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## **Systems Biology based studies on anti-inflammatory compounds**

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### **Citation**

Verhoeckx, K. C. M. (2005, November 14). *Systems Biology based studies on anti-inflammatory compounds*. Retrieved from <https://hdl.handle.net/1887/3744>

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# Chapter 4

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**In search of secreted protein biomarkers for the anti-inflammatory effect of  $\beta_2$ -adrenergic receptor agonists: application of DIGE technology in combination with multivariate and univariate data analysis tools.**

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*Accepted for publication in J Proteome Res*

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**In search of secreted protein biomarkers for the anti-inflammatory effect of  $\beta_2$ -adrenergic receptor agonists: application of DIGE technology in combination with multivariate and univariate data analysis tools.**

*Two-dimensional difference gel electrophoresis (DIGE) in combination with univariate (Student's t-test) and multivariate data analysis, principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) were used to study the anti-inflammatory effects of the  $\beta_2$ -adrenergic receptor ( $\beta_2$ -AR) agonist zilpaterol. U937 macrophages were exposed to the endotoxin lipopolysaccharide (LPS) to induce an inflammatory reaction, which was inhibited by the addition of zilpaterol (LZ). This inhibition was counteracted by addition of the  $\beta_2$ -AR antagonist propranolol (LZP). The extracellular proteome of the U937 cells induced by the three treatments were examined by DIGE. PCA was used as an explorative tool to investigate the clustering of the proteome dataset. Using this tool, the dataset obtained from cells treated with LPS and LZP were separated from those obtained from LZ treated cells. PLS-DA, a multivariate data analysis tool that also takes correlations between protein spots and class assignment into account, correctly classified the different extracellular proteomes and showed that many proteins were differentially expressed between the proteome of inflamed cells (LPS and LZP) and cells in which the inflammatory response was inhibited (LZ). The Student's t-test revealed 8 potential protein biomarkers, each of which was expressed at a similar level in the LPS and LZP treated cells, but differently expressed in the LZ treated cells. Two of the identified proteins, macrophage inflammatory protein-1beta (MIP-1 $\beta$ ) and macrophage inflammatory protein-1alpha (MIP-1 $\alpha$ ) are known secreted proteins. The inhibition of MIP-1 $\beta$  by zilpaterol and the involvement of the  $\beta_2$ -AR and cAMP were confirmed using a specific immunoassay.*

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**Introduction**

Inflammation occurs as a defensive response to invasion of the host by foreign intruders, often of microbial nature. This response normally involves a complex series of events including macrophage activation, secretion of inflammatory mediators e.g. cytokines and chemotactic cytokines (chemokines) and recruitment of leukocytes into the inflamed area<sup>1,2</sup>. Cytokines (e.g. TNF- $\alpha$ , IL-1 $\beta$ , and IFN- $\gamma$ ) and chemokines (e.g. IL-8 and RANTES) are small secreted proteins that are involved in regulation of the network of interactions between cells during inflammation. They are involved in the onset and development of inflammation, recruit, and

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activate a range of immune cells<sup>3</sup>. Regulation of the expression of these secreted inflammatory mediators is of therapeutic importance in many inflammatory diseases like asthma, rheumatoid arthritis and many others.

Beta<sub>2</sub>-adrenergic receptor ( $\beta_2$ -AR) agonists are widely used in the treatment of pulmonary diseases, e.g. asthma. Their effect on the airways primarily involves relaxation of airway smooth muscle<sup>4-6</sup>. Binding of  $\beta_2$ -AR agonists to the  $\beta_2$ -AR activates adenylate cyclase, which subsequently elevates the intracellular level of cyclic adenosine-3',5'-cyclic monophosphate (cAMP)<sup>7-12</sup>. Cyclic AMP is a second messenger that exerts its effects via many different metabolic pathways and regulates the production of various inflammatory mediators<sup>13, 14</sup>.

In the present study we examined proteins that are secreted by macrophages in response to LPS in combination with a  $\beta_2$ -AR agonist and that are regulated via the  $\beta_2$ -AR. These proteins are potential biomarkers in monitoring the effect of  $\beta_2$ -AR agonists in the treatment of inflammatory diseases and may further elucidate the mechanism of action of these compounds. For this purpose we used the human monocytic U937 cell line that expresses the  $\beta_2$ -AR<sup>15</sup> and that has previously proved to be a suitable model system to study the effect of  $\beta_2$ -AR agonists on the inflammation response induced by the endotoxin lipopolysaccharide (LPS)<sup>15-18</sup>. The extracellular proteome of U937 macrophages exposed to LPS, LPS in combination with zilpaterol ( $\beta_2$ -AR agonist)<sup>18, 19</sup>, and LPS in combination with zilpaterol and propranolol ( $\beta_2$ -AR antagonist) were compared using 2-D difference gel electrophoresis (DIGE). The DIGE technology enables the analysis of multiple protein samples within one gel. This is achieved through covalent modification of each protein with structurally similar but spectrally distinct fluorophores (CyDye2, CyDye3, and CyDye5). On each gel two samples and an internal standard comprising an equal amount of each sample within the study can be examined. This process reduces the gel-to-gel variation and allows more accurate and sensitive quantitative proteomics studies<sup>20-22</sup>. The proteomics data was analyzed using a classical univariate data analysis tool (Student's *t*-test) and two multivariate data analysis tools (principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA)). PCA<sup>23-27</sup> was used as an explorative tool to visualize differences between the complex datasets. PLS-DA<sup>28</sup> on two groups (inflammation and inhibition of inflammation), was used to discover potential biomarkers for anti-inflammatory effects of  $\beta_2$ -AR agonists. In the literature, little attention has been paid to the validation of potential biomarkers found by multivariate data analysis, especially the biological validation. In this study the PLS-DA was

validated by cross validation, and the permutation test. Finally a biological validation was performed using enzyme-linked immunoassay.

## Materials and methods

### *Chemicals*

Unless indicated otherwise, all reagents and equipment were obtained from Amersham Biosciences (Uppsala, Sweden). Lipopolysaccharide (LPS, *E.coli* 0111:B4), propranolol, formoterol, salbutamol, dibutyryl cAMP, prostaglandin E<sub>2</sub>, and forskolin were obtained from Sigma Aldrich (St. Louis, MO, USA) and zilpaterol from Intervet Inc. (Millsboro, US).

### *Cell cultures*

Human monocyte-like histiocytic lymphoma cells U937<sup>29</sup> obtained from the ATCC (CRL-1593.2) were grown in RPMI-1640 medium, supplemented with 10% (v/v) fetal calf serum and 2 mM L-glutamine (Life technologies, Breda, The Netherlands) at 37 °C, 5% CO<sub>2</sub> in a humidified atmosphere. U937 monocytic cells were differentiated into macrophages using phorbol 12-myristate 13-acetate (PMA, 10 ng/ml, overnight, Omnilabo, Breda, The Netherlands) as described previously<sup>18,30</sup>. The PMA-differentiated macrophages were allowed to recover from PMA treatment for 48 h, during which the culture medium was replaced daily.

Peripheral blood monocytes (PB-MO) were isolated from human EDTA-blood with Rosette Sep<sup>TM</sup> human monocyte enrichment cocktail (Stemcell Technologies Inc, Meylan, France) as described previously<sup>30</sup>. The monocytes were cultured in 24-well cell culture plates containing RPMI-1640 medium supplemented with 10% (v/v) human serum and 2 mM L-glutamine and were allowed to differentiate into peripheral blood macrophages (PB-MØ) for 8 days. Following this procedure, the macrophage maturation has been shown to give rise to the morphology and phenotype that is characteristic of primary macrophages<sup>31</sup>.

### *Incubations for proteome analysis*

Per incubation 40x10<sup>6</sup> U937 monocytic cells in a 175 cm<sup>3</sup> culture flask were differentiated into macrophages using PMA. At day three after PMA treatment, the macrophages were washed 5 times with serum free culture medium. Subsequently, the cells were exposed to 1 µg/ml LPS, LPS in combination with 1x10<sup>-6</sup> M zilpaterol or LPS in combination with zilpaterol and 1x10<sup>-6</sup> M propranolol respectively, for 16 h at 37 °C, 5% CO<sub>2</sub> in a humidified atmosphere. The incubation time of 16 h was chosen based on the results of microarray

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experiments<sup>18</sup> and time course experiments<sup>32</sup>. The incubations were performed in duplicate. Finally, a protease inhibitor cocktail was added and the culture medium was filtered over a 0.45  $\mu\text{M}$  filter. The samples were stored at  $-80\text{ }^{\circ}\text{C}$  until further analysis.

#### *Incubations for MIP-1 $\beta$ analysis*

PB-M $\emptyset$  and U937 cells ( $1 \times 10^6$  cells per well) were exposed for 16 h to 1  $\mu\text{g}/\text{ml}$  LPS in the presence or absence of  $\beta_2$ -adrenergic receptor agonists; clenbuterol ( $1 \times 10^{-7}$  M), formoterol ( $1 \times 10^{-8}$  M), salbutamol ( $1 \times 10^{-6}$  M), and zilpaterol ( $1 \times 10^{-6}$  M). Furthermore, U937 macrophages were incubated with other cAMP elevating compounds, dibutyryl cAMP ( $1 \times 10^{-4}$  M), forskolin ( $1 \times 10^{-5}$  M), and prostaglandin E<sub>2</sub> ( $1 \times 10^{-4}$  M) in the presence of 1  $\mu\text{g}/\text{ml}$  LPS. The concentrations of the various compounds were chosen as such to achieve similar levels of TNF- $\alpha$  inhibition. Culture medium was collected and diluted 500 fold. The concentration of MIP-1 $\beta$  in the culture supernatants was determined using the cytosect antibody pair kit for MIP-1 $\beta$  from Biosource (Etten-Leur, The Netherlands) according to the manufacturer's protocol. The cells were lysed in 0.1 M NaOH and used for protein determination by the modified method of Bradford (Bio-Rad, Veenendaal, The Netherlands). All incubations were performed in triplicate and were corrected for protein content.

#### *Two-dimensional gel electrophoresis*

Culture media (10 ml) was thawed on ice and the proteins were precipitated by adding 1.8 ml of a 100% (w/v) TCA solution. After 45 min, the mixture was centrifuged at 3000 g and  $4\text{ }^{\circ}\text{C}$ . The pellet was washed twice with 1 ml of cold acetone and air-dried for a few minutes. The proteins were dissolved in 100  $\mu\text{l}$  of DIGE lysis buffer (8M Urea, 4% w/v CHAPS and 30 mM Tris) and the pH was adjusted to 8.5. The protein content was determined using the modified method of Bradford. Each sample (50  $\mu\text{g}$ ) was labelled with 0.8  $\mu\text{l}$  of CyDye 3 and CyDye 5 CyDye<sup>TM</sup> DIGE fluors minimal dyes (400  $\mu\text{M}$ ). The experimental design is shown in Table 1. After 30 min, the incubation was stopped by adding 1  $\mu\text{l}$  of 10 mM Lysine. The labelled samples were further diluted with an equal volume of 2 x sample buffer containing 8 M urea, 4% w/v CHAPS, 130 mM DTT (Sigma Aldrich), and 2% Pharmalyte<sup>TM</sup> 3-10. The internal standard included 50  $\mu\text{g}$  of each sample (6 samples in total) labelled with CyDye 2. Two samples (CyDye 3 and CyDye 5) and the internal standard (CyDye 2) were run per gel. The three labelled samples were mixed and the volume was adjusted to 350  $\mu\text{l}$  with rehydration buffer containing 8 M urea, 4% w/v CHAPS, 2 mM tributyl phosphine (Fluka, Buchs SG, Switzerland), and 1% (v/v) IPG ampholytes pH 4-7.

All gels, 6 in total, were processed and analyzed simultaneously.

The first dimension was carried out on an IPGphor system using pH 4-7 IPG gel strips of 18 cm. The IEF was performed at 20 °C under the following conditions: 12 h at 30 V; 30 min at 150 V; 1 h at 300 V; 1 h at 1500 V and 6 h at 8000 V. After isoelectric focussing, the IPG strips were equilibrated for 15 min in reduction buffer (6 M urea, 30% (v/v) glycerol, 1% w/v DTT, and 2% (w/v) SDS in 0.05 M Tris-HCl buffer, pH 8.8) and subsequently alkylated for 15 min in alkylation buffer containing 6 M urea, 30% (v/v) glycerol, 4.7% (w/v) iodoacetamide, and 2% (w/v) SDS in 0.05 M Tris-HCl buffer, pH 8.8. The second dimensional separations were carried out on custom made 12% SDS-polyacrylamide gels and a Hoefer DALT electrophoresis system.

**Table 1** Experimental design of the 2-D DIGE experiment

Incubation	Labeling	Gel code	Progenesis analysis	Statistical analysis
1-LPS	CyDye-3	1	3 images:	2 data sets:
	CyDye-5	1	CyDye2, CyDye3, and CyDye5	CyDye3/CyDye2 and CyDye5/CyDye2
2-LPS	CyDye-3	2	3 images:	2 data sets:
	CyDye-5	2	CyDye2 CyDye3, and CyDye5	CyDye3/CyDye2 and CyDye5/CyDye2
1-LZ	CyDye-3	3	3 images:	2 data sets:
	CyDye-5	3	CyDye2 CyDye3, and CyDye5	CyDye3/CyDye2 and CyDye5/CyDye2
2-LZ	CyDye-3	4	3 images:	2 data sets:
	CyDye-5	4	CyDye2 CyDye3, and CyDye5	CyDye3/CyDye2 and CyDye5/CyDye2
1-LZP	CyDye-3	5	3 images:	2 data sets:
	CyDye-5	5	CyDye2 CyDye3, and CyDye5	CyDye3/CyDye2 and CyDye5/CyDye2
2-LZP	CyDye-3	6	3 images:	2 data sets:
	CyDye-5	6	CyDye2 CyDye3, and CyDye5	CyDye3/CyDye2 and CyDye5/CyDye2
1-LPS + 2-LPS + 1-LZ + 2-LZ + 1-LZP + 2-LZP	CyDye-2	1 - 6		
Total	13	6	18	12

### *Gel imaging and data analysis*

The gels were scanned using the Typhoon 9400 laser scanner at three different settings (CyDye2, blue laser 488 nm and 520 bp 40 filter; CyDye3, green laser 532 nm and 580 bp 30 filter; CyDye5, red laser 633 nm and 670 bp 30 filter). Three images per gel were obtained

(18 in total). The scanned images were analyzed using Progenesis workstation 2004 with the special cross stain analysis (CSA) module for analysing multi-labelled gels (Nonlinear Dynamics, Newcastle upon Tyne, UK). Spots were automatically detected and visually checked for undetected or incorrectly detected spots. The protein spots detected in each image were automatically linked between the three images per gel. All gels were matched to a digitized reference gel, containing all the protein spots present in all 6 internal standard images.

Per image the intensity levels were normalized by dividing the spot volume through the total intensity of all the spots in the image and multiplying it by the average of the total spot intensity of all 18 gel images. Subsequently, the CyDye3 and CyDye5 labelled spot volumes were divided by the spot volume of the corresponding protein spot in the internal standard (CyDye2) image. The differences in spot ratios were analyzed by multivariate data analysis tools and the Student's *t*-test (assuming normal distributions and equal variance) using MS Excel (Microsoft Corporation, Redmond, USA).

#### *Multivariate data analysis*

A list of spots with their normalized spot volumes per gel was mean-centered<sup>27</sup>.

Subsequently, the dataset was examined by PCA and PLS-DA. PCA was applied as an exploratory data analysis method that is able to visualize differences between complex samples. The dataset can be visualized as a cloud of data points, where each data point represents a sample in a multidimensional space. The coordinates of these data points are represented by the spot intensities (dimensions). PCA reduces the large number of dimensions of a dataset into a smaller number of dimensions in such a way that most of the variance of the dataset is described by the first principal component (PC)<sup>33,34</sup>.

PLS-DA was used to cluster the gels from LPS treated U937 cells and cells treated with LPS in combination with zilpaterol and propranolol. These two groups resemble the same biological state of the cell, namely inflammation. The second group contained the gels from cells treated with LPS in combination with zilpaterol (inhibition of inflammation). Using PLS-DA a regression model can be formed between the intensity of the proteins spots (X-block) and class assignment (Y-block). In PLS-DA the scores and loadings are described as latent variables (LV).

PCA and PLS-DA were performed using the PLS toolbox (3.0.2 (2003), Eigenvector Research, Inc.) in Matlab (Version 7.0 (R14) Service Pack 1 (2004), The Mathworks).

### *Validation*

Full leave one out cross-validation was used to validate statistically the PLS-DA model. The class assignment of one sample was predicted from a calibration model consisting of the rest of the samples (11 gels). This validation was repeated for every sample. The percentage misclassification was calculated from the class predictions for all individual samples for each LV.

A permutation test was performed to test if the separation between the two assigned groups (inflammation/no inflammation) was significant. Therefore only the Y-block (class assignment) was permuted 1000 times, whereas the X-block was left unchanged. For every permutation of the Y-block, a PLS-DA model was built between the X-block (protein spot intensities) and the new class assignment (Y-block) using the same number of LV's (lowest number of misclassifications) as was used with the correct Y-block (the 'real class' assignment). For every PLS-DA model built, a ratio of the distance between the two assigned groups (sum of squares between) and the distance within a group (sum of squares within) was calculated (B/W). The ratio of all class assignment permutations can be plotted, resulting in a distribution of nonsense. When the B/W value of the 'real class' assignment is positioned outside the distribution of nonsense, the separation between the assigned groups can be considered as significant.

The biological validation was performed using an ELISA.

### *In-gel digestion and nLC-MS/MS*

Preparative gels were run with 1 mg of protein and stained with RuBPS fluorescent staining using the protocol which is described by Rabilloud et al <sup>35,36</sup>. Spots were excised and sliced into small pieces. The gel pieces were washed twice with 100 mM ammonium bicarbonate and acetonitrile. Next, the pieces were dried in a vacuum centrifuge and digested overnight with 25 ng/ $\mu$ l trypsin (sequencing grade, Promega Benelux, Leiden, The Netherlands) in 50 mM ammonium bicarbonate and 2 mM dithiothreitol at 37 °C. The peptide fragments were extracted twice with 5  $\mu$ l water:acetonitrile:formic acid (5:14:1). After drying in a vacuum centrifuge, the lyophilized digest was dissolved in 25  $\mu$ l of 4 M Urea buffered at pH 8.0 with 25 mM tris.

Nanobore chromatography was performed on an Ultimate nano LC system from LC Packings (Amsterdam, The Netherlands). Ten microliters of the peptide mixture was injected on a 300  $\mu$ m ID X 0.5 mm Pepmap C<sub>18</sub> trap column (LC Packings) and washed at 30  $\mu$ l/min for 10 minutes with 0.05% trifluoroacetic acid in water before the RP trap was switched on-line in

back-flush mode to a 75  $\mu\text{m}$  X 150 mm Pepmap C<sub>18</sub> nano LC column. Gradient elution of peptides was achieved at 300 nl/min going from 95% mobile phase A (water:acetonitrile:formic acid 97.9:2:0.08 v/v/v) and 5% mobile phase B (water:acetonitrile:formic acid 19.9:80:0.1 v/v/v) to 45% B in 35 minutes, then to 60% B in 10 minutes.

The nano LC column was coupled to a LCQ DECA ion trap MS (Thermo Electron, San Jose, CA, USA) via a nano electrospray interface from Proxeon (Odense, Denmark). Electrospray was performed by applying 1.3 kV to the electrospray pico tip (20  $\mu\text{m}$  ID, 10  $\mu\text{m}$  tip ID, distal coated from New Objective, Cambridge, MA, USA) via a Pt wire; ions were introduced in the mass spectrometer through a heated capillary kept at 180° C.

The ion trap was operated in data-dependent mode, selecting top two ions for MS/MS scans at 35 % collision energy units. Protein identification was performed by searching MS/MS spectra against the NCBI nr database (accessed on August 2004) using Mascot search engine ([www.matrixscience.com](http://www.matrixscience.com)). Search parameters used were as follows: peptide mass tolerance 0.8 Da; MS/MS tolerance 1.2 Da; allowed missed cleavages 1; enzyme trypsin; taxonomy human; fixed modification, carbamidomethyl (C) variable modifications, oxidation (M).

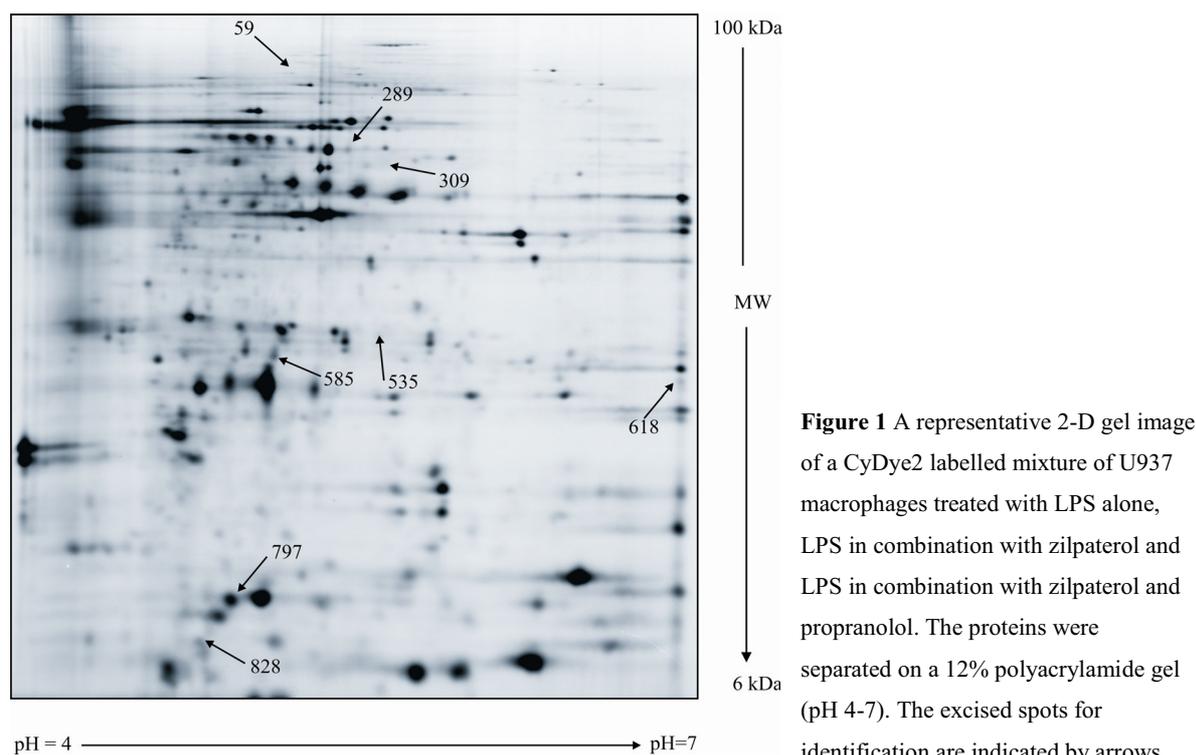
## Results

### *Proteomics*

The proteome of the secreted proteins of three different incubations, U937 macrophages treated with LPS (inflammation), treated with LPS and zilpaterol (inhibition of inflammation) and treated with LPS, zilpaterol and propranolol (antagonism of the inflammatory inhibition), were compared to each other by using the DIGE technology.

Figure 1 shows a representative 2-D gel image of the internal standard, which consisted of a CyDye2-labelled mixture of all samples analyzed in this experiment. In total 586 different spots could be detected on all the internal standard images of the experiment. The expression ratios were calculated for each spot by dividing the normalized spot intensity of the CyDye3 or CyDye5 labelled spot by the normalized spot intensity of the corresponding reference spot labelled with CyDye2.

The obtained dataset containing the expression ratios of each spot in each sample was analysed by multivariate data analysis tools, PCA and PLS-DA, and a univariate data analysis tool, Student's *t*-test.

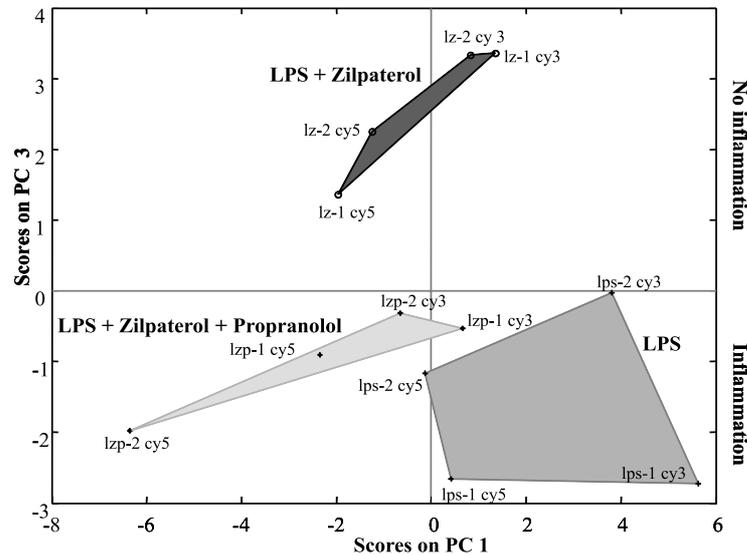


### *Multivariate data analysis*

PCA was used as an explorative data analysis tool. This unsupervised method resulted in a separation visible between the data of the gels from cells treated with LPS in combination with zilpaterol and those from cells treated with LPS alone or in the presence of zilpaterol and propranolol. Figure 2 shows that PC 1 mainly describes differences induced by the labelling with either CyDye3 or CyDye5. A similar result was obtained for PC 2 (data not shown). By contrast, PC 3 predominantly describes differences that appear to be due to the treatments of the U937 cells. Although the number of samples analysed is relatively small, it seems that there are no outliers present in the dataset. Because the number of variables (586) is much larger than the number of samples analysed, the interpretation of the loadings of the PCA for identification of potential biomarkers is limited and the observation can only serve as a first exploratory result.

In order to find specific protein biomarkers for the anti-inflammatory properties of zilpaterol mediated by the  $\beta_2$ -adrenergic receptor, PLS-DA was applied to the same proteome dataset. Two groups for PLS-DA were defined. Group one, labelled “inflammation”, consisted of the DIGE-gel images belonging to the U937 cells treated with LPS or LPS in combination with zilpaterol and propranolol (LZP), while the second group labelled, “no inflammation” consisted of the gel images from U937 cells treated with LPS in combination with zilpaterol

(LZ). A PLS-DA model was generated from the data, containing 586 protein spots and 12 gels (X-block) using the classes inflammation and no inflammation as the response variable (Y-block).



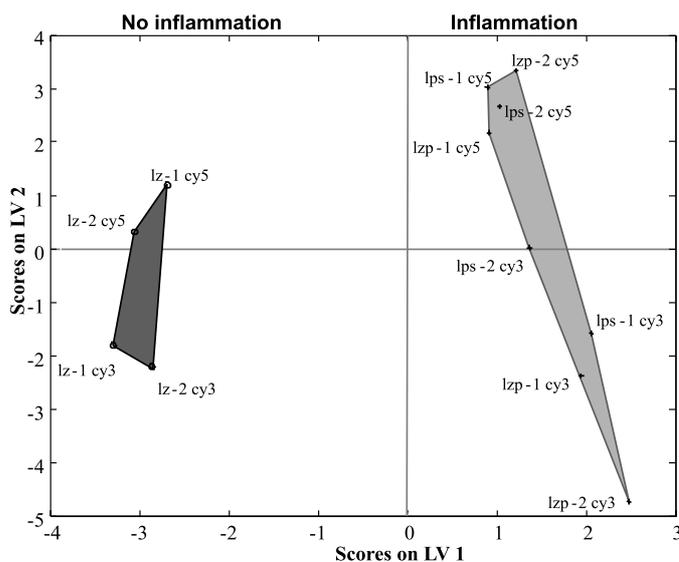
**Figure 2** Graphical presentation of the proteomics data analyzed with Principal Component Analysis (score plot). The gel images of U937 cells treated with LPS (LPS, +), LPS in combination with zilpaterol (LZ, ○) or LPS in combination with zilpaterol and propranolol (LZP, \*) are represented as crosses, dots or stars and image ID code. Each group consists of 4 data points, comprising two separate incubations (1 and 2) each labelled with CyDye 3 and CyDye 5. The images of the LZ group are clustered in the upper part of the score plot and the LPS and LZP group are positioned at the lower part. This indicates that the LPS and LZP group share a similar protein expression pattern whereas the LZ group has a distinct pattern.

The score plot of LV 1 versus LV 2 is presented in Figure 3. By using 4 LV's, no misclassifications were observed. The class assignment considered was reliable according to the permutation test results. The B/W value was positioned outside the distribution of nonsense. Figure 3 shows that LV 1 mainly describes differences that appear to involve the inflammatory status of the U937 cells, while LV 2 appears to describe differences related to the labelling of samples with either CyDye3 or CyDye5.

The influences of the individual protein spots on the PLS-DA model are described by regression values. In this particular case the high regression values indicated proteins that were down-regulated in the “no inflammation” group (LZ) with respect to the “inflammation” group (LPS and LZP), whereas low regression values indicated the up-regulated proteins. Figure 4 shows the mean expression ratios of the protein spots with successively the lowest and highest regression values.

From Figure 4 can be concluded that the differential expression of many proteins are assigned by PLS-DA as relevant to the separation of the proteomes of the “inflammation group” and

the “no inflammation” group. However, with PLS-DA we tried to identify proteins that were regulated in a similar manner in the LZP treated cells and LPS treated cells. Figure 4A and 4B show that some proteins do not meet this criterion (e.g. proteins in spots 21, 163, 310, 660, and 784). These discrepancies are most probably caused by the limited number of samples analysed in this study. Moreover, the differences in the expression levels of several other proteins do not appear to be significant (Fig. 4). Proper validation of these results is therefore necessary.

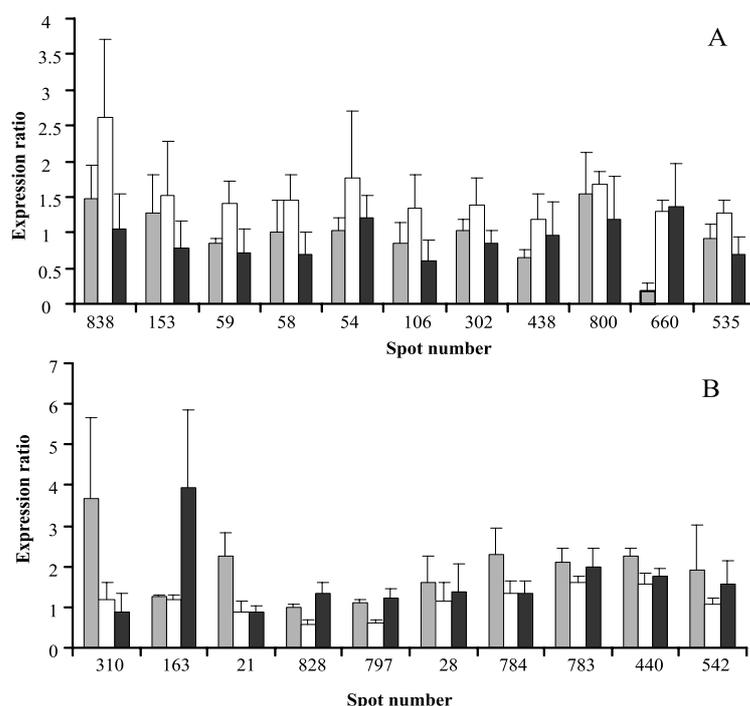


**Figure 3** Score plot of 12 protein samples. The samples were divided into two groups; “inflammation” containing all the gel images of the cells treated with LPS (LPS) or LPS in combination with zilpaterol and propranolol (LZP) and “no inflammation” containing only the gel images belonging to U937 cells treated with LPS in combination with zilpaterol (LZ). The gel images are represented as dots (no inflammation,  $\circ$ ) or crosses (inflammation,  $+$ ) and image ID code. The images of the “no inflammation” group are clustered in the left part, whereas the images of the “inflammation” group are positioned at the right of the score plot.

### Univariate data analysis

The differences in protein expression levels were also analyzed by the Student’s *t*-test to identify potential biomarkers for the anti-inflammatory effect of zilpaterol, which were regulated via the  $\beta_2$ -AR. For this purpose the following requirements were set: i) the protein had to be regulated in a similar manner ( $p > 0.05$ ) by LPS alone and by LPS in combination with zilpaterol and propranolol ii) the protein had to be significantly up- or down-regulated in U937 macrophages treated with LPS in combination with zilpaterol with respect to the other two treatments according to the Student’s *t*-test ( $p < 0.05$ ). Eight proteins met these requirements. Moreover, some of these proteins (59, 535, 797, and 828) were also marked as potential biomarkers by PLS-DA (Fig.4). The other protein spots (289, 309, 618, and 585)

were also important in the PLS-DA model (data not shown), but were not found in the top 10 of protein spots with the highest or lowest regression values.



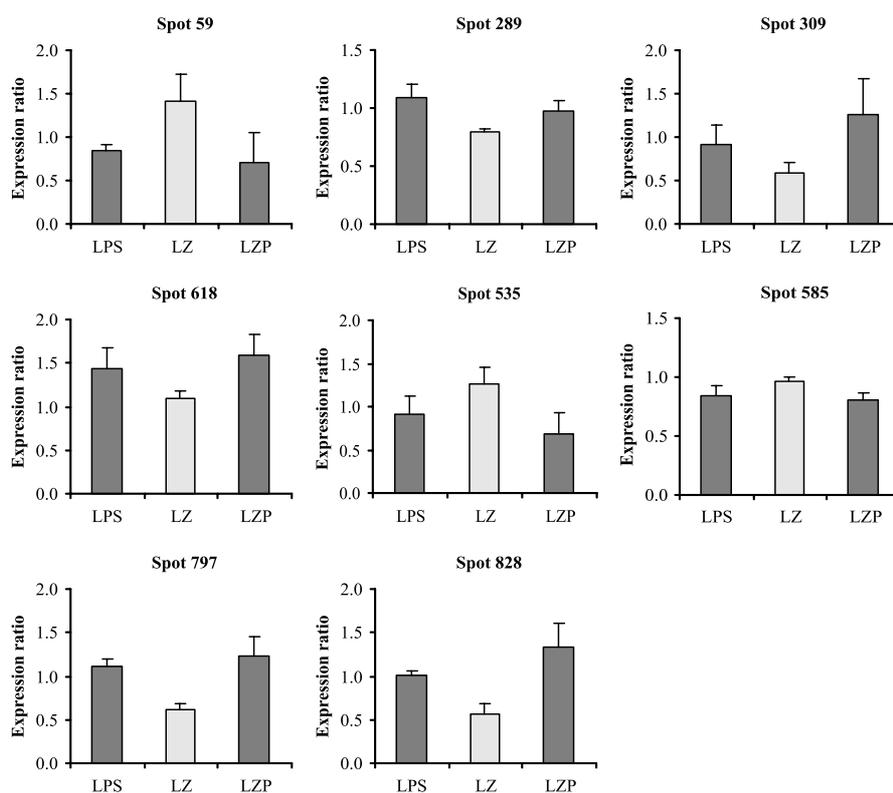
**Figure 4** Expression ratios of 10 protein spots with the A) lowest and B) highest regression values. Protein spots with high regression values are down-regulated in culture media of U937 macrophages treated with LPS and zilpaterol (white bars) with respect to LPS (light grey bars) and LPS in combination with zilpaterol and propranolol (dark grey bars), whereas the low regression values indicate up-regulation.

**Table 2** List of proteins up- or down-regulated in culture media from LPS plus zilpaterol treated U937 macrophages with respect to U937 macrophages exposed to LPS alone or LPS in combination with zilpaterol and propranolol according to the Student's *t*-test ( $p < 0.05$ ).

Spot no.	Protein identification	Accession nr.	Experimental MW	Experimental Ip	Theoretical MW	Theoretical Ip	Peptides identified	Coverage %	Total score*
59	Heat shock protein apg-2	P34932	83.3	5.2	95.1	5.2	7	8	344
289	60 kDa heat shock protein	P10809	62.1	4.8	61.2	4.8	5	18	372
309	No identification		59.3	5.6					
535	No identification		32.8	5.6					
585	Rho-gdp-dissociation inhibitor 2	P52566	29.4	5.1	23.0	5.1	10	67	378
618	Phosphoglycerate mutase	P18669	26.5	6.9	28.9	6.8	8	51	289
	Adenylate kinase iso enzym	P54819	26.5	6.9	26.6	7.9	4	17	177
797	Macrophage inflammatory protein 1-beta	P13236	11.9	4.9	10.5	5.3	1	25	81
828	Macrophage inflammatory protein 1-alpha	P10147	10.1	4.8	10.1	4.8	2	43	85

\*Total score is the sum of the individual peptide scores. Individual peptide score  $>39$  indicate identity or extensive homology ( $p < 0.05$ ) according to mascot database search program.

Figure 5 shows the differences between the expression levels of the eight proteins in the three different treatments of U937 cells, as represented by the mean spot intensity on the DIGE gels. In order to identify the proteins in the eight selected protein spots, the spots were excised and subjected to trypsin digestion followed by identification using nLC-MS/MS. Six out of 8 spots could be identified (Table 2). The identification was impossible for faint spots (spot 309, and 535). Spot 618 was found to consist of two proteins, phosphoglycerate mutase and adenylate kinase. The individual contribution of the two proteins to the spot intensity was not further investigated.

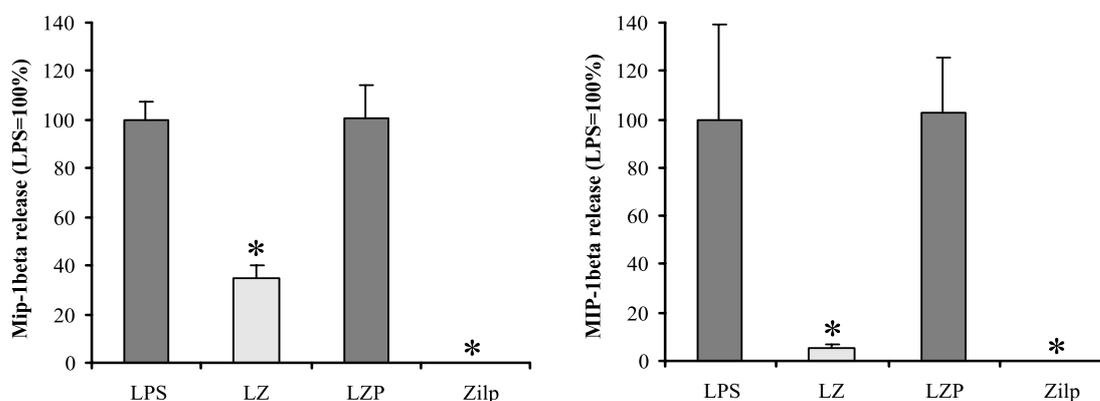


**Figure 5** Differential expressions of 8 protein spots in culture media of U937 macrophages treated with LPS alone (LPS), LPS in combination with zilpaterol (LZ) or LPS in combination with zilpaterol and propranolol (LZP). The protein spots were equally expressed in LPS treated macrophages and macrophages treated with LZP. The expressions of LZ treated macrophages were opposite to the above mentioned treatments. The expressions are given as ratios (spot intensity sample/spot intensity internal standard). The ratios are expressed as the mean of 4 expression ratios  $\pm$  SD.

Two out of eight spots were identified as a known secreted protein, macrophage inflammatory protein-1beta (MIP-1 $\beta$ ) and macrophage inflammatory protein-1alpha (MIP-1 $\alpha$ ). The other proteins are known to be involved in the maintenance of the cell, protein folding or development and are located in the cytoplasm or mitochondrial matrix (i.e. intracellular locations) (Table 3).

**Table 3** List of protein function and cellular location of proteins identified using nLC-MS/MS. More information can be found on <http://us.expasy.org>, using the accession no. from Table 2

Protein name	Function	Sub cellular location
Heat shock protein apg-2	ATP binding, protein folding	Cytoplasm
60 kDa heat shock protein	ATP binding, protein folding, mitochondrial matrix protein transport	Mitochondrial matrix
Rho-gdp-dissociation inhibitor 2	Actin cytoskeleton organisation, development	Cytoplasm
Phosphoglycerate mutase	Enzyme in glycolysis	Cytosol
Adenylate kinase iso enzym	Enzyme, maintenance and cell growth	Mitochondrial
Macrophage inflammatory protein -1beta	Chemokine, cell-cell signalling, inflammatory response	Secreted
Macrophage inflammatory protein -1alpha	Chemokine, cell-cell signalling, inflammatory response	Secreted

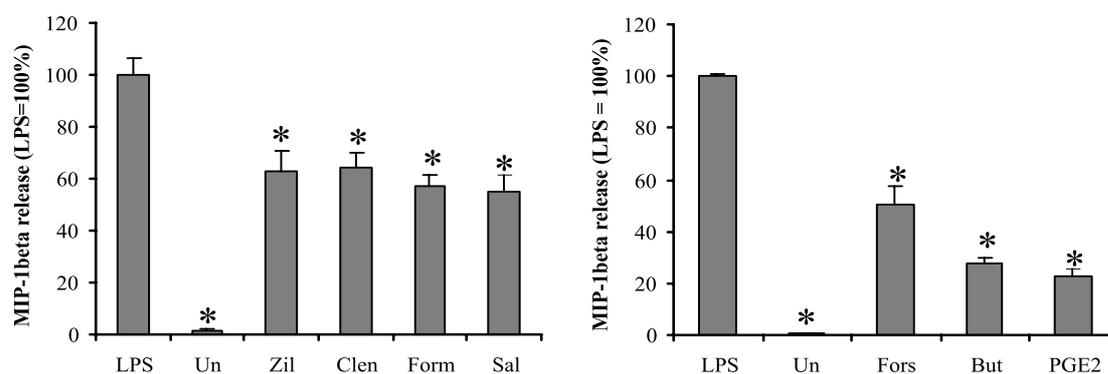


**Figure 6** Release of MIP-1 $\beta$  from U937 macrophages (left panel) and PB-M $\emptyset$  (right panel) incubated for 16 h with 1  $\mu$ g/ml LPS (LPS), LPS in combination with  $1 \times 10^{-6}$  M zilpaterol (LZ), LPS in combination with zilpaterol and  $1 \times 10^{-6}$  M propranolol (LZP) or  $1 \times 10^{-6}$  M zilpaterol alone (Zilp), respectively. The release of MIP-1 $\beta$  is corrected for protein content and represented as mean percentage of LPS induction (LPS = 100%)  $\pm$  SD of triplicate measurements. \* Different with respect to LPS (based on Student's *t*-test).

### Biological validation

The down-regulation of MIP-1 $\beta$  was confirmed by the analysis of the culture media using a specific ELISA for MIP-1 $\beta$  (Fig. 6, left panel). MIP-1 $\beta$  was up-regulated in LPS-treated U937 macrophages and this up-regulation was inhibited by the addition of zilpaterol. Using an antagonist for the  $\beta$ -AR, propranolol this inhibition could be blocked, indicating that the inhibitory effect of zilpaterol on MIP-1 $\beta$  production is mediated via the  $\beta$ -AR. When human peripheral blood macrophages were incubated with LPS, LPS in combination with zilpaterol and LPS in combination with zilpaterol and propranolol, the effect was more pronounced (Fig. 6, right panel).

In order to confirm that the inhibition of MIP-1 $\beta$  is mediated by the  $\beta_2$ -AR, U937 macrophages were exposed to LPS in combination with other known  $\beta_2$ -receptor agonists. Figure 7 shows the inhibitory effect of zilpaterol, clenbuterol, formoterol and salbutamol on the production of MIP-1 $\beta$ . Since the main pathway by which activation of the  $\beta_2$ -AR exerts its effect is related to the elevation of intracellular cyclic AMP, we determined the effect of other agents that elevate intracellular cAMP levels. From the right panel of Figure 7, it can be concluded that forskolin, dibutyryl cAMP and PGE<sub>2</sub> were also capable of inhibiting the LPS induced MIP-1 $\beta$  production in U937 macrophages.



**Figure 7** Release of MIP-1 $\beta$  from U937 macrophages incubated for 16 h with 1  $\mu$ g/ml LPS (LPS), no treatment (Un) or LPS in de presence of  $\beta_2$ -adrenergic agonists, zilpaterol  $1 \times 10^{-6}$  M (Zil), clenbuterol  $1 \times 10^{-7}$  M (Clen), formoterol  $1 \times 10^{-8}$  M (Form) and salbutamol  $1 \times 10^{-6}$  M (Sal), respectively (left panel). The right panel shows the release of MIP-1 $\beta$  from U937 macrophages exposed for 16 h with 1  $\mu$ g/ml LPS (LPS), no treatment (Un), or LPS in de presence of cAMP elevating agents, forskolin  $1 \times 10^{-5}$  M (Fors), dibutyryl cAMP  $1 \times 10^{-4}$  M (But), or prostaglandin E<sub>2</sub>  $1 \times 10^{-4}$  M (PGE2) respectively. The release of MIP-1 $\beta$  is corrected for protein content and represented as mean percentage of LPS induction (LPS = 100%)  $\pm$  SD of triplicate measurements. \* Different with respect to LPS according to Student's *t*-test.

## Discussion

Conventional two-dimensional gel electrophoresis (2-D PAGE) allows the resolution of several thousand proteins in a single gel<sup>37,38</sup>. The well known limitations of this technique are low sensitivity (Coomassie and Sypro Ruby), limited dynamic range (silver) and gel to gel variability. Difference gel electrophoresis (DIGE) circumvents these issues associated with traditional 2-D PAGE and allows sensitive and more accurate quantitative proteomics studies<sup>20-22</sup>. However, with minimal labelling, only 5 % of the proteins will be labelled, the bulk unlabelled proteins will run with a higher mobility during the electrophoresis<sup>39</sup>. Preparative gels, stained with less sensitive stains as Coomassie blue or Sypro Ruby are therefore necessary in order to be able to excise the protein spots out of the gel for spot identification.

Using this procedure we encountered that some of the protein spots of interest, identified by DIGE, could not be found back on the fluorescent-stained gels. This is in line with the previously published observation that approximately 40 % of the proteins spots from a Cydye labelled gel could not be found back on Coomassie stained gels, this was somewhat better with Sypro Ruby<sup>39</sup>. An additional limiting step in spot identification is the insufficient amount of peptides being generated after in-gel digestion and their low signal intensity. This is especially true for hydrophobic, low abundant proteins or proteins with low MW. Several recent reports have described improved digestion protocols, signal intensities and recoveries of peptides<sup>40-45</sup>. However, at present there is no universal protocol suitable for every type of protein that may be present on a 2-D gel. Improving the sensitivity of protein stains also requires a better recovery of proteins out of the gel and improved peptide signals for mass spectrometry.

In this study we compared the secreted protein pattern of U937 macrophages exposed to LPS (inflammation), LPS in combination with zilpaterol (LZ; inhibition of inflammation) and LPS in combination with zilpaterol and propranolol (LZP; counteraction of the anti-inflammatory effect of zilpaterol), by using 2-D DIGE. The datasets generated after scanning of the 2-D gels were analysed by univariate (Student's *t*-test) and multivariate data analysis tools (PCA and PLS-DA). PCA showed clustering of the gel images obtained from LPS and LZP group (inflammation) and separated these gels from the images obtained from the LZ group (no inflammation). The first two principal components mainly described the effect of the different CyDyes used (CyDye 3 and CyDye 5). This dye bias has also been observed by Karp et al<sup>34</sup> who analysed *Erwinia carotovora* samples using DIGE in combination with PCA and PLS-DA. It is therefore important to label samples with both dyes in order to counteract the dye bias effect.

PLS-DA was used to correlate class assignment ("inflammation" and "non-inflammation") with spot data to discover potential biomarkers for the anti-inflammatory effect of  $\beta_2$ -agonists. The use of PCA or PLS-DA to cluster data and discover potential biomarkers is not justified in cases where the number of samples is much smaller than the number of variables, as is the case with most proteomic studies. Nevertheless, cautious use for exploratory studies can be helpful as a first guidance in the biomarker discovery trajectory<sup>30, 33, 34, 46</sup>. Using the multivariate approach, up- or down-regulated markers are easily identified, although it should be noted that these would also have been identified by more straightforward univariate tools, such as the Student's *t*-test. In this study, we only identified the proteins that were assigned by PLS-DA as an important protein in the separation between the groups "inflammation" and

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“non-inflammation”, and that were significantly altered according to the Student’s *t*-test. A major drawback of the Student’s *t*-test is its sensitivity to false positive results ( $p < 0.05$ ), especially when large numbers of variables are analysed. Protein markers that are found to be correlated either to each other or to a class assignment (PLS-DA) or identified by the Student’s *t*-test have to be additionally validated, both statistically as well as biologically. In addition to the statistical arguments, another reason to biologically validate putative marker proteins is that uncertainties arise in each step of the proteomics workflow (e.g. accidental modifications introduced by sample preparation, spot shifts in gels which may result in missing values and false spot identification).

The aim of this study was to identify protein biomarkers for the anti inflammatory properties of zilpaterol mediated by the  $\beta_2$ -AR. By examining the secreted fraction of proteins, we identified 8 proteins as putative biomarkers. Two of the eight proteins were identified as secreted proteins, namely macrophage inflammatory protein-1beta (MIP-1 $\beta$ ) and macrophage inflammatory protein-1alpha (MIP-1 $\alpha$ ). The other 6 proteins were previously reported to be intra-cellular proteins. Most likely, the culture media samples that were examined in these experiments were contaminated with intracellular proteins. When comparing the protein pattern of the secreted proteins with the pattern of the cell lysate<sup>30</sup>, many similarities could be observed. Other studies performed on secreted proteins reported the same phenomenon<sup>47-49</sup>. This contamination is unfortunately inevitable, because a few dead cells are already enough to mask the very low abundant proteins present in the culture media.

The down-regulation of MIP-1 $\beta$  by zilpaterol was confirmed in U937 macrophages as well as human peripheral blood macrophages by using a specific ELISA. The  $\beta$ -adrenergic blockade by propranolol counteracted the inhibitory effect of zilpaterol on the MIP-1 $\beta$  production. Furthermore, the down-regulation of MIP-1 $\beta$  was also achieved by other  $\beta_2$ -AR agonists like clenbuterol, salbutamol and formoterol. These data demonstrate the involvement of the  $\beta$ -AR. The involvement of cAMP was investigated by incubating U937 macrophages with LPS in combination with cAMP elevating agents. The release of MIP-1 $\beta$  was inhibited by prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) which augments cyclic AMP by binding to its own receptor, forskolin, a direct activator of adenylate cyclase and the membrane permeable stable derivate of cAMP, dibutyryl cAMP. This observation suggests that the inhibition of MIP-1 $\beta$  production is most probably due to the elevation of intracellular cAMP. The  $\beta$ -AR belongs to the family of G-protein coupled receptors that activate Gs proteins. Gs proteins activate adenylate cyclase that subsequently catalyses the conversion of ATP into cAMP<sup>7-12</sup>. Cyclic

AMP is known to be involved in the induction and inhibition of many inflammatory genes encoding proteins involved in inflammation<sup>13, 14, 50</sup>. The results described above suggest that zilpaterol inhibits the LPS induced production of MIP-1 $\beta$  via the  $\beta_2$ -AR by elevating the cAMP production. This finding is in agreement with those of Martin et al<sup>51</sup>, who discovered that PGE<sub>2</sub> inhibited the production of MIP-1 $\beta$  mRNA levels in murine macrophage cell line partially via a cAMP mediated pathway of signal transduction.

MIP-1 $\beta$  belongs to the chemokine family and is involved in endotoxin induced inflammation. Chemokines play a major role in the recruitment of leukocytes to sites of infection. In addition they often activate these cells resulting in an enhanced local inflammatory response<sup>3</sup>. MIP-1 $\beta$  is a chemoattractant for natural killer cells and has been found in inflamed tissues of patients with rheumatoid arthritis, Multiple Sclerosis, and respiratory system disorders<sup>52-54</sup>. Interestingly, MIP-1 $\alpha$  (chemokine CC), which shows 68% homology with MIP-1 $\beta$ , was also inhibited by  $\beta_2$ -AR agonists and other cAMP elevating compounds<sup>52, 55, 56</sup>. This inhibition was also found in this report using 2-D DIGE. The regulation of MIP-1 $\alpha$ , MIP-1 $\beta$  and perhaps other chemoattractants by  $\beta_2$ -AR agonists could therefore be of therapeutic importance and could in part explain the mechanism of action of  $\beta_2$ -AR agonist in the treatment of asthma.

### **Acknowledgements**

The authors thank Age Smilde and Carina Rubingh for there helpful discussion on statistical data analysis and Ian Sanders and Louise Vickers from Nonlinear Dynamics (Newcastle upon Tyne, UK) for their support in the implementation of the Progenesis software

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