

Original Article

Targeting TNF α in severe psoriasis-mass spectrometry reveals a time-dependent specific inhibition of Factor H in responding patients

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Abstract: Drugs targeting TNF α (eg, Etanercept®) provide effective control of severe psoriasis. In absence of validated biological parameters of inflammation in psoriasis most decisions on therapeutics have relied mostly on clinical criteria, namely the “Psoriasis Area and Severity Index” (PASI). The purpose of this study was to assess by mass spectrometry alterations in concentrations of serum proteins that specifically correlated with effectiveness of Etanercept treatment. This prospective study enrolled 10 patients suffering from moderate to severe psoriasis (PASI score > 10 and < 17) and treated with Etanercept over a period of 24 weeks; 10 healthy, age-matched volunteers provided controls. Serum proteins sensitive to Etanercept treatment were identified using SELDI-TOF (surface-enhanced laser desorption and ionization - time of flight) coupled to nano LC-ESI/MS (nano liquid chromatography-electrospray ionization/tandem mass spectrometry) technologies. For comparisons between groups of individuals *p*-values (considered significant when < 0.01) were estimated with non-parametric tests, namely Mann-Whitney (for unpaired data) and Wilcoxon signed-rank (for paired data). In responding patients it could be shown using SELDI-TOF spectrometry that two proteins (134 kDa and 4.3 kDa) return to control levels by 24 weeks of treatment. Using nano LC-ESI/MS the 134 kDa species was identified as complement Factor H. These observations deserve further analyses utilizing larger cohorts of patients. Determination of Factor H levels may become a complementary tool to follow remission or predict the onset of relapse in the follow-up of patients under treatment with Etanercept.

Keywords: Etanercept®, TNF α , psoriasis, serum, mass spectrometry, complement factor H

Introduction

Patients with psoriasis require an individual management and long-term planning of therapeutic strategies. The therapy is chosen in accordance with clinical history, age, severity of psoriasis and response to previous treatments. Biological therapies are based on the role of inflammatory cytokines in the pathogenesis of this disease. Supporting a key role for tumor necrosis factor (TNF α) in psoriasis, the intro-

duction in recent years of anti-TNF α agents has had a strong impact in treatment [1-3].

Since 2004, several phase III trials have shown that Etanercept provides an effective and well-tolerated treatment against psoriasis and psoriatic arthritis [4-6]. Typical profiles for patients susceptible to benefit from Etanercept treatment have been defined [7]. Advantages of treatment with Etanercept are its flexibility which permits personalized adjustments ac-

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ording to disease severity and duration of remission [7]. The extent of these adjustments has, however, remained difficult to anticipate in the absence of validated biological parameters of disease activity [7]. For example, C reactive protein (CRP), a broadly used marker of inflammation in many clinical settings, has its use restricted in psoriasis to untreated patients [8].

The inflammatory burst inherent to psoriasis induces changes in the serum levels of pro-inflammatory cytokines [9, 10] and complement components [11]. We have thus searched for changes in serum protein components that correlated with effectiveness of Etanercept during patient follow-up. To this end, we used SELDI-TOF spectrometry to provide an overview of over-represented or under-represented serum constituents during therapy. Once characterized, the nano LC-ESI/MS has been subsequently used to identify such proteins. We have shown that in patients responding to Etanercept treatment factor H, a member of the complement system involved in inflammatory dermatoses [12], specifically correlates with disease severity.

Materials and methods

Overview of study design

This study aimed at identifying serum proteins in patients with moderate to severe psoriasis (n=10) whose levels correlated with disease improvement under therapy with Etanercept. Blood samples were collected at baseline (week 0/WO) and thereafter at week 12 (W12) and week 24 (W24) during treatment. Blood samples from age-matched healthy individuals (n=10) provided controls (C). Sera were initially fractionated in four fractions (F1 to F4) using Proteominer™ and a single-run sequential elution kit; non-fractionated (NF) serum was also used in parallel experiments. For each fraction serum proteins were captured on six different types of protein chips (ProteinChip®) and exposed to energy absorbing molecules (EAMs), either sinapinic acid (SPA) or α -cyano-4-hydroxycinnamic acid (CHCA), before subsequent SELDI-TOF analysis. This generated a total of 60 combinations, herein termed conditions (5 sample types, *i.e.* F1-F4 plus NF \times 6 protein chips \times 2 EAMs). Conditions allowing detection of the largest number of masses with the lowest redundancy were selected for each serum

fraction. SELDI-TOF data were acquired for low and high molecular masses as detailed below. Data for each condition were processed through Data Manager software 4.1 (Bio-Rad). For comparisons between groups of individuals (C, WO, W12 and W24) *p*-values were estimated with non-parametric tests. The Mann-Whitney test was used when comparing C to WO (unpaired data), and the Wilcoxon signed-rank test when comparing WO to W12, or WO to W24 (paired data). Values lower than 0.01 were considered as significantly different. Peptides showing statistically significant changes between C and WO, and restoration to normal values under Etanercept treatment from WO to W12 (or W24), were resolved by SDS-PAGE and further characterized by nano LC-ESI/MS aided by the Uniprot protein sequence database.

Patients and blood sample collection

No patient suffering from psoriasis had received systemic treatment or phototherapies for 3 months (washout period) prior to initiation of treatment with Etanercept. Patients presenting other skin diseases, diabetes, cardiovascular, hepatic or renal diseases and inflammatory or infectious diseases were not included in this study. Adult patients in an active phase of chronic plaque-type psoriasis were recruited after giving written consent.

The protocol used for this study was approved by the local Ethics Committee. Consecutive patients with inclusion criteria for biological treatment were enrolled to obtain a total of 10 patients. Etanercept was administered subcutaneously weekly at a dose of 50 mg. Blood samples from fasted subjects (12 mL) were collected before treatment (WO) and at week 12 (W12) and 24 (W24) during treatment. Controls (noted C) were matching healthy volunteers of similar age and gender with normal haematological and biochemical parameters. After clotting (30 min, 20°C) blood samples were centrifuged (2,200 g, 30 min, 4°C). One percent of Sigma P8340 anti-protease mixture was added to collected supernatants. Aliquots (1.2 mL) were kept frozen at -80°C. For serum fractionation, defrost serum samples were centrifuged at 10,000 g for 5 min. Five aliquots (20 μ L) of non-fractionated (NF) serum were stored at -80°C for further SELDI-TOF analysis while 1 mL was submitted to sequential serum frac-

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tiation. This was achieved using the Proteominer™ kit (Bio-Rad #163-3000) together with the sequential elution reagent kit (Bio-Rad #163-3003). Column conditioning, loading of samples on columns and sequential elution were performed according to the manufacturer guidelines. Thus, 4 fractions were obtained (hereafter called F1, F2, F3 and F4) and 20 µL aliquots were stored at -80°C for subsequent SELDI-TOF analysis. All the serum samples were fractionated within a single run.

SELDI-TOF procedure

For protein binding on ProteinChips the H50, CM10, Q10, IMAC 30 with either Cu²⁺ immobilized ions (IMAC/Cu) or with Zn²⁺ immobilized ions (IMAC/Zn) ProteinChip® arrays were used for SELDI-TOF analysis. Conditioning, sample incubation, and washings of the ProteinChip® arrays were performed with the Bio-Rad ProteinChip® Bioprocessor®. To immobilize Cu²⁺, Zn²⁺ or Ni²⁺ on the IMAC 30 surface, spots of the arrays were treated with 200 µL of 100 mM CuSO₄, ZnCl₂ or NiSO₄ respectively. Excess metal ions were removed by 2 washes with 200 µL of distilled water. All spots of the ProteinChip® arrays were similarly treated namely equilibration, neutralization when required, binding of the sample proteins, and washing steps, according to procedures recommended by the manufacturer. Binding buffers were 5% acetonitrile and 0.1% trifluoroacetic acid in 150 mM NaCl for H50, pH4 (100 mM sodium acetate) for CM10, pH9 (100 mM Tris buffer) for Q10 and PBS supplemented with 500 mM NaCl for IMAC/Cu or IMAC/Zn. Aliquots of samples were defrost and diluted 10 times with the appropriate binding buffer. Triplicate spots per sample received each 50 µL of the diluted sample and were incubated for 30 min at room temperature. The energy absorbing molecules (EAM) were either sinapinic acid (SPA) at 56 mM or α-cyano-4-hydroxycinnamic acid (CHCA) at 26 mM in acetonitrile-water (50/50, v/v) containing 0.5% trifluoroacetic acid. After protein binding, dried spots received twice 1 µL of the EAM solution.

ProteinChip®-captured proteins were detected using a ProteinChip® SELDI-TOF reader PCS4000 MS-TOF Enterprise (Bio-Rad). For ProteinChips® with CHCA EAM, one over 3 partitions was read up to 20 kDa, with matrix

attenuation of 500 Da and a focus mass of 5,000 Da. Data (m/z = molecular mass/charge) are the average of 30 shots with a laser intensity of ~200 nJ. Mass calibration was performed with the ProteinChip® peptide calibrating kit (Bio-Rad, C10-00002). For ProteinChips® with SPA EAM, one over 4 partitions was read up to 50 kDa, with matrix attenuation of 1,000 Da and a focus mass of 8,000 Da for acquisition of low MW mass spectra. A laser intensity of ~250 nJ was then used. A second partition was used for acquisition of high MW mass spectra from 30 to 300 kDa. Matrix attenuation was set at 10,000 Da with a focus mass of 35,000 Da and with a laser intensity of ~500 nJ. For both low and high laser intensities, thirty shots were averaged for signal acquisition. Mass calibration was obtained with the ProteinChip® protein calibrant kit (Bio-Rad, C10-00001). Each sample was analysed in triplicate, *i.e.* spotted in triplicates on the ProteinChip® arrays.

Among 60 conditions (5 sample types (F1-F4 and NF) × 6 ProteinChip® types × 2 EAM types), twelve conditions for which the largest number of masses was detected with the lowest redundancy from one condition to the other were determined from a preliminary study with normal serum. These conditions led to associate F1 with CM10 and SPA, IMAC/Cu and CHCA, IMAC/Zn and CHCA, F2 with IMAC/Ni and SPA, Q10 and SPA, H50 and CHCA, F3 with Q10 and SPA, F4 with Q10 and SPA, IMAC/Zn and CHCA, CM10 and SPA. Non-fractionated serum (NF) was analysed with IMAC/Cu and CHCA and IMAC/Cu and SPA.

Data analysis and statistical treatment

Data for each experimental condition (type of ProteinChip®, type of EAM) were individually processed according to manufacturer recommendations using the ProteinChip® Data Manager software 4.1 (Bio-Rad). To avoid matrix interferences, signals below about 1,000 Da for CHCA, below 1,500 Da for SPA at low laser intensities, and below 13,000 Da for SPA at high laser intensities were removed. Spectra were then analysed as per manufacturer recommendations, *i.e.* after baseline subtraction and spectra alignment, and normalized through use of the total ion current. A histogram of the normalization factors was built and spectra

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Table 1. Characteristics of patients included in this study

Patient Number	Sex	Age	Previous Treatments	PASI WO	PASI W12	PASI W24
1	M	33	MTX	11.0	0.9*	1.6*
2	F	61	PUVA	10.5	2.1*	2.1*
3	M	54	PUVA, MTX, Acitretin	12.9	4.3*	3.9*
4	F	35	PUVA, CsA	16.9	2.8*	5.4*
5	M	29	PUVA	12.0	4.9**	1.8*
6	M	61	PUVA, nbUVB, MTX	14.4	5.6**	2.4*
7	M	48	PUVA, Acitretin	10.5	10.1***	8.0***
8	M	36	CsA, Acitretin	12.9	12.2***	8.1***
9	F	30	Acitretin	11.8	4.1**	2.8
10	M	66	MTX	15.8	7.2**	3.0

M, male; F, female; PASI, Psoriasis Area Severity Index; W, week; MTX, methotrexate; CsA, cyclosporine; PUVA, psoralen and ultraviolet A light therapy; nbUVB, narrowband ultraviolet B light therapy. Age (years). *responder, **partial responder, ***non-responder.

with a normalization factor exceeding 2 SD from the mean were discarded. Then peaks were auto-detected and clustered with an S/N ratio equal to 5 and a valley depth equal to 3. A peak threshold percentage equal to 12.5% of the total number of spectra was chosen. All first pass peaks were preserved, and a cluster completion was achieved within a cluster mass window equal to 0.1% of the mass using a second pass with both S/N ratio and valley depth equal to 3. Estimated peaks were added to complete the clusters. Then, clusters with poorly resolved peaks or a poor baseline and those with a mean S/N ratio lower than 4 compared to the group exhibiting the largest mean intensity were discarded. For each cluster (*i.e.* each mass), and each patient (before and after treatment) and each control, the mean of the intensities of the triplicates was used for the following statistical analyses: WO vs C, WO vs W12 and WO vs W24. The *p*-values were calculated with the non-parametric Mann-Whitney test when comparing C to WO (unpaired data), and with the non-parametric Wilcoxon signed-rank test when comparing WO to W12, or WO to W24 (paired data; same individual over time). Only *p*-values lower than 0.01 have been considered as significantly different. For illustration, **Table 2** under “Results” shows masses for which at least one *p*-value is statistically significant for either WO vs C, or WO vs W12 or WO vs W24.

Nano LC-ESI/MS procedure

The F2 fractions from patients 4, 5, 7, 8, 9 and those from matching controls (C) previously

prepared for the SELDI-TOF study (see **Table 1** under “Results”) were pooled for the nano LC-ESI/MS. Three pooled F2 fractions gathering together either healthy controls (C), patients before treatment (WO), or after 24 weeks of treatment (W24) have thus been used to identify the species of 4.3 and 134 kDa. Briefly, each fraction F2 was divided into 2 parts. One part was utilized for identification of the protein at 134 kDa after prior separation by SDS PAGE. The subsequent nano LC-ESI/MS analysis with a Q Exactive Orbitrap was performed after gel digestion. The other part was saved for the tentative identification of the 4.3 kDa peptide by nano LC-MS and by MALDI-TOF/TOF (matrix assisted laser desorption and ionization - time of flight/time of flight) after separation by filtration with a 10 kDa filter. All procedures were done in duplicate.

SDS-PAGE

Sample proteins (20 µg) were resolved by SDS-PAGE using 12.5% polyacrylamide gels prepared as previously described [13]. Gels were stained with BlueSafe® solution for 15 minutes under agitation and then scanned with a Gel Doc XR System (Bio-Rad). Gel lanes in the rather broad mass range of 75-200 kDa that centred on ~135 kDa were manually excised from the gel, transferred to micro-tubes and further digested with trypsin.

In gel protein digestion

Gel pieces were washed once with 25 mM ammonium bicarbonate (AMBIC), three times with 25 mM AMBIC/50% acetonitrile (ACN; VWR Chemicals), at 37°C and 300 rpm and then once with 100% ACN at room temperature. Cysteine residues were reduced with 6.5 mM DTT for 60 min at 56°C. Next, they were washed with 100% ACN. Alkylation was performed with 54 mM iodoacetamide, in the dark, at room temperature. Gel pieces were subsequently washed twice with 25 mM AMBIC/50% ACN, once with 100% ACN and dried in a SpeedVac (Thermo Savant). Then, they were rehydrated with digestion buffer containing 12.5 µg/mL sequence grade modified por-

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Table 2. Peaks of SELDI-TOF clusters showing significant differences for WO vs C, WO vs W12, and WO vs W24

Mass (Da)	SELDI-TOF condition	WO vs C (ratio)	WO vs W12	WO vs W24	Group
2262	F1-IMAC/Zn-CHCA	0.003 (1.10)	NS	NS	G1
4151	F2-Q10-SPA-Low laser	0.007 (1.40)	NS	NS	G1
6527	F2-Q10-SPA-Low laser	0.01 (0.75)	NS	NS	G1
6639	F1-IMAC/Zn-CHCA	0.008 (1.06)	NS	NS	G1
9118	F1-CM10-SPA-Low laser	0.0002 (1.23)	NS	NS	G1
9138	F1-CM10-SPA-Low laser	0.001 (1.15)	NS	NS	G1
1803	F4-IMAC/Zn-CHCA	NS (1.35)	NS	0.01	G2
2611	F4-IMAC/Zn-CHCA	NS (1.30)	NS	0.007	G2
3951	F2-Q10-SPA-Low laser	NS (2.50)	NS	0.01	G2
28081	F3-Q10-SPA-Low laser	NS (0.94)	0.005	0.007	G2
43407	F2-Q10-SPA-High laser	NS (0.79)	NS	0.006	G2
44103	F2-Q10-SPA-High laser	NS (0.80)	NS	0.006	G2
45463	F2-Q10-SPA-High laser	NS (0.86)	NS	0.007	G2
2874	F2-H50-CHCA	0.008 (1.50)	NS	NS	G3
4138	F4-CM10-CHCA	0.006 (1.35)	NS	NS	G3
4710	F3-Q10-SPA-Low laser	0.002 (0.81)	NS	NS	G3
9144	F2-IMAC/Ni-SPA-Low laser	0.003 (1.29)	NS	NS	G3
14041	F2-Q10-SPA-Low laser	0.008 (1.22)	NS	NS	G3
14138	F2-Q10-SPA-Low laser	0.01 (1.17)	NS	NS	G3
33440	F2-Q10-SPA-Low laser	0.007 (2.51)	NS	NS	G3
33451	F2-Q10-SPA-High laser	0.005 (2.30)	NS	NS	G3
34405	F2-Q10-SPA-Low laser	0.004 (2.15)	NS	NS	G3
34421	F2-Q10-SPA-High laser	0.003 (1.82)	NS	NS	G3
35328	F2-Q10-SPA-Low laser	0.005 (2.33)	NS	NS	G3
35367	F2-Q10-SPA-High laser	0.002 (1.74)	NS	NS	G3
37457	F3-Q10-SPA-High laser	0.003 (0.73)	NS	NS	G3
95351	F2-Q10-SPA-High laser	0.01 (1.27)	NS	NS	G3
176952	F1-CM10-SPA-High laser	0.007 (0.55)	NS	NS	G3
4326	F2-IMAC/Ni-SPA-Low laser	0.0002 (9.45)	0.01	0.006	G4
6632	F4-Q10-SPA-Low laser	0.008 (0.79)	0.005	0.007	G4
133977	F2-Q10-SPA-High laser	0.007 (8.38)	NS	0.01	G4

WO, W12 and W24 correspond to duration of Etanercept treatment in weeks. *P*-values were calculated with the non-parametric Mann-Whitney test when comparing C (controls) to WO (unpaired data), and with the non-parametric Wilcoxon signed-rank test when comparing WO to W12, or WO to W24 (paired data). Values lower than 0.01 were considered as significantly different. Within brackets are ratios of peak intensities (3rd column). The second column depicts specific experimental SELDI-TOF conditions described in the following order: fraction (F) number - ProteinChip® type - EAM (energy absorbing molecule) type - Laser intensity (cf experimental section for further details on nomenclature). Note that laser intensity was specified only when SPA (sinapinic acid) was used as the EAM. The last column corresponds to groups used for classifying intensity peaks (cf results).

cine trypsin (Promega) in 25 mM AMBIC. After 60 min on ice the excess supernatant was removed and discarded, 100 µL of 25 mM AMBIC were added and samples were incubat-

ed overnight at 37°C. Extraction of tryptic peptides was performed by the sequential addition, and incubation at room temperature, of 10% formic acid (FA, Fluka), three times with 10% FA/50% ACN, and finally with 100% ACN. Recovered peptides after each step were pooled in one microtube and lyophilized in a SpeedVac (Thermo Savant).

Nano LC-ESI/MS analysis

Samples were re-suspended in 20 µL of 5% ACN/0.1% FA solution and analysed using a QExactive Orbitrap (Thermo Fisher Scientific, Bremen) that was coupled to an Ultimate 3000 (Dionex, Sunnyvale, CA) HPLC (high-pressure liquid chromatography) system. The trap (5 mm × 300 µm I.D.) and analytical (150 mm × 75 µm I.D.) columns used were C18 Pepmap100 (Dionex, LC Packings), the latter having a particle size of 3 µm. Peptides were trapped at 30 µL/min in 95% solvent A (0.1% FA/5% ACN v/v). Elution was achieved with solvent B (0.1% FA/100% ACN v/v) at 300 nL/min. The 50 min gradient used was as follows: 0-3 min, 95% solvent A; 3-35 min, 5-45% solvent B; 35-38 min, 45-80% solvent B; 38-39 min, 80% solvent B; 39-40 min, 20-95% solvent A, and 40-50 min, 95% solvent A. Nanospray was achieved using an uncoated fused silica emitter (New Objective, Cambridge, MA) (o.d. 360 µm; i.d. 50 µm, tip i.d. 15 µm) biased to 1.8 kV. The mass spectrometer was operated in the data-dependent acquisition mode. A MS2 method was used with a FT survey scan

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from 375 to 1600 m/z (resolution 35.000; AGC target 3E6). The 10 most intense peaks were subjected to HCD fragmentation (resolution 17.500; AGC target 5E4, NCE 25%, max. injection time 120 ms, dynamic exclusion 35 s).

Data processing

Spectra were processed and analysed using Proteome Discoverer (version 2.0, Thermo), with the MS Amanda search engine (version 2.1.4.3751, University of Applied Sciences Upper Austria, Research Institute of Molecular Pathology). Uniprot (TrEMBL and Swiss-Prot) protein sequence database (version of May 2016) was used for all searches under *Homo sapiens* (Human). Database search parameters were as follows: carbamidomethylation and carboxymethyl of cysteine as a variable modification as well as oxidation of methionine, and allowance for up to two missed tryptic cleavages. The peptide mass tolerance was 10 ppm and fragment ion mass tolerance was 0.05 Da. To achieve a 1% false discovery rate, the Percolator (version 2.0, Thermo) node was implemented for a decoy database search strategy. Peptides were filtered for high confidence and a minimum length of 6 amino acids whereas proteins were filtered for a minimum number of peptide sequences of 2 and only rank-1 peptides. Protein quantitation was performed using the precursor ion area detection which calculates the protein peak area on the basis of the top three unique peptides for the given protein.

Results

Patient status

All patients had been diagnosed with psoriasis at least 5 years earlier and had been previously treated as described in **Table 1**. In this study psoriasis severity was evaluated using PASI. Current guidelines for Etanercept establish a primary endpoint at 12 weeks. Patients were considered responders whenever there was a reduction in 75% of baseline PASI after 12 weeks of treatment or partial responders when PASI was $\geq 50\%$ and $< 75\%$. We note that it is accepted that some subgroups of patients not reaching remission at week 12 may still show some improvement at week 24.

SELDI-TOF

In this research, serum samples from psoriasis patients and matching healthy controls were sequentially fractionated yielding four fractions (F1-F4); non-fractionated (NF) serum was also utilized. Subsequently, proteins in fractions were made to bind to protein chip arrays before exposure to the energy absorbing molecules (EAM) sinapinic acid/SPA or α -cyano-4-hydroxycinnamic acid/CHCA. Protein chip-captured proteins were then detected using a ProteinChip® SELDI-TOF reader. This protocol generated 60 combinations, hereafter termed conditions (5 sample types (F1-F4 plus NF) \times 6 ProteinChip® types \times 2 EAM types), from which twelve conditions showing the highest number of masses with the lowest redundancy between conditions were selected (cf “Materials and methods”).

Using these twelve conditions about 1,180 clusters were detected, each cluster corresponding to a molecular mass detected in at least one experimental group comprising either patients at specific time-points of Etanercept treatment or healthy controls. As expected, redundancies have been found since some clusters appeared on the same protein chip array with either CHCA or SPA as the EAM, or when scanned at low or high laser energy (see experimental section). Among these clusters we selected those exhibiting a significant difference for C (healthy controls) vs W0, W0 vs W12 and W0 vs W24 (cf “Materials and methods”), with W0 corresponding to patient samples before treatment, and W12 and W24 to samples obtained after 12 and 24 weeks of treatment onset, respectively (**Table 2**).

Thirty one masses could be schematically classified into 4 groups. Group 1 (G1) comprises masses for which a significant difference was found for C vs W0 with no further changes observed after 12 or 24 weeks. The difference between C and W0, corresponding to either an increase or a decrease in the peak intensity, was always low (cf **Table 2**). Therefore, these masses were considered of minor interest.

In a second group (G2), an increase or a decrease in the peak intensity was observed from C to W0. With the exception of the 3.9 kDa mass, these changes were always modest and

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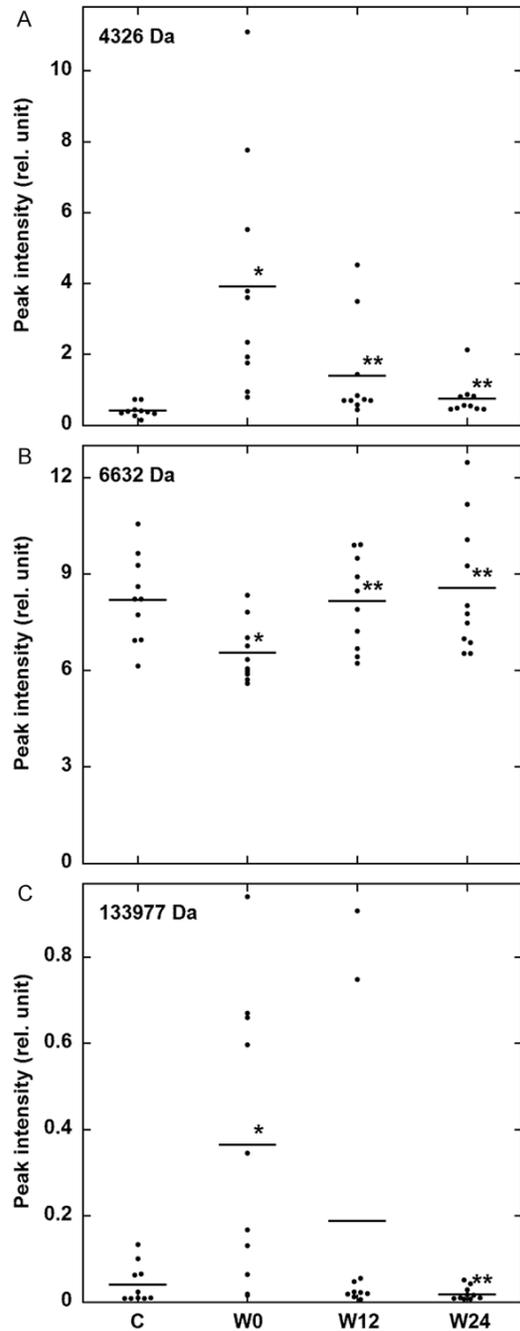


Figure 1. Progression of specific intensity peaks within mass profiles during Etanercept treatment. Shown is the progression of intensities (relative units) of the 4326 Da (A), 6632 Da (B) and 133977 Da (C) peaks of the mass profiles over time. W0, W12 and W24 correspond to duration of Etanercept treatment expressed in weeks. Samples exhibiting a significant difference for C (healthy controls) vs W0, W0 vs W12 and W0 vs W24 are shown. Horizontal lines correspond to the mean of the 10 samples for each condition. *P*-values were calculated using the non-parametric Mann-Whitney test when comparing C to W0 (unpaired data), and the non-parametric Wilcoxon signed-rank test when comparing W0 to W12,

or W0 to W24 (paired data). *Statistically significant difference between C and W0. **Statistically significant difference between W0 and W12, or between W0 and W24. The 4.3 and 134 kDa masses were detected using fraction 2, and the 6.6 kDa mass was detected using fraction 4. ProteinChip®, matrix and laser intensity conditions are detailed in Table 2.

not statistically significant (Table 2). Nevertheless, this group was considered relevant since we observed a return to basal level of the peak intensity in patients after 12 or 24 weeks of Etanercept treatment. This restoration was progressive over time and statistically significant (W0 vs W24 or even W0 vs W12; Table 2). In the third group (G3) a statistically significant but moderate increase or decrease in the peak intensity was found between C and W0. As in group 2, a return to control level was clearly observed after 12 or 24 weeks of treatment but this was not statistically significant (data not shown). An illustration of the redundancies mentioned above is provided in this group (G3) for the 33.4, 34.4 and 35.3 kDa masses obtained using fraction F2 plus a Q10 ProteinChip® at low laser energy, and the 33.4, 34.4 and 35.4 kDa masses at high laser energy. Finally, the 4.3, 6.6 and 134 kDa masses comprised a fourth and more interesting group (G4). In this group, changes from C to W0, and restoration to baseline from W0 to W12, and from W0 to W24 were statistically significant as shown in Figure 1. The 4.3 and 134 kDa masses were particularly interesting since intensities in controls (C) were close to zero and the peak intensity ratios (W0/C in Table 2) were 9.45 and 8.4, respectively. The 6.6 kDa mass was less interesting since the ratio between C and W0 although significant was not far from 1 (Table 2 and Figure 1).

Figure 2A illustrates the changes in mass spectral profiles recorded in the 120-180 kDa region. For clarity, the spectral profiles are only shown for patients 7 (P7) and 10 (P10). It can be observed that the 134 kDa peak intensity is readily reduced to control levels by 24 weeks of treatment. It is of note that the same behaviour holds for the 4.3 kDa mass (Figure 2B), with the exception of patients 7 and 8 whose PASI score had not yet changed after 12 weeks under Etanercept treatment. We note that we could not identify the 4326 m/z value through the direct analysis of fraction F2 with a 4800 MALDI-TOF/TOF mass spectrometer (AB Sciex) using two different matrices, SPA and CHCA.

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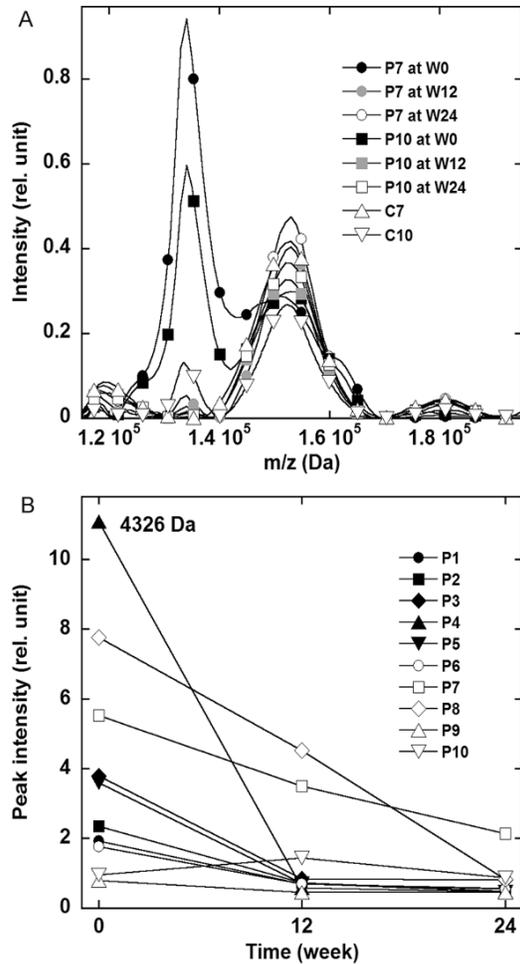


Figure 2. Protein profiling by SELDI reveals that intensities of the 134 kDa and 4.3 kDa peaks decline to baseline levels under Etanercept treatment. A. Mass profiles in the range of 120,000 (1.2×10^5) Da to 200,000 (2.0×10^5) Da were obtained from fractionated serum samples of two patients (P7 and P10) at specific times of treatment with Etanercept. Corresponding spectra are also shown for matching controls (C7 and C10). The shown spectra are the average of triplicate SELDI-TOF measurements for each condition. Spectra were analysed after baseline subtraction and spectra alignment, and normalized using the total ion current values. Note that for P7 and P10 the 134 kDa peak intensity is reduced to control levels by 24 weeks of treatment. B. Progression of the intensity of the 4.3 kDa peak for all patients as a function of treatment duration. Data are the average of replicate SELDI-TOF measurements in triplicates and have been processed as described above. Note that with exception of patients 7 and 8 (P7 and P8), whose PASI score had not yet changed after 12 weeks of Etanercept treatment, baseline levels are reached already at 12 weeks of treatment.

Nano LC-ESI/MS

To further characterize fraction 2 (F2) by nano LC-ESI/MS and to identify the protein of 134

kDa mass suggested by the SELDI-TOF analysis, we performed a prior separation by SDS-PAGE (**Figure 3**). Gel bands were excised and digested with trypsin, and the proteins subsequently identified by nano LC-ESI/MS. Seventy-two distinct proteins common to all groups were retrieved within this rather broad mass range (~ 75 -250 kDa; $P < 0.05$). The majority of identified proteins were serum proteins belonging to the complement system, the coagulation system and immunoglobulins (data not shown). Ten proteins were detected in patient samples in significant amounts (**Table 3**).

It was possible to observe a tendency to a decline in the relative abundance of several human immunoglobulin lambda light chain constant regions (IGLC2, IGLC3, IGLC6 and IGLC7) identified in both W0 and W24 patient samples. Of note, these proteins have a MW which is almost 11 kDa deviant from the MW range excised from gels. It is, however, well established that IGLCs have a strong tendency for dimerization and oligomerization [14]. This may explain the discrepancy between their predicted MW [15] and that obtained here, even considering the strong reducing conditions of SDS-PAGE.

Of all identified proteins, the 169 kDa alpha-2-macroglobulin featured the highest (12 times higher) increase from healthy controls to patients with psoriasis (data not shown). Only two out of the 10 serum proteins reported in **Table 3** have a molecular mass within a range consistent with the 134 kDa species found by SELDI-TOF. Using the protein sequence database resource these two proteins correspond to complement factor H (139 kDa) and ceruloplasmin (122 kDa) whose accession numbers are provided in **Table 3**. These proteins may harbour post-translational modifications, and a broad range of masses has been reported for serum proteins due to the presence of many isoforms [16]. Ceruloplasmin and complement factor H are glycosylated species and, as such, match the expected MWs. The concentration of ceruloplasmin in serum is about 0.3 g/l in healthy subjects and is only slightly increased in moderate and severe psoriasis [17]. Little variation in the peak intensity detectable with mass spectrometry is thus anticipated. Therefore, the only protein which shows a clear time-dependent decrease in its intensity and a return to low, baseline levels during Etanercept treatment in the m/z region extending

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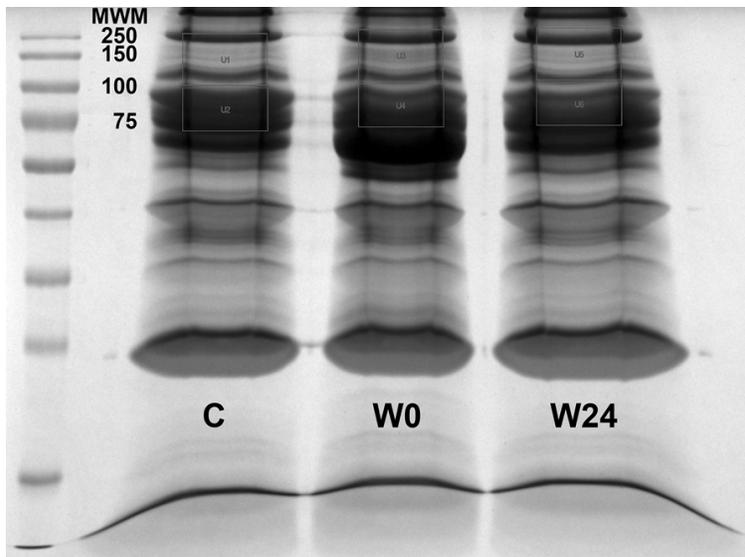


Figure 3. SDS-PAGE profiles of proteins before identification by nano LC-ESI/MS. Pooled protein samples of serum fraction 2 (F2; 20 mg of protein) were resolved by SDS-PAGE (12.5% acrylamide) and are shown for healthy controls (C), patients before initiation of treatment with Etanercept (W0), and patients after 24 weeks of treatment (W24). Gel bands comprised within a range of molecular masses of interest (~75-250 kDa) were excised (U1-6) and digested with trypsin in preparation for subsequent identification of the 134 kDa species by nano LC-ESI/MS. MWM - Molecular Weight Marker.

Table 3. Proteins identified in patient groups according to Uniprot protein sequence database

Accession	Description	MW (Da)
P01023	alpha-2-macroglobulin	163,188
P08603-1	Complement factor H	139,005
P00450	Ceruloplasmin	122,128
P05155-3	Isoform 3 of Serum protease C1 inhibitor	55,734
P05155	Serum protease C1 inhibitor	55,119
P05155-2	Isoform 2 of Serum protease C1 inhibitor	49,726
P01857	Ig gamma-1 chain C region	36,083
P01859	Ig gamma-2 chain C region	35,878
P02751	Fibronectin	262,46
P02751-8	Isoform 8 of Fibronectin	252,652

from 120 to 170 kDa is complement factor H (Figure 2A).

Discussion

Psoriasis is a chronic T-cell-mediated inflammatory skin disease. Mediators of inflammation found in skin plaques in psoriasis have been identified in the blood of patients with mild and severe disease [18]. As a result, correlations between concentrations of C-reactive protein (CRP), alpha-2-macroglobulin, C3 and C4 com-

plement proteins, and of complement-modulating proteins such as factor F and factor I, and disease severity have been attempted [12, 19]. Unfortunately, although CRP is a useful marker of inflammation in several clinical settings, in psoriasis CRP provides a measure of disease activity only in untreated patients [7].

Etanercept is expected to modulate several pathways in which TNF α is involved in psoriasis [20]. Also expectedly, the concentration of numerous serum components should be altered during the course of treatment. Herein, we have searched for those that specifically and unambiguously changed during the follow-up of patients treated with Etanercept.

The mean PASI score of patients enrolled in this study was 12.9 ± 2.9 (Table 1). Their psoriasis can therefore be considered as moderate to severe. Among 31 serum components revealed by SELDI-TOF, which deals with intact proteins, we have shown that protein species of 4.3 kDa, 6.6 kDa and 134 kDa were downregulated in a time-dependent manner during treatment (Table 2; also Figure 2A, 2B). The notable peak intensities of the 4.3 and 134 kDa proteins prompted us to undertake their identification using nano LC-ESI/MS coupled to a protein sequence database after prior separation by SDS-PAGE (Figure 3). For unknown reasons the 4.3 kDa species could not be identified by nano LC-ESI/MS, but this species deserves future special attention since it also showed a consistent, time-dependent decay during the follow-up period.

Among the specific proteins detected in higher amounts in the region of high molecular masses (Table 3), but outside the range of interest, stands the constant region of several immuno-

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globulin light chains, consistent with a role for B-cells in psoriasis [21]. Also, using nano LC-ESI/MS we could detect a high level of the 169 kDa alpha-2 macroglobulin, a carrier of immunological mediators such as cytokines and growth factors in serum. However, its levels do not return to baseline after 24 weeks of Etanercept treatment. Indeed, high alpha-2 macroglobulin levels may rather reflect the general health status of the patient [22].

The detection of ceruloplasmin - the Cu^{2+} carrier in blood - in the 134 kDa region was not surprising [17] since the molecular mass attributed to ceruloplasmin and its isoforms ranges from 122 kDa to 129 kDa [23]. However, serum concentrations of ceruloplasmin are hardly altered in psoriasis [17]. SELDI-TOF spectrometry clearly established that among multiple peaks the 134 kDa peak is the only one in the m/z region extending from 120 to 170 kDa (**Figure 2A**). Interestingly, this peak featured a clear time-dependent decrease in intensity and a return to a low baseline level during treatment with Etanercept (**Figure 2A**). Using a protein database this 134 kDa peak could be identified as complement factor H. We note that Ohkohchi *et al.* have previously shown a relationship between disease activity, extent of skin lesions, and factor H levels before and after successful treatment of psoriasis with steroids [12].

Factor H is the main soluble negative regulator of the alternative complement pathway by inhibiting the assembly of C3 and C5 convertases and facilitating their disassembly, and by acting as a cofactor for factor I in the inactivation of C3b [24, 25]. Moreover, factor H also has the capacity to bind to the surface of host cells and to tissues to limit local complement-mediated inflammation [24, 25]. Factor H is constitutively expressed in the liver being systemically distributed in body fluids [24, 25]. Besides hepatocytes, low levels of factor H are further synthesised by several other cell types including monocytes [26], B-cells [27] and fibroblasts [28], but also keratinocytes, the most abundant cells in the epidermis [24, 25].

Interferon gamma (IFN γ), along with other cytokines namely IL-17, IL-23, IL-22 and TNF α , plays a major role in the pathogenesis of psoriasis [20]. Interestingly, IFN γ stimulates the produc-

tion of factor H in cultured keratinocytes [29]. In the context of the pathophysiology of psoriasis epidermal keratinocytes may thus be induced to synthesize increased amounts of factor H in response to the locally high levels of IFN γ produced by infiltrating Th1 cells [20]. Conversely, blockade of TNF α with Etanercept imposes a reduced activity of the dendritic cell-Th1-IFN γ axis [20] that could lead to down-regulation of factor H production by epidermal keratinocytes. Although this could explain both the higher levels of factor H found here in patients with psoriasis and their reduction under treatment with Etanercept it must remain a hypothetical scenario. Indeed, the relative contribution of hepatic vs extra-hepatic (eg, skin) sources of factor H remains to be established in psoriasis. In either case, factor H may act as protective against inflammation-associated tissue damage in psoriasis.

The number of patients enrolled in our analysis is still reduced, which represents a limitation of this pilot study. Clearly, further studies using larger cohorts shall tell whether the observed decrease in factor H levels in the serum of responders is maintained after longer periods of treatment. It will also be relevant to study whether a similar response is observed with other currently available therapies for psoriasis. Also, whether after disease remission progressively increased levels of factor H herald the onset of recurrence. If so, since simple and reliable biological tests for assessment of factor H are already available, determination of factor H levels may become a useful tool to follow remission or the onset of relapse in the follow-up of patients under treatment with Etanercept.

Conclusions

Herein, we have shown using mass spectrometry techniques that in patients suffering from moderate to severe psoriasis complement factor H returns to baseline levels after 24 weeks of treatment with Etanercept, a TNF α -targeting drug. These observations deserve further analyses utilizing larger cohorts of patients. In the future, determination of factor H levels may become a complementary tool to follow remission or anticipate the onset of relapse in the follow-up of patients undergoing treatment for psoriasis.

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Adult patients in an active phase of chronic plaque-type psoriasis were recruited after giving written consent. The protocol used for this study was approved by the local Ethics Committee (Hospital Santa Maria-Centro Hospitalar Lisboa Norte, Lisbon).

Disclosure of conflict of interest

None.

Abbreviations

PASI, psoriasis area and severity index; SELDI-TOF, surface enhanced laser desorption and ionization-time of flight; nano LC-ESI/MS, nano liquid chromatography-electrospray ionization/tandem-mass spectrometry; MALDI-TOF/TOF, matrix assisted laser desorption and ionization-time of flight/time of flight; SPA, sinapinic acid; CHCA, α -cyano-4-hydroxycinnamic acid; EAM, energy absorbing molecule.

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