Effects of inhaled corticosteroids on clinical and pathological outcomes in COPD - Insights from the GLUCOLD study
Kunz, L.I.Z.

Cover Page

The handle http://hdl.handle.net/1887/44522 holds various files of this Leiden University dissertation.

Author: Kunz, L.I.Z.
Title: Effects of inhaled corticosteroids on clinical and pathological outcomes in COPD - Insights from the GLUCOLD study
Issue Date: 2016-11-30

CHAPTER 3

Regulation of YKL-40 Expression by Corticosteroids: Effect on Pro-inflammatory Macrophages *in vitro* and its modulation in COPD *in vivo*
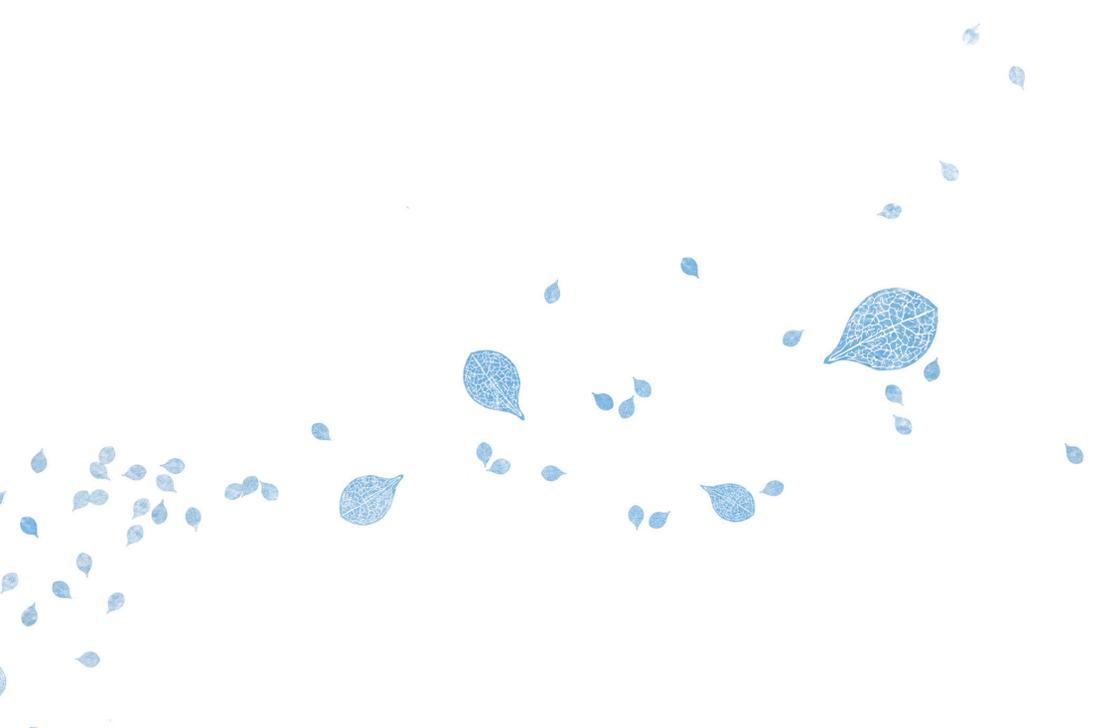
Lisette I.Z. Kunz*, Emily F.A. van 't Wout*, Annemarie van Schadewijk,
Dirkje S. Postma, Huib A.M. Kerstjens, Peter J. Sterk and Pieter S. Hiemstra
* authors contributed equally to this study

ABSTRACT

Background: Macrophages constitute a heterogeneous cell population with pro- (MΦ1) and anti-inflammatory (MΦ2) cells. The soluble chitinase-like-protein YKL-40 is expressed in macrophages and various other cell types, and has been linked to a variety of inflammatory diseases, including COPD. Dexamethasone strongly reduces YKL-40 expression in peripheral blood mononuclear cells (PBMC) *in vitro*. We hypothesized that: a) YKL-40 is differentially expressed by MΦ1 and MΦ2, b) is decreased by corticosteroids and c) that long-term treatment with inhaled corticosteroids (ICS) affects YKL-40 levels in serum and sputum of COPD patients.

Methods: Monocytes of healthy subjects were cultured *in vitro* for 7 days with either GM-CSF or M-CSF (for MΦ1 and MΦ2, respectively) and stimulated for 24h with LPS, TNFα, or oncostatin M (OSM). MΦ1 and MΦ2 differentiation was assessed by measuring secretion of IL-12p40 and IL-10, respectively. YKL-40 expression in macrophages was measured by quantitative RT-PCR (qPCR) and ELISA; serum and sputum YKL-40 levels were analyzed by ELISA.

Results: Pro-inflammatory MΦ1 cells secreted significantly more YKL-40 than MΦ2, which was independent of stimulation with LPS, TNFα or OSM (P<0.001) and confirmed by qPCR. Dexamethasone dose-dependently and significantly inhibited YKL-40 protein and mRNA levels in MΦ1. Serum YKL-40 levels of COPD patients were significantly higher than sputum YKL-40 levels but were not significantly changed by ICS treatment.

Conclusions: YKL-40 secretion from MΦ1 cells is higher than from MΦ2 cells and is unaffected by further stimulation with pro-inflammatory agents. Furthermore, YKL-40 release from cultured monocyte-derived macrophages is inhibited by dexamethasone especially in MΦ1, but ICS treatment did not change YKL-40 serum and sputum levels in COPD. These results indicate that YKL-40 expression could be used as a marker for MΦ1 macrophages *in vitro*, but not for monitoring the effect of ICS in COPD.

INTRODUCTION

YKL-40 (or chitinase 3-like-1 [CHI3L1], breast regression protein [BRP]-39 or human cartilage glycoprotein-39 [HCgp-39]) is a chitinase-like protein which is found in humans [1]. It is expressed by various cell types, including neutrophils and macrophages [2-4], while macrophages have been identified as its main cellular source [2, 5]. Monocytes do not express YKL-40, and YKL-40 expression appears to be associated with later stages of macrophage differentiation [2, 6]. Although its biological function is largely unknown, YKL-40 has been suggested to play a major role in a variety of processes, including epithelial-mesenchymal transition, migration and proliferation of (malignant) cells, angiogenesis, tissue remodeling and inflammation [7-9]. Furthermore, YKL-40 has been implicated in several acute and chronic inflammatory diseases, including asthma and COPD [10, 11]. Several studies have shown that YKL-40 levels are higher in sputum and serum of COPD patients compared to asymptomatic smokers [12, 13].

These data suggest that macrophages may also be a major source of YKL-40 in inflammatory lung diseases such as COPD, a disease in which macrophages play an important role [14]. It is however unknown whether YKL-40 expression is restricted to a subset of macrophages. This is a relevant question, since macrophages constitute heterogeneous cell populations with various functions. Pro-inflammatory, or classically activated macrophages (type I, MΦ1), show pro-inflammatory properties by secreting pro-inflammatory cytokines such as interleukin-12 (IL-12), have antigen presenting capacity and promote Th1 immunity. In contrast, anti-inflammatory, or alternatively activated macrophages (type II, MΦ2), demonstrate anti-inflammatory characteristics with expression and secretion of anti-inflammatory cytokines, such as IL-10 and promote development of regulatory T cells [15]. Furthermore, Di Rosa *et al*. observed higher expression of YKL-40 in monocyte-derived MΦ1 [16].

Dexamethasone strongly suppresses YKL-40 expression in cultured monocyte-derived macrophages [17]. In line with these findings, rheumatoid arthritis patients treated with methotrexate and prednisolone had lower serum YKL-40 concentrations compared to patients treated without prednisolone [18]. However, it is unknown if YKL-40 levels are influenced by treatment with inhaled corticosteroids (ICS) in COPD and could therefore be used to monitor treatment response. Furthermore, it is not known whether modulation by steroids is a direct genomic anti-inflammatory effect or due to e.g. epigenetic mechanisms [19-22].

Based on these observations we hypothesized that YKL-40 expression is directed by macrophage polarization, with a higher expression in monocyte-derived MΦ1 compared to MΦ2. Furthermore, we hypothesized that steroids decrease YKL-40 secretion and expression in MΦ1 and that YKL-40 in serum and sputum of COPD patients is decreased by treatment with ICS.

METHODS

Cell culture

Monocytes were isolated from buffy coats of healthy blood donors (Sanquin Blood Bank, Leiden, The Netherlands) using anti-CD14 microbeads (Miltenyi Biotec, Auburn, CA, USA) according to the manufacturer's protocol. MΦ1 and MΦ2 were derived as described previously [23, 24]. Briefly, cells were cultured for six days in RPMI 1640 medium in 48 well plates (Invitrogen, Life Technologies, Bleiswijk, The Netherlands) containing 10% fetal calf serum (FCS, Invitrogen), 2mM L-glutamine, 100U/ml penicillin and 100µg/ml streptomycin (all Bio Whittaker, Walkersville, MD, USA) at 37°C in 5% CO_2 atmosphere with either GM-CSF (5ng/ml, Invitrogen) or M-CSF (50ng/ml, R&D Systems, Minneapolis, MN, USA) to obtain MΦ1 and MΦ2, respectively (13, 14). Differentiated macrophages were stimulated with the pro-inflammatory stimuli lipopolysaccharide (LPS, from Pseudomonas aeruginosa, 100ng/ml, Sigma-Aldrich, St. Louis, MO, USA), TNF-α (10ng/ml, Peprotech, Rocky Hill, NJ, USA) or oncostatin M (OSM, 100ng/ml, R&D Systems) for 24 hours. Dexamethasone (0.1, 0.3 and 1nM, Sigma) was added during differentiation at day 0, 3 and/or day 7. The demethylating agent 5-AZA-2'-deoxycytidine (5-AZA, 0.1, 1 and 10 µM, Sigma) was added during differentiation. Every day 100 µl per well was removed and replaced by fresh medium containing growth factors and 5-AZA.

GLUCOLD study

Serum and sputum supernatants were obtained from patients with moderate to severe COPD who participated in the GLUCOLD (Groningen and Leiden Universities Corticosteroids in Obstructive Lung Disease) study [25]. Patients were steroid-naive at baseline and were subsequently randomized to one of four inhaled treatments, all twice daily: 6- or 30-month

fluticasone propionate dry-powder inhaler (500µg, group 1 and 2, respectively), 30-month fluticasone with salmeterol (500/50µg, group 3) or 30-month placebo (group 4). In this mechanistic study, we only used data from the compliant patients (≥70% of the prescribed dose of treatment) of groups 2 and 3 combined to increase power, and group 4. At baseline and after 30 months of treatment, serum and sputum samples were collected. Sputum induction and processing were performed as previously described [26, 27]. Cell free supernatants of serum and sputum were stored at -80°C. The ethics committees of Leiden University Medical Center and University Medical Center Groningen approved the study and all patients provided written informed consent.

Enzyme-linked Immunosorbent Assay (ELISA)

Commercially available ELISA kits were used to detect IL-12p40 (IL-12/ IL-23p40, R&D Systems; sensitivity 62.5 pg/ml) and IL-10 (Sanquin, Amsterdam, The Netherlands; sensitivity 4.1 pg/ml), to confirm that the monocytes were adequately differentiated towards MΦ1 and MΦ2, respectively (Figure 1A). YKL-40 ELISA (R&D Systems; sensitivity 16 pg/ml [10]) was performed on cell culture supernatant, serum and sputum supernatant. The absorbance was measured at 450nm using a Microplate reader (model 680; Bio-Rad, Hercules, CA, USA) and Microplate Manager software (version 5.2.1, Bio-Rad).

Flow cytometry

Cell surface markers were assessed by standard flow cytometry using a FACS Calibur cytometer (Becton and Dickinson, La Jolla, CA, USA) and CellQuest Pro software after staining with specific APC-conjugated CD163 and CD14 (both BD Biosciences/ Pharmingen (Temse, Belgium), FITC-conjugated CD68 (eBioscience, Vienna, Austria) and/or goat-anti-human YKL-40 (R&D systems) detected by Alexa Fluor 594 F(ab')2 fragment of goat anti-mouse (Invitrogen) as secondary antibody. Cells were incubated with the antibodies for 30 minutes on ice in PBS containing 0.5% BSA (w/v) and 0.2% sodium azide (w/v) (both Sigma). After fixation with Cytofix/ Cytoperm buffer, intracellular staining was performed in Perm/ Wash buffer (both BD Biosciences). Flow cytometric analysis confirmed that the differentiated monocytes were CD14^{+} and CD68^{+}, and that the majority of MΦ2 were CD163^{+} (Figure 1B). Therefore, we concluded that the monocytes were properly differentiated into MΦ1 and MΦ2 cells.
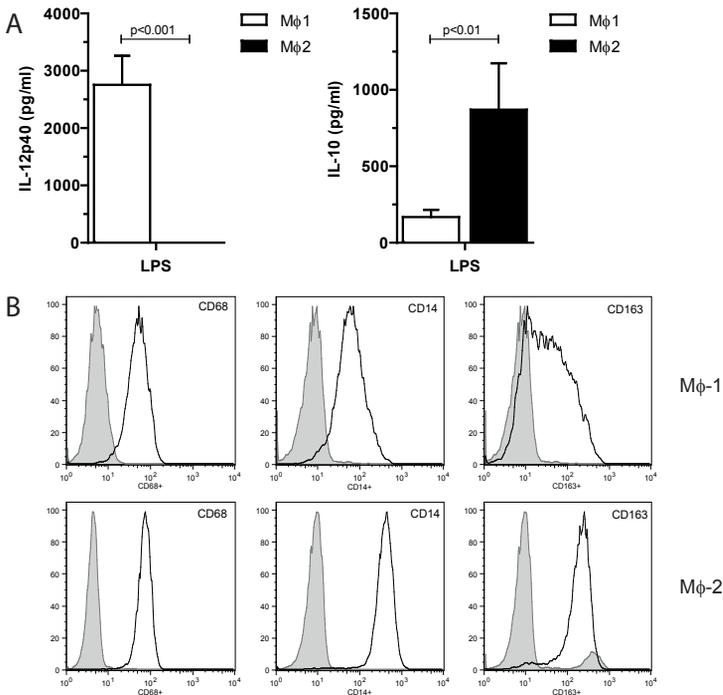
Figure 1: Characterization of differentiated MΦ1 and MΦ2. Figure 1A: IL-12p40 (left panel) and IL-10 (right panel) in supernatants of MΦ1 and MΦ2, respectively, stimulated with LPS for 24 hours. Data represent means with SEM of 6 donors. Figure 1B: Flow cytometry analysis for CD68, CD14 and CD163 (left, middle and right panel, respectively) for MΦ1 and MΦ2 (upper and lower panel, respectively).

Immunofluorescence staining

YKL-40 and CD68 expression in sputum cytospins was demonstrated using immunofluorescence. Cytospins were fixed in 4% formaldehyde in PBS for 30 minutes, followed by antigen retrieval in citrate solution pH6.0 (DAKO, Glostrup, Denmark) for 30 minutes and cooled on ice. The primary antibodies goat-anti-human YKL-40 (R&D systems, dilution 1:25) and mouse-anti-human CD68 (clone PG-M1, DAKO, dilution 1:50), diluted in PBS/ 1% BSA (w/v) were incubated together overnight. Alexa Fluor568 donkey-anti-goat and Alexa Fluor 488 donkey-anti-mouse (Invitrogen, Eugene, OR, dilution of both 1:200) were incubated in a dark environment for 30 minutes. The cytospins were covered with Vectashield with DAPI (Vector Laboratories, Inc. Burlingame, CA, USA). Photographs were taken with a confocal microscope.

Quantitative reverse-transcriptase polymerase chain reaction (qPCR)

RNA was isolated using Qiagen RNeasy mini kit (Qiagen, Venlo, The Netherlands) and cDNA was synthesized in equal amounts per experiment. Quantitative reverse-transcriptase polymerase chain reaction (qPCR) was performed with the primers for YKL-40 and LL-37 as presented in Table 1. QPCR was performed on the iCycler PCR device using iQ SYBR Green Supermix (Bio-Rad) for 40 cycles at 58°C. Relative mRNA concentrations of ACTB and ATP5B (GeNorm, PrimerDesign Ltd., Southampton, UK) were used as housekeeping genes for human genes.

Table 1: Primer pairs used for quantitative reverse-transcriptase polymerase chain reaction (qPCR).

	Forward	**Reverse**
YKL-40 (CHI3L1) (154 base pairs)	CTG TGG GGA TAG TGA GGC AT	CTT GCC AAA ATG GTG TCC TT
LL-37 (CAMP) (249 base pairs)	TCA TTG CCC AGG TCC TCA G	TCC CCA TAC ACC GCT TCA C

Statistical analysis

Differences within one cell type and between cell types were analyzed by one-way and two-way ANOVA, respectively. Paired and unpaired tests were used for evaluating YKL-40 levels in serum and sputum within and between treatments, respectively, using only data from subjects with availability of samples from both baseline and 30 months. Statistical analysis was performed with SPSS 22.0 software (SPSS Inc., Chicago, IL). Data are presented as means with standard error of the mean (SEM) for *in vitro* experiments and means with standard deviations (SD) for serum and sputum samples. Differences at p-values ≤0.05 were inferred as statistically significant.

RESULTS

MΦ1 produce more YKL-40 compared to MΦ2

Secretion of YKL-40 by MΦ1 was markedly higher compared to MΦ2 (P<0.001) and not further increased by stimulation for 24 hours with 100ng/ml LPS compared to medium (P<0.001; Figure 2A, left panel). qPCR confirmed these results, i.e. MΦ1 express more YKL-40 than MΦ2 with and without LPS stimulation (10-fold higher in MΦ1 vs MΦ2, P<0.001; Figure 2A, right panel), with a trend towards more YKL-40 expression in LPS-stimulated MΦ1 (P<0.1). Flow cytometry analysis also showed that MΦ1 cells express more YKL-40 compared to MΦ2 (Figure 2B). In addition, MΦ1 secreted more YKL-40 compared to MΦ2 which was irrespective of further stimulation with TNF-α, and OSM for 24 or 48 hours, respectively (all P<0.001, Figure 2C). Immunofluorescence staining showed that only a minority of macrophages in sputum of COPD patients was positive for YKL-40 as shown by co-staining with CD68 (Figure 2D).

YKL-40 expression is inhibited by dexamethasone

To investigate the effect of steroids on macrophage YKL-40 expression, we first assessed its effect on expression of the MΦ1 and MΦ2 markers IL-12p40 and IL-10. Whereas dexamethasone did not affect IL-10 secretion, it inhibited IL-12p40 secretion, especially when added at day 0 (Figure 3A and B, respectively). In line with these findings, dexamethasone also dose-dependently inhibited YKL-40 expression and secretion mainly in MΦ1 (Figure 4A and B, respectively). Again this effect was most pronounced when dexamethasone was added from the start of the differentiation at day 0 (Figures 4A and B, left panel). Adding dexamethasone at later time points (day 3 or day 7) showed a trend for lower YKL-40 expression and secretion (Figures 4A and B, middle and right panel, respectively), but failed to reach significance.

We have previously demonstrated that the human cathelicidin antimicrobial peptide hCAP/LL-37 directs macrophage differentiation towards MΦ1 cells [28]. In the course of the latter studies we also noted that hCAP18/LL-37 is preferentially expressed in MΦ1 cells (unpublished results). In the present study, we therefore used LL-37 expression for a comparison with YKL-40 expression. In line with the findings for IL-12p40 and YKL-40, the expression of LL-37 was also dose-dependently reduced by increasing doses of dexamethasone when added on day 0, but not on day 3 or day 7 (Figure 4C).

Collectively these data indicate that dexamethasone inhibits YKL-40 expression, but that this effect is most likely explained by a more generic effect on MΦ1 development and is not selective for YKL-40 expression.
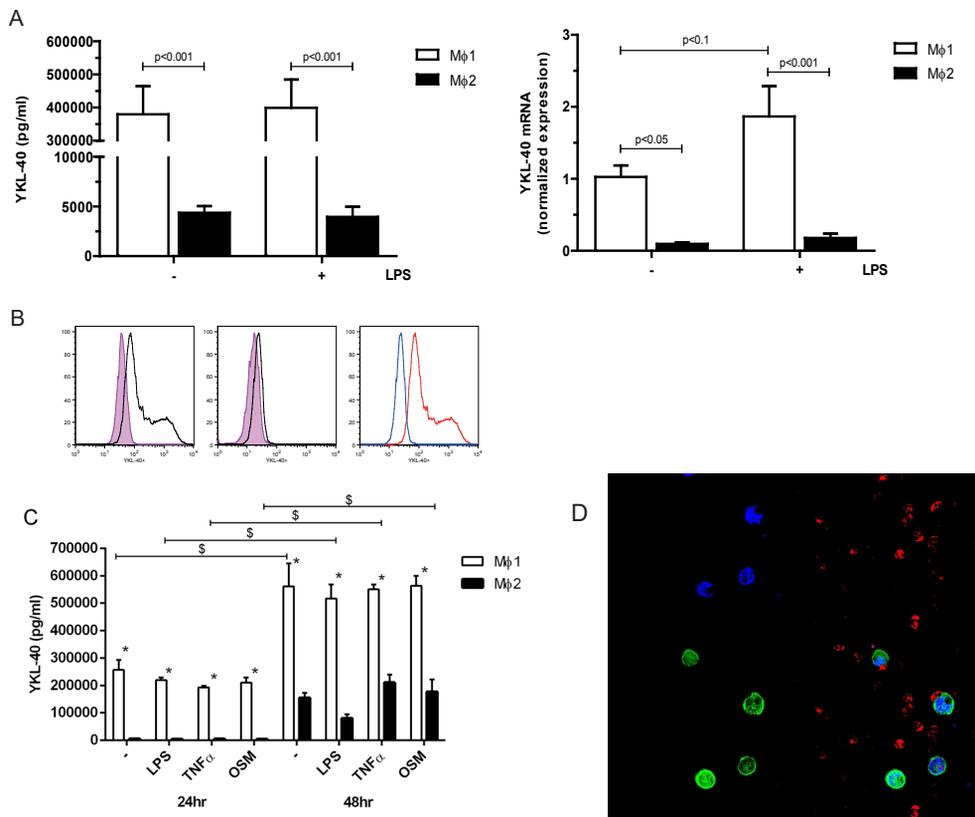
Figure 2: MΦ1 produce more YKL-40 compared to MΦ2. Figure 2A: YKL-40 secretion measured by ELISA (left panel, 6 donors) and mRNA levels (normalized expression, right panel, 5 donors) in MΦ1 and MΦ2 after medium and LPS stimulation for 24 hours. Figure 2B: Flow cytometry analysis of MΦ1 and MΦ2 with and without anti-YKL-40 and secondary antibody. Left panel: MΦ1 with secondary antibody (pink) and MΦ1 with anti-YKL-40 and secondary antibody (black); middle panel: MΦ2 with secondary antibody alone (pink) and MΦ2 with anti-YKL-40 and secondary antibody (black); right panel: MΦ1 and anti-YKL-40 with secondary antibody (red) and MΦ2 and anti-YKL-40 with secondary antibody (blue). Figure 2C: YKL-40 secretion in MΦ1 and MΦ2 after 24 and 48 hours of stimulation with medium, LPS, TNF-α and oncostatin M (OSM). Data represent mean and SEM of 4 and 3 donors (24 and 48 hours, respectively). *: P<0.001 between MΦ1 and MΦ2 for corresponding stimulus and time. $: P<0.001 between corresponding stimulus at different time points. Figure 2D: Immunofluorescence staining on sputum cytospin of a COPD patient (blue: DAPI; red: YKL-40; green: CD68 and overlay).
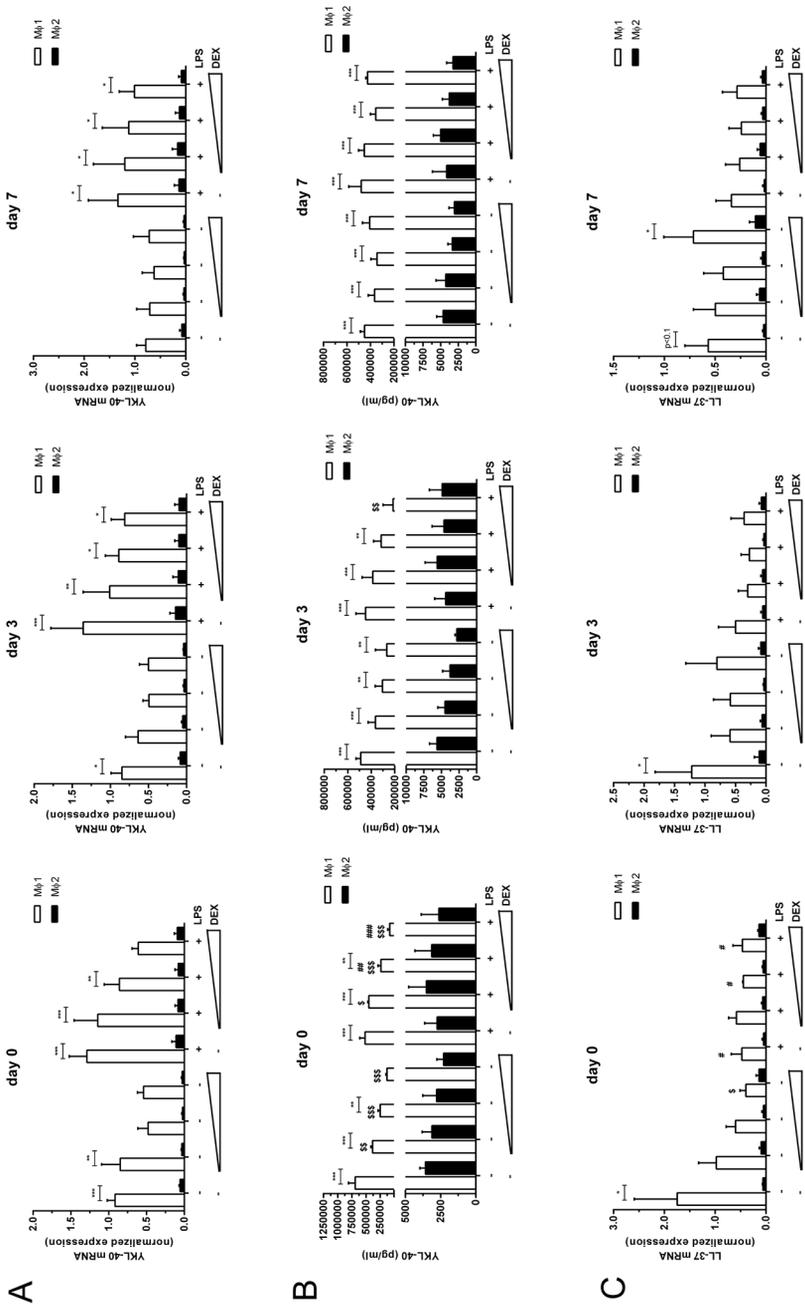
Figure 3: IL-12p40 secretion is dose-dependently inhibited by dexamethasone when added on day 0.

IL-10 and IL-12p40 secretion with addition of dexamethasone at day 0, 3 and 7 of differentiation (0.1, 0.3 and 1nM) (Figure 3A and B, left, middle and right panel, respectively). Data represent means and SEM (n= 3 donors). * p≤0.05, ** P<0.01 or *** P<0.001: between MΦ1 to MΦ2 with corresponding stimulus. $ p≤0.05 or $ P<0.001 between MΦ1 at different concentrations of dexamethasone.

YKL-40 expression is not affected by the demethylating agent 5-AZA

Epigenetic mechanisms may contribute to CHI3L1 expression. This is supported by the finding of a single-nucleotide polymorphism (SNP) localized near the CpG island in the promoter region of the CHI3L1 gene, which is associated with YKL-40 expression [19, 20]. To evaluate whether YKL-40 expression in monocyte-derived macrophages is influenced by the methylation status of the CHI3L1 gene, macrophages were generated in the presence of the demethylating agent 5-AZA. We first observed that 5-AZA inhibited IL-12p40 secretion, and to a smaller extent also IL-10 secretion (Figures 5A and B, respectively). In contrast, YKL-40 protein secretion and expression was not significantly affected by 5-AZA, although it needs to be noted that the highest concentration resulted in cell toxicity (Figures 5C and D, respectively).
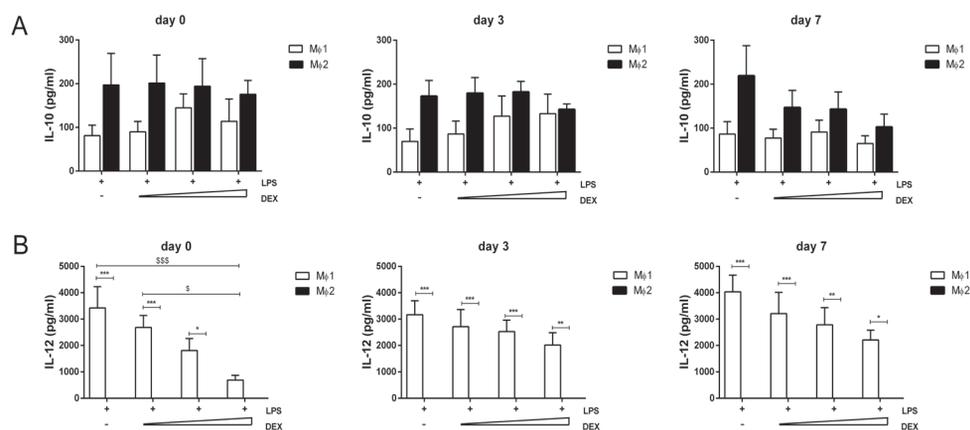
Figure 4: YKL-40 expression and protein secretion is inhibited by dexamethasone. YKL-40 mRNA levels (normalized expression) and YKL-40 protein secretion (figure 4A and B, respectively) in MΦ1 and MΦ2 with addition of dexamethasone at day 0, 3 and 7 of differentiation (0.1, 0.3 and 1nM) (left, middle and right panel, respectively). LL-37 mRNA levels (normalized expression) after 0, 3 and 7 days of differentiation with dexamethasone (0.1, 0.3 and 1nM) (figure 4C left, middle and right panel, respectively). Data represent means and SEM (n= 3 donors). * P≤0.05, ** P<0.01 or *** P<0.001 compared to MΦ2 with corresponding stimulus. $= p≤0.05, $$ P<0.01 or $$$ P<0.001 compared to corresponding unstimulated cells. ##: P<0.01 or ### P<0.001 compared to corresponding LPS stimulated cells.
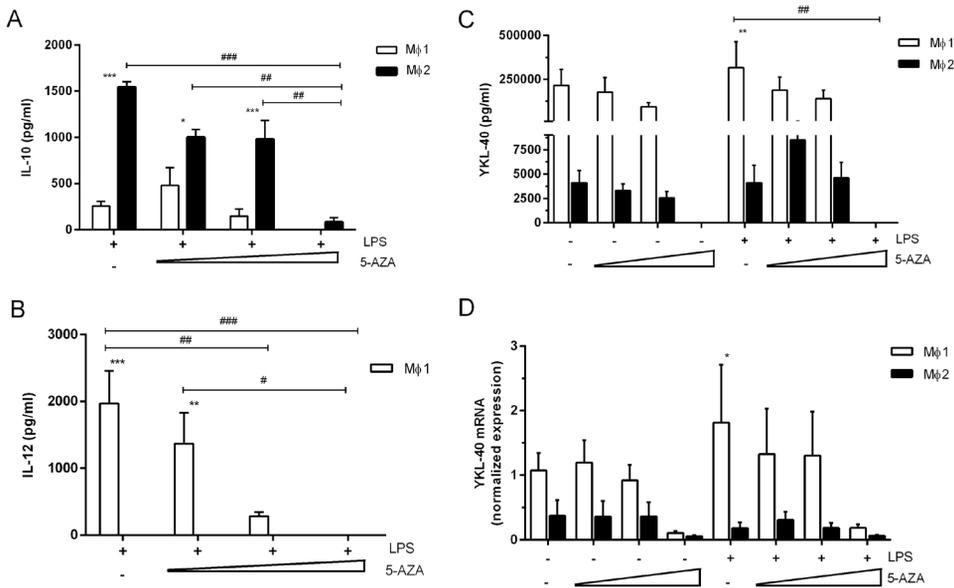
Figure 5: YKL-40 protein secretion and mRNA expression is inhibited by the demethylating agent 5-AZA. IL-10 and IL-12p40 secretion after stimulation with 5-AZA (concentration 0.1, 1 and 10μM) (Figures 5A and B, respectively). YKL-40 protein secretion and mRNA expression in MΦ1 and MΦ2 after culturing with 5-AZA (concentration 0.1, 0.3 and 1nM) (Figure 5C and D, respectively). Differentiated cells are stimulated with 100ng/ml LPS for 24 hours. Data represent mean and SEM (n=3 donors). * P≤0.05, ** P<0.01 or *** P<0.001 compared to MΦ2 with corresponding stimulus. # P≤0.05, ##: P<0.01 or ### P<0.001 compared to corresponding LPS stimulated cells.

YKL-40 levels in serum and sputum of COPD patients are not changed by treatment with inhaled corticosteroids

We next used samples of the GLUCOLD study to investigate the effects of ICS treatment on serum and sputum YKL-40 levels in COPD patients. Baseline characteristics between the group of moderate to severe COPD patients treated with ICS and placebo were not significantly different as shown in Table 2. Of the 75 compliant patients, 70 serum samples and 59 induced sputum samples at baseline were available and suitable for analysis. Serum YKL-40 levels at baseline were significantly higher compared to sputum levels at baseline (respectively median 71 ng/ml versus 29 ng/ml, P<0.001). ICS treatment did not significantly change YKL-40 levels in serum and sputum compared to placebo (both p>0.05; Figure 6).

Table 2: Patient characteristics of compliant patients at baseline of the GLUCOLD study. The ICS group is a combination of the original 30-month fluticasone and 30-month fluticasone with salmeterol groups.

Data represent mean with SD, median with interquartile range or numbers.

GLUCOLD: Groningen and Leiden Universities Corticosteroids in Obstructive Lung Disease. ICS: Inhaled corticosteroids. FEV_1: Forced Expiratory Volume in 1 second. IVC: Inspiratory vital capacity. Pred: predicted.

	ICS (n=51)	Placebo (n=24)
Gender (M/F) (n)	45/6	20/4
Age (yr)	61.7 (7.8)	59.4 (8.1)
Smoking (y/n) (n)	33/18	17/7
Packyears (yr)	48 (31-56)	42 (34-54)
Post-bronchodilator FEV_1 (% pred)	62.5 (9.2)	61.2 (8.3)
Post-bronchodilator FEV_1 (L)	2.02 (0.41)	2.00 (0.55)
Post-bronchodilator FEV_1/IVC (%)	47.3 (8.8)	46.7 (9.0)
Serum YKL-40 (ng/ml)	66 (49-119)	78 (60-118)
Sputum YKL-40 (ng/ml)	52 (20-79)	18 (12-40)
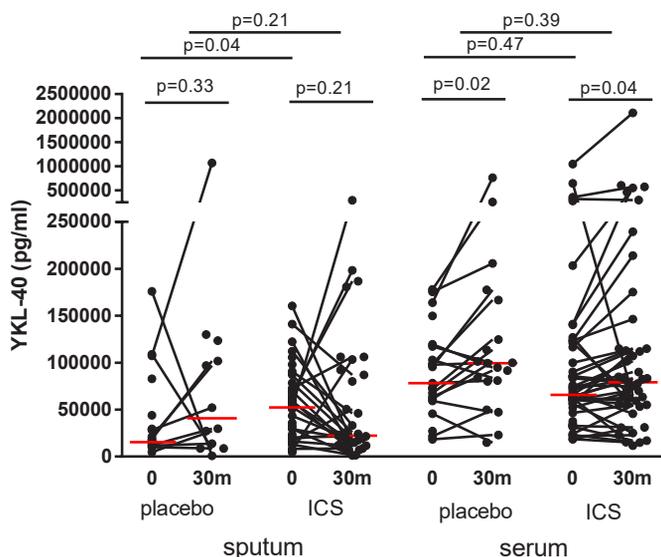
Figure 6: Sputum and serum YKL-40 protein levels of COPD patients before and after ICS treatment. YKL-40 levels in sputum and serum at baseline (0) and after 30 months (30m) of inhaled corticosteroids (ICS) and placebo. For comparison of the levels between baseline and 30 months, we only included patients from whom samples were available at both time points (paired data). Each dot represent a single patient, red horizontal bars represent medians.

DISCUSSION

This study shows that secretion and expression of YKL-40, a chitinase-like protein, is higher in *in vitro* generated monocyte-derived MΦ1 than in MΦ2, and that YKL-40 expression is not further increased upon stimulation with several pro-inflammatory stimuli. In addition, YKL-40 release *in vitro* is strongly inhibited by dexamethasone especially in MΦ1, most likely due to an effect on differentiation. Addition of the demethylating agent 5-AZA did not significantly decrease YKL-40 release, but did decrease IL-12p40 production by MΦ1 and to a smaller extent IL-10 production by MΦ2 cells. YKL-40 levels in serum were significantly higher in serum than in sputum of COPD patients. Treatment of these patients for 2.5 years with inhaled corticosteroids did not significantly change serum and sputum YKL-40 levels compared to placebo. These results suggest that YKL-40 is a promising pro-inflammatory marker in *in vitro* cultured pro-inflammatory macrophages, but is less suitable for monitoring *in vivo* effect of treatment with steroids on YKL-40 in serum and sputum of COPD patients.

We show that YKL-40 is a novel marker of *in vitro* cultured monocyte-derived MΦ1, which is independent of LPS, OSM and TNF-α. This is an important observation, since many established MΦ1 markers require additional stimulation to induce expression. Our data confirm and extend previous results [16, 17, 29], reporting higher CHI3L1 expression in classically activated macrophages compared to alternatively activated macrophages. In the latter studies, in contrast to our study, interferon-gamma (IFN-γ) and IL-4 were used for MΦ1 and MΦ2 polarization, respectively [16, 17]. We extended these data by differentiating monocytes with GM-CSF and M-CSF into MΦ1 and MΦ2, respectively, and explored the effect of further stimulation after differentiation with several pro-inflammatory stimuli. We found that dexamethasone efficiently suppressed YKL-40 expression and secretion in MΦ1, but that this was mainly explained by an inhibitory effect of dexamethasone on MΦ1differentiation, thus extending previous results [17].

We found that YKL-40 levels in serum were higher than in sputum of COPD patients. Serum YKL-40 levels of our group of patients were comparable with previous studies [13, 30, 31]. However, sputum YKL-40 levels with sputum processed using the whole sample method, were considerably lower than in studies using the selected plug method [12]. This is most likely due to dilution which is inherent to the whole sample method. After long-term treatment with ICS, we did not detect a significant change in YKL-40 levels in serum and sputum compared to placebo. To our knowledge, this is the first study that evaluates the

long-term effect of ICS in serum and sputum YKL-40 levels of COPD patients. Therefore, this study presents new *in vitro* and *in vivo* data that may help to provide insight in the function of YKL-40.

The mechanisms that regulate YKL-40 expression in health and disease are partly understood. It has been demonstrated that YKL-40 expression is absent in monocytes, and markedly induced during macrophage differentiation, especially during the later stages of differentiation [2]. Promotor analysis of the *CHI3L1* gene revealed that especially Sp1, an ubiquitous transcription factor, is important for *CHI3L1* gene expression [6]. Possibly epigenetic mechanisms also contribute to CHI3L1 expression. This is supported by the finding of a SNP localized near the CpG island in the promoter region of the *CHI3L1* gene, which is associated with YKL-40 expression [19, 20, 32]. Furthermore, hypomethylation of the *CHI3L1* gene in rheumatoid arthritis is associated with increased expression of YKL-40 [33, 34]. Therefore, *CHI3L1* gene expression may be regulated by transcription factors such as Sp1 and by DNA methylation status. Our observations with 5-AZA treatment do not support a role for DNA methylation in the expression of YKL-40 in MΦ1. Further studies into methylation status of the promotor of YKL-40, the role of histone modification and microRNAs are needed to define a role of epigenetic mechanisms in the expression of YKL-40 in (lung) macrophages.

The strength of our study is that it describes a thorough evaluation of a novel, potential pro-inflammatory macrophage marker using both *in vitro* and *in vivo* approaches. Well-characterized patients with COPD used long-term, randomized, placebo-controlled treatment with ICS. However, we were unable to detect an effect of a randomized treatment with ICS on YKL-40 serum and sputum levels. Nevertheless, our study has some limitations. First, we used *in vitro* cultured monocyte-derived macrophages from whole blood of healthy subjects that were differentiated towards MΦ1 and MΦ2 instead of lung-derived (e.g. alveolar) macrophages that were differentiated under the influence of the local environment. Since the culture systems do not fully reflect *in vitro* differentiation of macrophage subsets [35, 36], it needs to be noted that the effect of steroids on lung macrophages may differ from that on *in vitro* differentiated macrophages. Furthermore, *in vivo* a heterogeneous and intermediate macrophage population exists [37], which complicates the comparison with *in vitro* generated MΦ subsets. We therefore cannot formally exclude the possibility that this has contributed to our inability to detect an effect of inhaled corticosteroids on serum and sputum YKL-40 levels. Second, the demethylating agent 5-AZA demonstrated cell toxicity which might have influenced our results. However, we found a dose-dependent inhibition of YKL-40 expression and secretion, suggesting that DNA methylation status may contribute to regulation of YKL-40 expression in MΦ1, which is in line with studies posing that methylation

of a part of the CpG island of the *CHI3L1* gene is associated with YKL-40 levels [19, 32].

How can we explain that serum and sputum YKL-40 levels of COPD patients were not significantly changed after long-term ICS treatment compared to placebo? This is unexpected since serum YKL-40 levels of rheumatoid arthritis patients rapidly decreased after one week of prednisolone [18]. However, the amount of inhaled fluticasone that reaches the systemic circulation is low [38], which could explain why serum YKL-40 levels did not significantly change with ICS therapy. In addition, lung macrophages in COPD have reduced glucocorticoid sensitivity [39, 40].

CONCLUSION

YKL-40 is mainly expressed and secreted by MΦ1 and is not further increased by pro-inflammatory stimuli. YKL-40 release is inhibited by dexamethasone in MΦ1 *in vitro*, whereas long-term treatment of COPD patients with inhaled corticosteroids did not significantly change YKL-40 levels in serum and sputum. This suggests that YKL-40 is a potential marker for *in vitro* cultured pro-inflammatory macrophages and is not a valuable biomarker in serum and sputum of patients with COPD treated with inhaled corticosteroids.

REFERENCES

1. Johansen JS. Studies on serum YKL-40 as a biomarker in diseases with inflammation, tissue remodelling, fibroses and cancer. Dan Med Bull 2006; 53 (2): 172-209.

2. Krause SW, Rehli M, Kreutz M, Schwarzfischer L, Paulauskis JD, Andreesen R. Differential screening identifies genetic markers of monocyte to macrophage maturation. J Leukoc Biol 1996; 60 (4): 540-545.

3. Rehli M, Krause SW, Andreesen R. Molecular Characterization of the Gene for Human Cartilage gp-39 (CHI3L1), a Member of the Chitinase Protein Family and Marker for Late Stages of Macrophage Differentiation. Genomics 1997; 43 (2): 221-225.

4. Volck B, Price PA, Johansen JS, Sorensen O, Benfield TL, Nielsen HJ, Calafat J, Borregaard N. YKL-40, a mammalian member of the chitinase family, is a matrix protein of specific granules in human neutrophils. Proc Assoc Am Physicians 1998; 110 (4): 351-360.

5. Kirkpatrick RB, Emery JG, Connor JR, Dodds R, Lysko PG, Rosenberg M. Induction and Expression of Human Cartilage Glycoprotein 39 in Rheumatoid Inflammatory and Peripheral Blood Monocyte-Derived Macrophages. Exp Cell Res 1997; 237 (1): 46-54.

6. Rehli M, Niller H-H, Ammon C, Langmann S, Schwarzfischer L, Andreesen R, Krause SW. Transcriptional Regulation of CHI3L1, a Marker Gene for Late Stages of Macrophage Differentiation. J Biol Chem 2003; 278 (45): 44058-44067.

7. Johansen JS, Jensen BV, Roslind A, Nielsen D, Price PA. Serum YKL-40, A New Prognostic Biomarker in Cancer Patients? Cancer Epidemiol Biomarkers Prev 2006; 15 (2): 194-202.

8. Lee CG, Da Silva C, Dela Cruz CS, Ahangari F, Ma B, Kang MJ, He CH, Takyar S, Elias JA. Role of Chitin, Chitinase/Chitinase-Like Proteins in Inflammation, Tissue Remodeling, and Injury. Annu Rev Physiol 2011; 73: 479-501.

9. Jefri M, Huang Y-N, Huang W-C, Tai C-S, Chen W-L. YKL-40 regulated epithelial-mesenchymal transition and migration/invasion enhancement in non-small cell lung cancer. BMC Cancer 2015; 15 (1): 590.

10. Sakazaki Y, Hoshino T, Takei S, Sawada M, Oda H, Takenaka Si, Imaoka H, Matsunaga K, Ota T, Abe Y, Miki I, Fujimoto K, Kawayama T, Kato S, Aizawa H. Overexpression of Chitinase 3-Like 1/YKL-40 in Lung-Specific IL-18-Transgenic Mice, Smokers and COPD. PLoS ONE 2011; 6 (9): e24177.

11. Chupp GL, Lee CG, Jarjour N, Shim YM, Holm CT, He S, Dziura JD, Reed J, Coyle AJ, Kiener P, Cullen M, Grandsaigne M, Dombret MC, Aubier M, Pretolani M, Elias JA. A Chitinase-like Protein in the Lung and Circulation of Patients with Severe Asthma. N Engl J Med 2007; 357 (20): 2016-2027.

12. Otsuka K, Matsumoto H, Niimi A, Muro S, Ito I, Takeda T, Terada K, Yamaguchi M, Matsuoka H, Jinnai M, Oguma T, Nakaji H, Inoue H, Tajiri T, Iwata T, Chin K, Mishima M. Sputum YKL-40 Levels and Pathophysiology of Asthma and Chronic Obstructive Pulmonary Disease. Respiration 2012; 83 (6): 507-519.

13. Letuve S, Kozhich A, Arouche N, Grandsaigne M, Reed J, Dombret MC, Kiener PA, Aubier M, Coyle AJ, Pretolani M. YKL-40 Is Elevated in Patients with Chronic Obstructive Pulmonary Disease and Activates Alveolar Macrophages. J Immunol 2008; 181 (7): 5167-5173.

14. Brusselle GG, Joos GF, Bracke KR. New insights into the immunology of chronic obstructive pulmonary disease. Lancet 2011; 378 (9795): 1015-1026.

15. Gordon S, Taylor PR. Monocyte and macrophage heterogeneity. Nat Rev Immunol 2005; 5 (12): 953-964.

16. Di Rosa M, Malaguarnera G, De Gregorio C, Drago F, Malaguarnera L. Evaluation of CHI3L-1 and CHIT-1 Expression in Differentiated and Polarized Macrophages. Inflammation 2013; 36 (2): 482-492.

17. Kzhyshkowska J, Mamidi S, Gratchev A, Kremmer E, Schmuttermaier C, Krusell L, Haus G, Utikal J, Schledzewski K, Scholtze J, Goerdt S. Novel stabilin-1 interacting chitinase-like protein (SI-CLP) is up-regulated in alternatively activated macrophages and secreted via lysosomal pathway. Blood 2006; 107 (8): 3221-3228.

18. Johansen JS, Stoltenberg M, Hansen M, Florescu A, Hørslev-Petersen K, Lorenzen I, Price PA. Serum YKL-40 concentrations in patients with rheumatoid arthritis: relation to disease activity. Rheumatology 1999; 38 (7): 618-626.

19. Ober C, Tan Z, Sun Y, Possick JD, Pan L, Nicolae R, Radford S, Parry RR, Heinzmann A, Deichmann KA, Lester LA, Gern JE, Lemanske RF, Nicolae DL, Elias JA, Chupp GL. Effect of Variation in CHI3L1 on Serum YKL-40 Level, Risk of Asthma, and Lung Function. N Engl J Med 2008; 358 (16): 1682-1691.

20. Michel S, Busato F, Genuneit J, Pekkanen J, Dalphin JC, Riedler J, Mazaleyrat N, Weber J, Karvonen AM, Hirvonen MR, Braun-Fahrländer C, Lauener R, von Mutius E, Kabesch M, Tost J, the Psg. Farm exposure and time trends in early childhood may influence DNA methylation in genes related to asthma and allergy. Allergy 2013; 68 (3): 355-364.

21. Matsuda A, Asada Y, Takakuwa K, Sugita J, Murakami A, Ebihara N. DNA Methylation Analysis of Human Trabecular Meshwork Cells During Dexamethasone StimulationDNA Methylation Analysis of Trabecular Meshwork Cells. Invest Ophthalmol Vis Sci 2015; 56 (6): 3801-3809.

22. Kagoshima M, Wilcke T, Ito K, Tsaprouni L, Barnes PJ, Punchard N, Adcock IM. Glucocorticoid-mediated transrepression is regulated by histone acetylation and DNA methylation. Eur J Pharmacol 2001; 429 (1–3): 327-334.

23. Verreck FAW, de Boer T, Langenberg DML, van der Zanden L, Ottenhoff THM. Phenotypic and functional profiling of human proinflammatory type-1 and anti-inflammatory type-2 macrophages in response to microbial antigens and IFN-{gamma}- and CD40L-mediated costimulation. J Leukoc Biol 2006; 79 (2): 285-293.

24. van 't Wout EFA, van Schadewijk A, Savage NDL, Stolk J, Hiemstra PS. a1-Antitrypsin Production by Proinflammatory and Antiinflammatory Macrophages and Dendritic Cells. Am J Respir Cell Mol Biol 2012; 46 (5): 607-613.

25. Lapperre TS, Snoeck-Stroband JB, Gosman MM, Jansen DF, Van Schadewijk A, Thiadens HA, Vonk

JM, Boezen HM, Ten Hacken NH, Sont JK, Rabe KF, Kerstjens HA, Hiemstra PS, Timens W, Postma DS, Sterk PJ. Effect of fluticasone with and without salmeterol on pulmonary outcomes in chronic obstructive pulmonary disease: a randomized trial. Ann Intern Med 2009; 151 (8): 517-527.

26. Kunz LIZ, Lapperre TS, Snoeck-Stroband JB, Budulac S, Timens W, van Wijngaarden S, Schrumpf JA, Rabe KF, Postma DS, Sterk PJ, Hiemstra PS. Smoking status and anti-inflammatory macrophages in bronchoalveolar lavage and induced sputum in COPD. Respir Res 2011; 12 (1): 34.

27. in 't Veen JC, de Gouw HW, Smits HH, Sont JK, Hiemstra PS, Sterk PJ, Bel EH. Repeatability of cellular and soluble markers of inflammation in induced sputum from patients with asthma. Eur Respir J 1996; 9 (12): 2441-2447.

28. van der Does AM, Beekhuizen H, Ravensbergen B, Vos T, Ottenhoff THM, van Dissel JT, Drijfhout JW, Hiemstra PS, Nibbering PH. LL-37 Directs Macrophage Differentiation toward Macrophages with a Proinflammatory Signature. J Immunol 2010; 185 (3): 1442-1449.

29. Hashimoto S, Suzuki T, Dong HY, Yamazaki N, Matsushima K. Serial analysis of gene expression in human monocytes and macrophages. Blood 1999; 94 (3): 837-844.

30. Matsuura H, Hartl D, Kang MJ, Cruz CSD, Koller B, Chupp GL, Homer RJ, Zhou Y, Cho WK, Elias JA, Lee CG. Role of Breast Regression Protein-39 in the Pathogenesis of Cigarette Smoke-Induced Inflammation and Emphysema. Am J Respir Cell Mol Biol 2011; 44 (6): 777-786.

31. Holmgaard D, Mygind L, Titlestad I, Madsen H, Pedersen S, Johansen J, Pedersen C. Plasma YKL-40 and all-cause mortality in patients with chronic obstructive pulmonary disease. BMC Pulm Med 2013; 13 (1): 77.

32. Konradsen JR, James A, Nordlund B, Reinius LE, Söderhäll C, Melén E, Wheelock Å, Lödrup Carlsen KC, Lidegran M, Verhoek M, Boot RG, Dahlén B, Dahlén SE, Hedlin G. The chitinase-like protein YKL-40: A possible biomarker of inflammation and airway remodeling in severe pediatric asthma. J Allergy Clin Immunol 2013; 132 (2): 328-335..

33. Nakano K, Boyle DL, Firestein GS. Regulation of DNA Methylation in Rheumatoid Arthritis Synoviocytes. J Immunol 2013; 190 (3): 1297-1303.

34. Nakano K, Whitaker JW, Boyle DL, Wang W, Firestein GS. DNA methylome signature in rheumatoid arthritis. Ann Rheum Dis 2013; 72 (1): 110-117.

35. Gordon S, Mantovani A. Diversity and plasticity of mononuclear phagocytes. Eur J Immunol 2011; 41 (9): 2470-2472.

36. Sica A, Mantovani A. Macrophage plasticity and polarization: *in vivo* veritas. J Clin Invest 2012; 122 (3): 787-795.

37. Hiemstra PS. Altered Macrophage Function in Chronic Obstructive Pulmonary Disease. Ann Am Thorac Soc 2013; 10 (Supplement): S180-S185.

38. Falcoz C, Oliver R, McDowall JE, Ventresca P, Bye A, Daley-Yates PT. Bioavailability of orally administered micronised fluticasone propionate. Clinical pharmacokinetics 2000; 39 Suppl 1: 9-15.

39.   Culpitt SV, Rogers DF, Shah P, De Matos C, Russell REK, Donnelly LE, Barnes PJ. Impaired inhibition by dexamethasone of cytokine release by alveolar macrophages from patients with chronic obstructive pulmonary disease. Am J Respir Crit Care Med 2003; 167 (1): 24-31.

40.   Ito K, Lim S, Caramori G, Chung KF, Barnes PJ, Adcock IM. Cigarette smoking reduces histone deacetylase 2 expression, enhances cytokine expression, and inhibits glucocorticoid actions in alveolar macrophages. FASEB J 2001; 15 (6): 1110-1112.