shown for the range between 3300 and 3600 m/z. Protein peaks with m/z values of 3371, 3443, and 3486 represent HNP 2, HNP 1, and HNP 3, respectively.

were significantly different between the 2 patient groups (Fig. 1). Sixteen peaks resulted in *P*-values less than 0.01 (Table 2). The most dramatic difference was detected for a 3371 m/z protein, the level of which was increased in the sera of UC patients compared with healthy controls.

Identification of HNP 1–3

A previous study of colon tumor tissue identified a similarly increased signal at 3371 m/z using ProteinChip

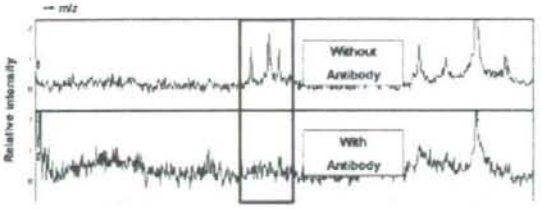

Figure 2. Immunodepletion assay using Protein A beads. Anti-HNP 1–3 antibodies (Hycult Biotechnology) were bound to 30 μL of Protein A beads. Sera from UC patients were incubated with these beads. After clearing by centrifugation, 3 μL of each supernatant was analyzed using an NP20 ProteinChip array.

arrays.[12,14] The peak was confirmed to correspond to HNP 2 with an immunodepletion assay. Peaks at 3443 and 3486 m/z, reported to correspond to HNP 1 and HNP 3 in the previous report, were also found to be significantly increased in analyses of the sera of UC patients compared to results observed for control samples. HNP 1, 2, and 3 have similar structures consisting of 30, 29, and 30 amino acids, respectively; 29 of the amino acids are identical among the peptides.[12,15] We also subjected the serum samples to immunodepletion assays using monoclonal antibodies against HNP 1–3 and found that the 3371, 3443, and 3486 m/z protein peaks in the SELDI-TOF MS spectra were no longer observed for the sera from patients with UC (Fig. 2). These peaks were clearly observed for negative control samples, which underwent immunodepletion assays in the absence of specific antibodies. These results indicate that the 3371, 3443, and 3486 m/z

TABLE 2. Discriminatory Peaks and Mean Values in Samples from Patients with Ulcerative Colitis and Healthy Volunteers

Mass to Charge (m/z)	Ulcerative Colitis (n = 11)	Healthy Subject (n = 7)	P-value
3371	1.42 ± 0.66	0.40 ± 0.10	4.8×10^{-4}
4789	0.51 ± 0.82	0.05 ± 0.03	4.8×10^{-4}
5421	0.34 ± 0.24	0.09 ± 0.02	4.8×10^{-4}
8688	0.65 ± 0.41	1.70 ± 0.38	6.8×10^{-4}
5838	0.79 ± 0.85	0.21 ± 0.05	9.4×10^{-4}
4351	0.82 ± 0.62	2.21 ± 0.56	1.3×10^{-3}
5620	0.11 ± 0.05	0.39 ± 0.23	1.7×10^{-3}
6881	1.00 ± 0.59	2.24 ± 0.46	1.7×10^{-3}
9358	0.17 ± 0.06	0.80 ± 0.52	1.7×10^{-3}
7023	0.12 ± 0.07	0.66 ± 0.46	2.4×10^{-3}
4469	3.31 ± 2.16	1.02 ± 0.59	3.2×10^{-3}
4542	0.39 ± 0.17	0.16 ± 0.02	4.3×10^{-3}
4590	0.86 ± 0.45	1.63 ± 0.26	4.3×10^{-3}
4287	0.68 ± 0.37	1.26 ± 0.39	5.7×10^{-3}
2900	0.18 ± 0.12	0.37 ± 0.14	9.8×10^{-3}
2979	1.00 ± 0.88	0.26 ± 0.15	9.8×10^{-3}

Statistical significance was determined using a Mann-Whitney U-test.

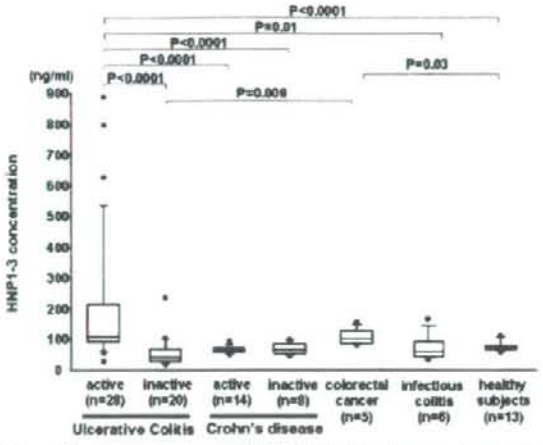

Figure 3. Concentrations of HNP 1–3 in the plasma of patients with UC, CD, colorectal cancer, infectious colitis, and healthy controls. Boxes indicate the median ± 25th percentile. The lower bar indicates the 10th percentile and the upper bar indicates the 90th percentile.

protein peaks, which were larger in the spectra for sera of UC patients, corresponded to HNP 1–3.

Concentrations of HNP 1–3 in Plasma

It was not possible to determine the individual concentrations of HNP 1, 2, or 3 using commercially available ELISA kits; therefore, we evaluated the total concentration of HNP 1, 2, and 3 in plasma. We found that there was a clear correlation between the serum HNP 1–3 peak intensities determined using the SELDI system and the plasma HNP 1–3 concentration measured using ELISAs in 11 patients with UC and 7 normal controls ($r = 0.68$, $P < 0.01$). We then determined the plasma concentrations of HNP 1–3 in 48 UC patients, 22 CD patients (Table 1), 5 CRC patients, 6 infectious colitis patients, and 13 healthy controls (Fig. 3). The plasma concentrations of HNP 1–3 were significantly higher in patients with active UC (203.1 ± 215.5 ng/mL) than in patients with inactive UC (58.3 ± 49.5 ng/mL), CD (active; 65.5 ± 11.2 ng/mL, inactive; 70.4 ± 20.0 ng/mL), infectious colitis (72.2 ± 16.5 ng/mL), or the healthy controls (77.5 ± 16.4 ng/mL). In addition, HNP 1–3 concentrations in patients with active UC tended to be higher in patients with CRC at Duke's stage A (100.8 ± 27.6 ng/mL), but not significantly. HNP 1–3 concentrations in CRC patients were also higher than those in patients with inactive UC and healthy controls.

Expression of HNP 1–3 in Intestinal Tissue

We examined the localization of HNP 1–3 in normal tissues and those from patients with active-phase CD or UC using immunohistochemistry. The colonic mucosa, lamina propria, muscle layer, and crypt abscesses of patients with active UC exhibited strong staining with anti-HNP 1–3 antibodies (Fig. 4). These sections contained a number of infiltrating neutrophils (Fig. 4B,C), which may provide a source of the secreted HNP 1–3 near the colonic epithelium. Positive staining for neutrophils, however, was seen in the blood vessels of both normal and abnormal colon tissues. In addition, small numbers of neutrophils with positive staining were seen in submucosal tissue of patients with CD (Fig. 4D). Epithelial cells in colon samples from patients with inflamed CD or from normal healthy subjects did not exhibit staining with anti-HNP 1–3 antibodies (Fig. 4D,E).

HNP 1–3 as a Biomarker in UC Patients

We investigated the association between the HNP 1–3 concentration and the clinical course of UC. We determined the HNP 1–3 concentrations in pairs of plasma samples from 15 patients with active UC obtained before and after induction therapy with corticosteroids (Table 3). Eight UC patients in the responder group were successfully treated by induction therapy. The elevated HNP 1–3 levels of UC patients in the responder group were reduced after induction therapy (Fig. 5). In contrast, 7 patients in the nonresponder group, 2 of whom had a total colectomy and 5 who quickly relapsed, were not effectively treated. The HNP 1–3 levels of patients in the nonresponder group before treatment were lower than those in the responder group and were not changed after treatment (Fig. 5). Additionally, although plasma HNP 1–3 levels (means ± SD) of responder active UC patients (273.0 ± 224.8 ng/mL) were higher than those with active CD (65.5 ± 11.2 ng/mL) ($P < 0.001$), those with nonresponder active UC (84.6 ± 26.5 ng/mL) were similar to those with active CD. These results indicate that patients with active UC and low HNP 1–3 levels do not respond well to treatment.

We evaluated the relationship between the HNP 1–3 levels and the clinical activity of UC. There was a significant correlation between the HNP 1–3 levels and the UCDAI score or the white blood cell count (WBC) of UC patients ($r = 0.54$, $P < 0.01$; $r = 0.55$, $P < 0.01$, respectively), although no correlation between the HNP 1–3 levels and the C-reactive protein (CRP) levels was noted ($r = 0.24$). In addition, ROC analysis was performed to estimate the efficiency of induction therapy for patients with active-phase UC; we calculated the sensitivity and specificity of HNP 1–3 levels for discriminating responder UC patients from nonresponders. We obtained a sensitivity of 89% and a specificity of 80% using a cutoff value of 100 ng/mL HNP 1–3; the ROC AUC was 0.89 between the responder and nonresponder groups of UC patients. For evaluations of the activity of UC, we compared such inflammatory markers as the CRP level and the WBC to the HNP 1–3 level in patients with UC. ROC AUC of the CRP level and WBC were 0.76 and 0.56, respectively. Thus,

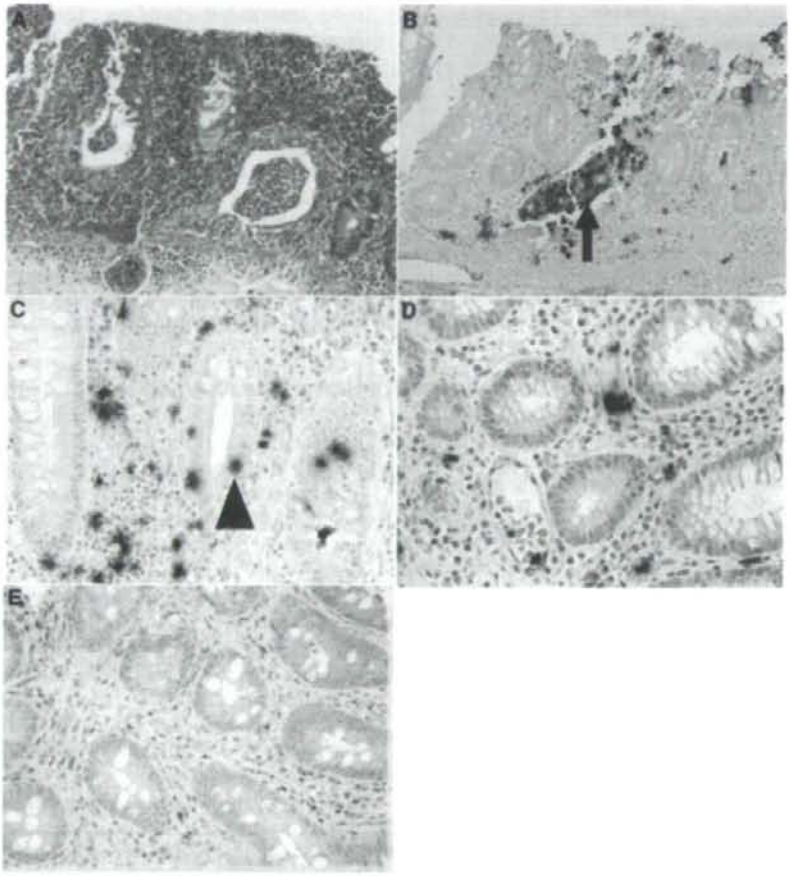

Figure 4. Expression of HNP 1–3 in the tissue of patients with active UC or CD and in normal colon tissue. (A) HE staining of colon tissues from patients with UC. (B,C) Immunohistochemical staining demonstrated extensive HNP 1–3 expression in the colon tissues of patients with UC. Many HNP 1–3-positive cells were observed in the crypt abscesses (B: arrow) and in neutrophils that had migrated into the epithelial layers (C: arrowhead). In addition, an ulcer lesion observed in the colon sample stained positive for HNP 1–3. (D,E) Although small numbers of neutrophils in the blood vessels and submucosal tissues were positive for HNP 1–3, epithelial cells in colon samples from patients with inflamed CD or normal subjects were not positive for HNP 1–3. Original magnification: 100× (A,B) and 200× (C–E).

the level of HNP 1–3 had a high discriminatory power for estimating the efficacy of treatment in patients with UC.

DISCUSSION

We identified 27 proteins that showed significant differences in the serum protein profiles of patients with UC compared with those of healthy controls using SELDI-TOF/MS analysis. Of these proteins, 3 signals around 3400 m/z were confirmed to correspond to HNP 1, 2, and 3. In addition, we observed an increase in HNP 1–3 plasma levels in patients with active-phase UC compared with that seen in patients with remission-phase UC or CD; these levels were higher in the plasma of UC patients who showed better therapeutic outcomes than in samples from nonresponder patients.

Several studies have suggested that the development of IBD requires the interaction of genetic factors with both specific luminal bacterial antigens and environmental triggers that break the mucosal barrier.[16–18] Although the principle treatment for IBD is the suppression of inflammation, treatment strategies for the 2 diseases, UC and CD, are somewhat different. Whereas these differences may address the different biomarkers of the 2 conditions, a specific biomarker for IBD remains unknown. To discover a biomarker of UC, we

TABLE 3. Characteristics of Patients with Active UC in the Responder Group and Nonresponder Group

	Responder	Nonresponder	*P*-value
Number	8	7	
Gender (M/F)	5/3	5/2	0.7
Age (yr)	33.5 ± 13.8 [14–50]	42.3 ± 19.8 [16–68]	0.4
CRP (mg/dl)	1.7 ± 1.7	3.3 ± 4.5	0.4
WBC (cells/ul)	12714 ± 4604	7657 ± 3423	*0.04*
Platelets × 10^4/ul	40.4 ± 7.4	36.2 ± 11.1	0.3
HNP 1–3 (ng/ml)	273.0 ± 224.8	84.6 ± 26.5	*0.002*
Type of UC			
Pancolitis/Left-side colitis	7/1	5/2	0.6
UCDAI score	9.4 ± 4.6	8.6 ± 1.9	0.7
Duration	6.7 ± 6.5 [1–19]	5.7 ± 5.1 [2–16]	0.8

Data are shown as the means ± SD [ranges]. Statistical significance was determined using a Mann-Whitney *U*-test or Fisher's exact test, as appropriate. UC, ulcerative colitis; UCDAI, Ulcerative Colitis Disease Activity Index.

employed ProteinChip technology. The likelihood of finding reliable tumor markers by analyzing tissue may be higher than in analyses of serum[12]; malignant cells may produce proteins that are useful biomarkers. In nonmalignant diseases, such as UC, protein profiling of serum or plasma may be more informative than that of tissue samples. Additionally, fluid samples, such as serum, are easier to obtain than tissue samples. Thus, we used serum samples to identify new biomarkers for UC.

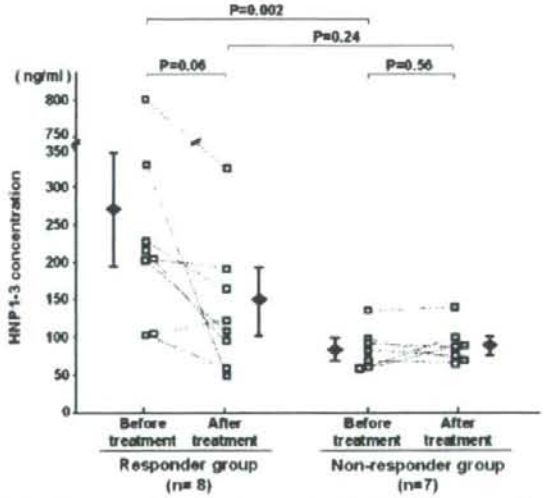

Figure 5. HNP 1–3 levels in the responder and nonresponder groups before treatment predicted therapeutic outcomes in UC patients; changes in the HNP 1–3 levels in UC patients in response to treatment are presented. The mean concentration of HNP 1–3 in the responder group before treatment was significantly higher than that seen in the nonresponder group, which indicates that HNP 1–3 levels may be an effective predictor of therapeutic outcomes. HNP 1–3 levels tended to decrease after treatment in the responder group, whereas no changes were observed for the nonresponder group. Patients whose plasma was not obtained after treatment were excluded from analysis.

Defensins are one of the most extensive peptide families of naturally occurring antibiotics. These peptides exhibit microbicidal activities against Gram-positive and Gram-negative bacteria, mycobacteria, fungi, and certain enveloped viruses. HNP 1–3 are part of the α-defensin family and components of the innate immune response. HNP 1–3 are synthesized by neutrophil precursor cells and released at inflammatory sites by mature circulating neutrophils.[9,19] The expression of HNP 1–3 has been observed in epithelial cells of the ileum and colon in patients with active UC or CD.[20] Whether neutrophils within inflamed colon tissue express HNP 1–3 in IBDs, however, is not known. In this study, we demonstrated that the colon mucosal tissue of patients with active UC or CD displayed minimal immunoreactivity for HNP 1–3, whereas the infiltrating neutrophils were stained strongly. These results indicate that HNP 1–3 were secreted from neutrophils, leading to increased plasma levels in patients with UC. High concentrations of HNP 1–3 can be cytotoxic for epithelial cells due to cytolysis and can induce apical conduction in Cl^- secretory epithelia.[21,22] Thus, whereas HNP 1–3 have antibacterial activities in the early phase of UC, they also may injure the colon if they are overexpressed by infiltrating neutrophils. High concentrations of HNP 1–3 may adversely affect colon tissues in UC patients, potentially contributing to diarrhea.[23] HNP 1–3 are secreted from the azurophilic granules of neutrophils following stimulation with IL-8.[24] Epithelial-derived IL-8 is thought to mediate neutrophil migration and infiltration during the inflammatory process of UC.[25,26] IL-8 mRNA levels are

significantly higher in UC patients with crypt abscesses.[27] Although HNP 1–3 have been reported to be expressed by surface enterocytes in the mucosa of patients with active IBD,[28] we observed only minimal staining of the colonic surface mucosa from patients with active UC using anti-HNP 1–3 antibodies. Moreover, Caco-2 and HT-29 cells, 2 colon epithelial cell lines, do not express HNP 1–3 (data not shown). Therefore, we hypothesized that HNP 1–3 are expressed by neutrophils following stimulation with IL-8, which suggested a correlation between the IL-8 and HNP 1–3 levels. We did not, however, observe a correlation between the IL-8 and HNP 1–3 levels in the plasma from active UC patients, and there was no association between the disease activity score and plasma IL-8 concentrations (data not shown). These results indicate that HNP 1–3 expression may be affected by other factors and HNP 1–3 values appear to be more useful to measure clinical UC disease activity than IL-8 levels.

Neutrophils are critical cellular mediators of the inflammation observed in UC. Neutrophils increase in number and display augmented activation during active-phase UC, but not inactive-phase UC.[28] Neutrophils extensively infiltrate colon tissue in patients with UC, and can be detected in the inflamed mucosa during even the early stages of inflammation.[29,30] Platelets are also important in the pathophysiology of UC.[31] Cytapheresis therapy (including LCAP) in combination with steroid therapy can be an effective treatment option for patients with active UC.[32] LCAP may remove and modulate both leukocytes and platelets, thereby altering the expression of proinflammatory cytokines.[33,34] The effect of LCAP on HNP 1–3 levels, however, has not been examined, and further studies are needed to determine whether HNP 1–3 levels decrease in response to LCAP. In addition, we showed that HNP 1–3 levels in the plasma were higher in patients with active UC than in those with infectious colitis, and HNP 1–3 levels were similar between patients with infectious colitis and healthy controls. In contrast, it was reported that HNP 1–3 levels in patients with severe infectious diseases, such as sepsis, were higher than those in healthy controls.[35] The disease severity of the enrolled patients with infectious colitis in this study may have affected our results. Cytapheresis therapy, however, may not be effective for severe infectious diseases, including infectious colitis, and high concentrations of HNP 1–3 in patients with active UC may be associated with disease characteristics. Further examination, including cases of infectious colitis with sepsis, will be necessary.

As previously reported, we found that several inflammatory makers, including the CRP level, WBC, and platelet count, decreased after treatment. Changes in these inflammatory markers did not predict the treatment outcome of patients with UC, whereas plasma levels of HNP 1–3 correlated with UC disease activity and predicted the therapeutic outcome. There were no correlations between plasma HNP 1–3 levels and inflammatory markers, such as platelet counts and CRP levels. These results may suggest that high levels of HNP 1–3 independently indicate the activity of disease and the feasible treatment outcome in patients with UC. However, there is a limitation in the use of HNP 1–3 measurement as a biomarker; low levels of HNP 1–3 in colitis patients did not diagnose whether they had nonresponder UC or active CD. Therefore, low levels of HNP 1–3 in colitis patients should be assessed by clinical symptoms, stool for bacterial examination, and endoscopic and radiographic examination of the gastrointestinal tract for diagnosis. Other proteins and peptides that were detected by SELDI/TOF-MS in this study are now under investigation and may serve as additional biomarkers for the assessment of IBD, especially in nonresponder UC patients.

The levels of HNP 1–3 in tumor tissue and serum were reported to increase in patients with CRC.[12] It was also reported that plasma HNP 1–3 concentrations determined using ELISA increased in Duke's stages C and D, but not in A or B compared to healthy controls.[14] In contrast, we showed that HNP 1–3 concentrations in CRC patients at Duke's stage A were higher than those seen in patients with inactive UC and healthy controls. Although HNP 1–3 concentrations in CRC patients at Duke's stage A seem to be similar between our study and a previous study[14] (100.8 ± 27.6 versus 105.4± 80.6 ng/mL, respectively), the concentrations in the healthy controls were different between the 2 studies (77.5 ±16.5 versus 96.6 ± 36.2 ng/mL). In addition, Albrethsen et al[14] mentioned that in addition to Duke's C and D, HNP 1–3 expression in CRC tissues at Duke's A and B was higher than in normal tissue by SELDI ProteinChip. It is controversial whether the increased HNP 1–3 in tumors is localized to cancer cells or to neutrophilic leukocytes. There is the possibility that the plasma HNP 1–3 levels will increase in patients with CRC at Duke's stage A and that HNP 1–3 concentration is a potential marker for the assessment of CRC patients with advanced disease.[12,14] In addition, these results indicate that HNP 1–3 levels may not be able to distinguish between active UC and colon cancer. In the clinical setting, however, UC can typically be distinguished from colon cancer by various clinical features, such as diarrhea, fever, and colonoscopic findings. On the other hand, colon cancer commonly occurs in patients with UC, especially those who have suffered from the disease for a long period of time; such colon cancers are difficult to detect using colonoscopy. HNP 1–3 levels may help to signal the occurrence of colon cancer in UC patients when high concentrations of HNP 1–3 are detected in the absence of active colitis; these patients should be extensively examined, including total colonoscopy and random biopsies.

In conclusion, we used SELDI-TOF/MS to perform serum protein profiling and determined that HNP 1–3 levels increase in patients with active-phase of UC. We also con-