European Journal of Pharmacology 696 (2012) 136–142

Contents lists available at SciVerse ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Pulmonary, gastrointestinal and urogenital pharmacology

# Multidrug resistance-associated protein 1 and lung function decline with or without long-term corticosteroids treatment in COPD

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ARTICLE INFO

*Article history:*
Received 30 January 2012
Received in revised form
17 August 2012
Accepted 27 August 2012
Available online 12 September 2012

*Keywords:*
Chronic Obstructive Pulmonary Disease (COPD)
Lung function decline
Multidrug resistance-associated protein-1
Protein expression
Single nucleotide polymorphisms (SNPs)
Treatment

ABSTRACT

Multidrug resistance-associated protein-1 (MRP1) reduces the oxidative stress generated by smoking, a risk factor for Chronic Obstructive Pulmonary Disease (COPD). We previously showed that *MRP1* variants are associated with the level and decline of annual forced expiratory volume in one second ($FEV_1$) in the general population. Moreover, we showed that *MRP1* variants are also associated with $FEV_1$ level and inflammatory markers in COPD patients.We investigate in the current study the association of MRP1 protein expression in bronchial biopsies with $FEV_1$ decline in COPD patients using placebo, or inhaled corticosteroids (ICS) with or without long-acting β2-agonists. Additionally we investigate the association of *MRP1* variants with $FEV_1$ decline. *MRP1* variants (rs212093, rs4148382, rs504348, rs4781699, rs35621) were genotyped in 110 COPD patients. Associations of *MRP1* variants and MRP1 protein expression in bronchial biopsies (obtained at baseline, 6 and 30 months) with $FEV_1$ decline were analyzed using linear mixed-effect models. During 30-month ICS treatment, subjects with a moderate staining for MRP1 had less $FEV_1$ decline than those with a weak staining. In subjects stopping ICS after 6 months followed by 24-month placebo, moderate staining for MRP1 was associated with faster $FEV_1$ decline than in those with a weak staining. None of the variants was associated with $FEV_1$ decline. Our unique study suggests a role of MRP1 protein expression in bronchial biopsies in $FEV_1$ decline occurring selectively in COPD patients with long-term (30-month) ICS therapy.



## 1. Introduction

Chronic Obstructive Pulmonary Disease (COPD) is an inflammatory disease characterized by airflow limitation that is not fully reversible, progressive in nature and associated with an abnormal inflammatory response of the lungs to cigarette smoke (Celli and MacNee, 2004). Smoking is associated with accelerated lung function decline, which can be tempered by quitting smoking. Smoking generates oxidative stress in the lung, which can be reduced by proteins of the ATP-binding cassette (ABC) superfamily such as multidrug resistance-associated protein 1 (MRP1) (Cole et al., 1992). MRP1 (official name ABCC1, ABC subfamily C, member 1) is highly expressed in human lung tissue (Cole et al., 1992), especially at the basolateral side of bronchial epithelium (Van der Deen et al., 2005). MRP1's contribution varies from protecting cells within the body against drugs, environmental toxins and heavy metals, to its involvement in the cellular oxidative defense system and inflammation (Hipfner et al., 1999 and Leslie et al. 2001).

In a recent study we showed that the *MRP1* gene is associated with the lung function in two independent general population-based cohorts (Siedlinski et al., 2009). In these cohorts five single nucleotide polymorphisms (SNPs) in the *MRP1* gene (rs212093, rs4148382, rs504348, rs4781699 and rs35621) were significantly associated with level of forced expiratory volume in one second ($FEV_1$) or with annual $FEV_1$ decline (Siedlinski et al., 2009). In a subsequent study in COPD patients we have shown that the *MRP1* gene plays a role in the severity of COPD, since rs212093 and rs4148382 in *MRP1* were associated with less respectively more airway wall inflammation and a higher respectively lower level of lung function (Budulac et al., 2010). Moreover, rs4148382 was significantly associated with a higher MRP1 protein expression in bronchial biopsies from subjects with established COPD (Budulac et al., 2010). Due to these novel results with respect to the *MRP1*

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http://dx.doi.org/10.1016/j.ejphar.2012.08.015

gene and severity of COPD, we aim in the current study to investigate in depth MRP1 protein expression and lung function in the same group of COPD patients.

Thus, consequently, our current study will focus on the associations MRP1 protein expression in airway wall biopsies with the decline of lung function in the same population of COPD patients we previously studied with respect to anti-inflammatory and clinical effects of inhaled corticosteroids (ICS) with or without long-acting β-agonists (LABAs) (Lapperre et al., 2009). We investigated whether MRP1 protein expression in bronchial biopsies obtained at baseline and at 6- and 30-month treatment, are associated with the $FEV_1$ level at baseline, 6 and 30 months and whether MRP1 protein expression in bronchial biopsies is associated with the course of $FEV_1$, with and without ICS ± LABA treatment. Additionally, we investigated whether *MRP1* SNPs are associated with the course of $FEV_1$.

## 2. Materials and methods

### 2.1. Study population

We included 114 patients with COPD who participated in a two-center trial (Groningen Leiden Universities and Corticosteroids in Obstructive Lung Disease; GLUCOLD study). Patient characteristics and methods have been described in detail previously (Lapperre et al., 2004). In brief, all patients had irreversible airflow limitation, chronic respiratory symptoms (Lapperre et al., 2007), did not use a course of oral steroids during the previous 3 months and had no maintenance treatment with inhaled or oral steroids during the previous 6 months. They were current or ex-smokers with a smoking history of ≥10 pack-years, aged between 45 and 75 years without a history of asthma. The study was approved by the medical ethics committees of the University Medical Centers of Leiden and Groningen. All patients gave their written informed consent.

#### 2.1.1. Clinical characteristics

Reversibility to salbutamol was measured at baseline, 6 and 30 months and $FEV_1$ postbronchodilator was measured every three months using standardized protocols (Lapperre et al., 2006).

#### 2.1.2. Intervention and follow-up procedures

Patients were randomly assigned to receive either: (1) fluticasone propionate, 500 μg twice daily, for the first 6 months followed by placebo, twice daily, for 24 months; (2) fluticasone, 500 μg twice daily for 30 months; (3) fluticasone, 500 μg twice daily and salmeterol, 50 μg twice daily, in a single inhaler for 30 months; (4) placebo, twice daily, for 30 months (Lapperre et al., 2009).

### 2.2. MRP1 tagging SNPs and genotyping

We selected from all *MRP1* tagging SNPs rs212093, rs4148382, rs35621, rs4781699 and rs504348 based on our previous studies showing a significant associations with $FEV_1$ level and/or annual $FEV_1$ decline in the general population (Siedlinski et al., 2009), as well as with $FEV_1$ level and inflammatory markers in COPD patients (Budulac et al., 2010). Genotyping was performed by K-Bioscience (UK) using their patent-protected competitive allele specific PCR system (KASPar).

### 2.3. Biopsies and immunohistochemistry on bronchial biopsies

Details on fiberoptic bronchoscopy, biopsy processing and immunohistochemical staining are described in the data supplement (additional file 1; subsections 2.3.1, 2.3.2 and 2.3.3). MRP1 protein was scored for staining intensity in intact epithelium of bronchial biopsies with a semiquantitative score: 1 = no or weak; 2 = moderate; 3 = strong staining intensity.

### 2.4. Statistics

Differences between treatment groups in MRP1 staining intensity at baseline, 6 and 30 months were analyzed using Chi-square tests (see additional file 1 for details; subsection 2.4.1).

We assessed the association of MRP1 protein expression at baseline, 6 months and 30 months with the $FEV_1$ levels at those time points using linear mixed-effect models (LME) stratified for treatment group, with adjustment for gender, height and age at baseline.

We used LME models to assess the association of MRP1 protein expression at baseline with $FEV_1$ decline stratified for treatment groups, separately for the period till 6 months and the time after 6 months because of the change in treatment for one of the groups. We adjusted our analyses for height and age at baseline, gender and the interaction of time with age at baseline and gender.

We assessed the associations of *MRP1* SNPs with $FEV_1$ decline using LME models (see additional file 1 for details on the genetic model; subsection 2.4.1). Analyses were adjusted for height and age at baseline, gender, treatment and the interactions of time with age at baseline, gender and treatment.

Analyses were performed using SPSS version 16.0 for Windows and values of $p < 0.05$ (tested 2-sided) were considered statistically significant. Linkage Disequilibrium (LD) plots a threshold of 0.8 for the correlation coefficient ($r^2$) and Hardy Weinberg Equilibrium (HWE) tests were performed with Haploview (version 4.2) (Barrett et al., 2005).

## 3. Results

The clinical characteristics of COPD patients are presented in Table 1.

All 5 *MRP1* SNPs were in Hardy Weinberg Equilibrium (HWE, $p > 0.05$) and were only weakly correlated with each other; the highest $r^2$ in our population being 0.34 (Fig. 1).

**Table 1**
Clinical characteristics of COPD patients.

| **Baseline characteristics, *n* = 114** | |
|---|---|
| Males, *n* (%) | 99 (86.8) |
| Age (years) | 61.6 (7.7) |
| Height (cm) | 175.5 (7.8) |
| Packyears[a] | 41.8 (31.2–54.7) |
| Current smoker, *n* (%) | 72 (63.2) |
| $FEV_1$/FVC (%) postbronchodilator | 50.3 (8.9) |
| $FEV_1$ (L) postbronchodilator | 2.03 (0.5) |
| $FEV_1$% predicted postbronchodilator | 62.7 (9.1) |
| **Treatment groups, *n* = 101 (%)[b]** | |
| Placebo | 24 (23.8) |
| Fluticasone 6 months, Placebo 24 months | 26 (25.7) |
| Fluticasone 30 months | 26 (25.7) |
| Fluticasone/Salmeterol | 25 (24.8) |

Data are presented as mean (S.D.) or

[a] median (25th–75th percentile);

[b] number of patients who adhered to therapy (used >70% medication); $FEV_1$ = forced expiratory volume in one second; $FEV_1$/FVC = $FEV_1$/forced vital capacity.

Table 2 shows the distribution of MRP1 protein expression within treatment groups.

There were no consistent differences between treatment groups in MRP1 staining intensity at baseline, 6 and 30 months. There were no differences in age, lung function and packyears between subjects with at least 1 biopsy with intact epithelium ($n$=91 (79.8%)) and those without any biopsy ($n$=23 (20.2%)). There were fewer women with at least 1 biopsy with intact epithelium ($n$=9 (64.3%)) than men ($n$=77 (88.5%)).

### 3.1. MRP1 protein expression and $FEV_1$ level at 0, 6 and 30 months

MRP1 protein expression at 0, 6 and 30 months was not significantly associated with $FEV_1$ levels at those time points (detailed results are presented in the table S1; additional file 1).

### 3.2. MRP1 protein expression at baseline and $FEV_1$ decline (0–6 months and 6–30 months)

Subjects with a moderate or strong intensity of staining for MRP1 protein expression at baseline did not have a slower or faster $FEV_1$ decline compared to those with weak staining for MRP1 protein expression in any of the treatment groups for the ensuing period of 6-month treatment (Fig. 2).

In the group with 30-month ICS treatment, subjects having a moderate intensity of staining at baseline had less $FEV_1$ decline between 6 and 30 months than those with a weak intensity of staining for MRP1 protein (Fig. 2). Conversely, in the treatment group with discontinuation of ICS therapy after 6 months, subjects having a moderate intensity of staining had a faster $FEV_1$ decline compared to those with a weak intensity of staining for MRP1 protein (Fig. 2).

MRP1 protein expression at baseline was not significantly predictive of 30-month $FEV_1$ decline in the group using placebo or combination of LABAs with ICS (Fig. 2).

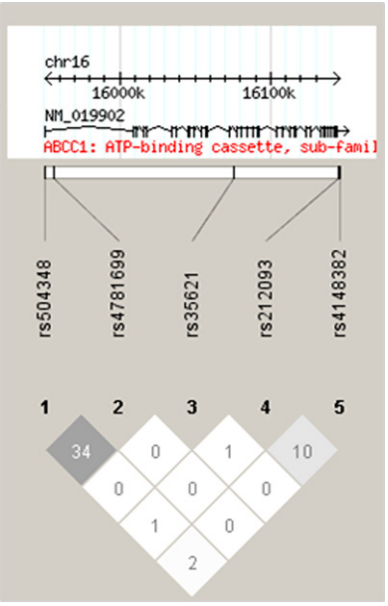


**Fig. 1.** Linkage disequilibrium plot and correlation coefficients ($r^2$) for the 5 *MRP1* polymorphisms genotyped in COPD patients ($n$=110). *MRP1=multidrug resistance-associated protein-1* (official name *ABCC1, ATP-binding cassette (ABC) subfamily C, member 1*); the location of the single nucleotide polymorphisms is given for the HapMap Data Release February 2009.

### 3.3. MRP1 SNPs and $FEV_1$ decline (0–6 months and 6–30 months)

None of the *MRP*1 SNPs was significantly associated with the $FEV_1$ decline for either the period till 6 months or the time after 6 months (Fig. 3).

Additional adjustment for the initial $FEV_1$ and its interaction with time did not change the size or significance of the estimated effect of the genotypes on $FEV_1$ decline.

## 4. Discussion

This is the first study showing significant associations of a higher MRP1 protein expression with less accelerated $FEV_1$ decline in COPD patients using long-term therapy with ICS and a significant association of higher MRP1 protein expression with a faster $FEV_1$ decline after withdrawal of ICS. These associations were not likely due to the genetic background in *MRP1*, since the SNPs in *MRP1* that were previously associated with the level of $FEV_1$ (Budulac et al., 2010), in the current study are not significantly associated with $FEV_1$ decline in the same group of COPD patients. This is in line with our previous findings showing that the same two SNPs are not significantly associated with the decline of lung function in the general population (Siedlinski et al., 2009) suggesting that these SNPs may play a role in COPD severity, without any further effects on lung function over time.

Current international guidelines recommend maintenance therapy with LABAs and ICS in moderate to severe COPD with exacerbations (Rabe et al., 2007). Variable findings have been published regarding the effect of ICS on lung function (decline) and exacerbations in COPD. In one study, fluticasone propionate and salmeterol significantly improved lung function after 2-week treatment and this effect was sustained during 12-month treatment (Calverley et al., 2003). Although some other studies showed no effect of fluticasone propionate or budesonide on lung function in COPD patients (Burge et al., 2000; Hattotuwa et al.,

**Table 2**
Frequencies of MRP1 staining intensity levels within treatment groups.

| Treatment group | MRP1 scores baseline | | | | MRP1 scores 6 months | | | | MRP1 scores 30 months | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1[a] | 2[b] | 3[c] | Nt[d] | 1[a] | 2[b] | 3[c] | Nt[d] | 1[a] | 2[b] | 3[c] | Nt[d] |
| Placebo, N | 3 | 8 | 2 | 13 | 3 | 6 | 4 | 13 | 1 | 9 | 3 | 13 |
| Fluticasone 6 months, N | 3 | 8 | 3 | 14 | 3 | 10 | 5 | 18 | 3 | 13 | 2 | 18 |
| Fluticasone 30 months, N | 3 | 8 | 4 | 15 | 1 | 12 | 4 | 17 | 3 | 8 | 1 | 12 |
| Fluticasone/Salmeterol, N | 8 | 2 | 1 | 11 | 3 | 11 | 1 | 15 | 1 | 12 | 6 | 19 |

MRP1=multidrug resistance-associated protein 1; MRP1 scores

[a] 1=weak staining,
[b] 2=moderate staining,
[c] 3=strong staining,
[d] Nt=total number of subjects per treatment group for each time point.

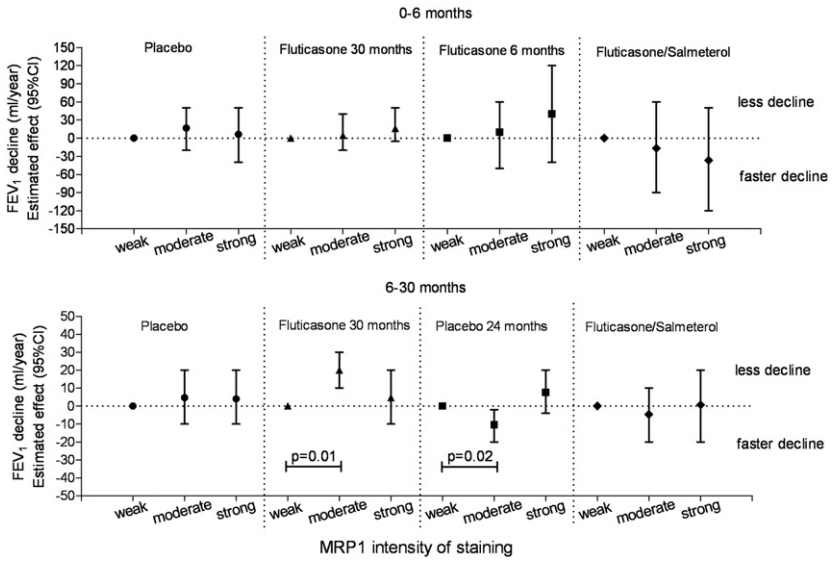


**Fig. 2.** The effect of MRP1 protein level at baseline on $FEV_1$ decline. The upper graph is for the first 6 months and the lower graph is for the period from 6 to 30 months. $FEV_1$ = forced expiratory volume in one second. Squares represent the regression coefficient (B) and vertical bars the 95% confidence interval (CI). The weak staining for MRP1 was set to zero as the reference category. The analyses are adjusted for height, age at baseline, gender and the interaction of time with gender and age at baseline.

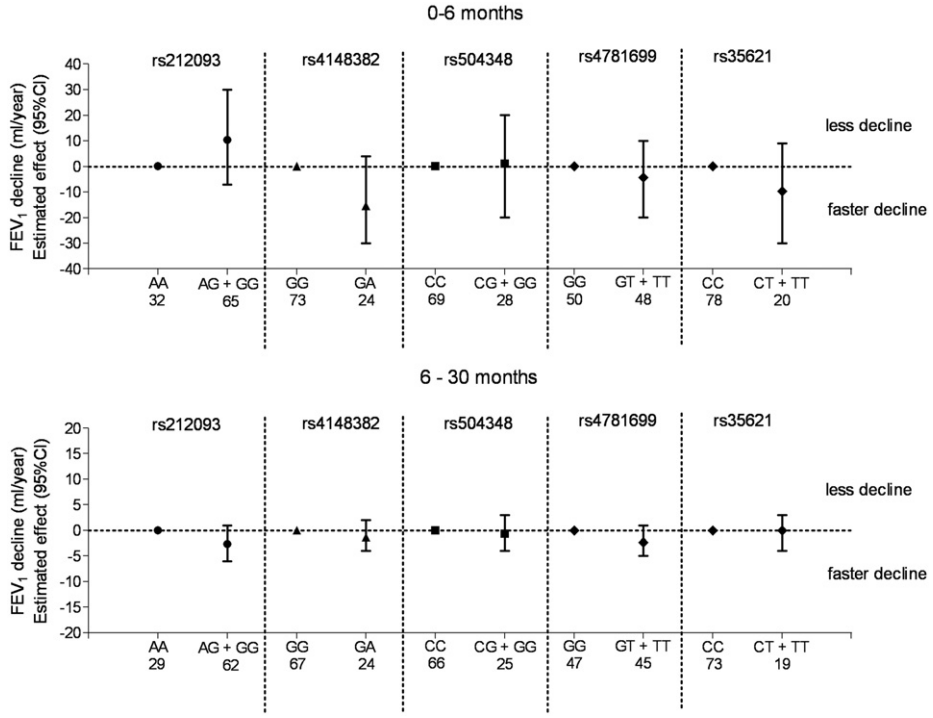


**Fig. 3.** The effect of *MRP1* SNPs on $FEV_1$ decline (0–6 months, 6–30 months). The upper graph is for the first 6 months and the lower graph is for the period from 6 to 30 months. $FEV_1$ = forced expiratory volume in one second. Squares represent the regression coefficient (B) and vertical bars the 95% confidence interval (CI). Wild type was set to zero as the reference category. Different numbers for the SNP genotypes are due to missing data on genotype or $FEV_1$ measurements at every time point. The analyses are adjusted to height and age at baseline, gender, treatment and the interactions of time with age at baseline, gender and treatment.

2002; Pauwels et al., 1999 and Vestbo et al., 1999), recent data suggest that disease progression can be modified by ICS (Celli et al., 2008; Lapperre et al., 2009; Van der Valk et al., 2002 and Wouters et al., 2005). Our current study provides further information in that those subjects with a moderate staining for MRP1 level had less lung function decline compared to those with a weak staining in the presence of long-term (30-month) therapy with fluticasone propionate. Importantly, subjects with a moderate intensity of MRP1 staining who stopped fluticasone treatment at 6 months and subsequently used 24-month placebo, had a significantly faster lung function decline compared with those with a weak staining for MRP1. This suggests that continuing therapy with ICS may positively influence the association of MRP1 protein with $FEV_1$ decline. Furthermore, an in vitro study showed that FP accelerated the conversion of leukotriene C4 (LTC4) to LTD4 and finally leaded to the accelerated degradation of LTC4 to less active LTE4 via LTD4 and this action was observed only after cells were exposed to FP for more than 2 days, suggesting new

protein synthesis (Zaitsu et al., 2001). The authors speculated that regular use of FP up-regulates the catabolic process of LTC4 to less active LTE4, and therefore contributes to the antiasthma actions (Zaitsu et al., 2001). Although the inflammatory processes occurring in asthma and COPD are different, we can speculate that in the current in vivo study, stopping ICS therapy after 6 months could have a negative effect on the action of the MRP1 protein substrate LTC4. Also this is suggestive of an interaction between long-term therapy with ICS and MRP1 function. We did not investigate the effect of specific treatment on MRP1 protein expression in bronchial biopsies (Van der Deen et al., 2008 and Bandi and Kompella, 2002), therefore it is uncertain whether this association of MRP1 protein with lung function decline in the presence of long-term therapy with fluticasone results from the effect of treatment or from MRP1 functionality and/or activity. Further studies, that might include smoking animal models, should also focus on the effect of bronchial epithelium MRP1 protein level in the presence of COPD medication such as fluticasone propionate. Whereas in previous studies in a smoking animal model we studied the association of bronchial MRP1 expression and inflammation (Van der Deen et al., 2007), it would be of interest in future studies to also focus in COPD/smoking-related animal models on the association of bronchial MRP1 protein and lung function.

A previous study showed that the therapy with salmeterol plus fluticasone propionate, or with salmeterol and fluticasone propionate separately reduces the rate of decline of $FEV_1$ in patients with moderate-to-severe COPD (Celli et al., 2008). Prior, we showed in the current COPD patients (GLUCOLD) that combination therapy of fluticasone propionate and salmeterol when compared with fluticasone alone has no additional long-lasting effects on inflammatory cells and that it improves $FEV_1$ level without further influencing $FEV_1$ decline (Lapperre et al., 2009). These observations may indicate that disease modification may occur in particular sub phenotypes of patients with COPD (Lapperre et al., 2009). Our data suggest that MRP1 protein expression may reflect one of the COPD phenotypes that is sensitive to inhaled corticosteroid therapy. In fact that we did not find a significant association of MRP1 protein expression with 30-month $FEV_1$ decline in the group using combination of LABA with ICS is likely due to lack of power. Unfortunately, only 2 subjects had analyzable biopsies with a moderate staining for MRP1 protein at baseline in the group randomly selected to use combination of LABAs with ICS.

MRP1 is located in bronchial epithelium, which is the first line of defense against invading microorganisms and inhaled toxic substances like cigarette smoke. Moreover, epithelial cells are the first cells that pulmonary drugs have to come across to reach the underlying tissue and act on it. Thus MRP1 may have a role in development and severity of COPD. We have previously shown that patients with moderate and severe COPD have a lower MRP1 expression in bronchial biopsies than healthy individuals. Furthermore, patients with severe and very severe COPD had again a lower intensity of MRP1 protein expression than those with mild and moderate severe COPD (Van der Deen et al., 2006). Thus the spectrum of MRP1 protein expression reaches from higher in healthy subjects to lower in severe disease. Although our previous study (Van der Deen et al., 2006) is not comparable with our current study due to differences in study design and type of biopsies used for analyses (e.g. frozen vs. paraffin), it is tempting to speculate that the observed association of a higher MRP1 protein expression with a less rapid $FEV_1$ decline fits with our COPD patients having mild to moderate COPD. This might also explain why we did not observe any significant association of an even higher MRP1 protein expression (coded as strong) with $FEV_1$ decline (Fig. 1). The ultimate effect may, as our data suggest, depend on the medication prescribed, as likely as on doses and time of administration. These findings are of importance since they can contribute to pharmacogenetics of COPD management in mild to moderate COPD patients, which will result in more accurate and targeted therapy.

We showed previously in an in vitro study that cellular concentrations of budesonide affect negatively MRP1 function, likely by inhibiting MRP1 mediated transport that protects against oxidative stress and toxic compounds (Van der Deen et al., 2008). How can we reconcile this with our current findings that a higher MRP1 protein expression is associated with less accelerated lung function decline in subjects using 30-month fluticasone propionate therapy? We can speculate on some possible explanations. First, the deposition of drugs may influence its effects. A human in vivo study demonstrated that fluticasone propionate concentrations are three times higher in central than peripheral lung tissue (Esmailpour et al., 1997). Moreover, the same study showed (Esmailpour et al., 1997) that fluticasone concentrations in peripheral lung tissue following inhalation of a 1.0 mg dose were higher compared to those reported for budesonide (Van den Bosch et al., 1993). Thus, there may be dose differences between our previously published in vitro study on effects of budesonide and the doses present in the airways and lung tissue with fluticasone in vivo.

Second, the action of different inhaled corticosteroids on epithelial tissue may depend on the pharmacokinetic and pharmacodynamic properties of their components. For example, budesonide is less lipophilic than fluticasone and therefore is dissolving freely in airway mucus and is more rapidly absorbed into the airway tissue (Brattsand and Miller-Larsson, 2003 and Thorsson et al., 2001) whereas fluticasone propionate is released slower from the lung lipid compartment with a longer local duration of action (Johnson, 1998). Thus the duration of action of each inhaled steroid may affect the outcome on MRP1 protein association and lung function decline.

Third, it has been previously shown that MRP1 substrates identified by vesicle transport studies compete with each other for transport, even if there are no similarities between the compounds and they are conjugated to different anionic moieties, e.g. LTC4 or $E_2$17βG (Loe et al., 1996a, 1996b). This suggests that each substrate is creating an exclusive interaction with MRP1 and it might be that fluticasone propionate and budesonide act also differently in the presence of this protein by being involved in different competing activities with other transported substrates.

We believe that our unique study, due to its clear design and including lung function measurements and biopsy data, has brought new ideas on a possible role of MRP1. This needs further investigation, since is seems possible that MRP1 is differentially influenced by different types of ICS. Our data suggest further exploration of MRP1 in a larger group to establish whether MRP1 could be a candidate marker for individualized ICS prescription in COPD.

## 5. Conclusions

In conclusion, this is the first study showing a protective association of a moderate MRP1 protein level in bronchial biopsies with lung function decline in COPD patients using long-term maintenance therapy with fluticasone propionate, when compared with COPD patients having a low intensity of MRP1 protein staining. Conversely, a deleterious association was present when ICS therapy was discontinued after 6-month treatment with fluticasone.

## Sources of support

Graduate School for Drug Exploration (GUIDE), Groningen, The Netherlands

The GLUCOLD study was supported by the Netherlands Organization for Scientific Research (NWO), the Netherlands Asthma Foundation (NAF), GlaxoSmithKline (NL), Leiden University Medical Center (LUMC), and University of Groningen (RUG). Funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

## Statement of interest

SEB has no competing interests. DSP received funding for research from AstraZeneca, GSK, Nycomed. Travel to ERS or ATS has been partially funded by AstraZeneca, GSK, Chiesi, Nycomed. She has been consultant to AstraZeneca, Boehringer Ingelheim, Chiesi, GSK, Nycomed, TEVA. PSH has received unrestricted research grants for the Department of Pulmonology (Leiden University Medical Center) from Amgen, Boehringer Ingelheim, Centocor, Galapagos and GlaxoSmithKline. TSL has no competing interests. LIZK has no competing interests. JMV has no competing interests. HMB has no competing interests. WT has received an unrestricted grant from GlaxoSmithKline and a sponsored grant from Netherlands Asthma Funds.

## Acknowledgments

Members of the GLUCOLD Study Group: H.F. Kauffman, D. de Reus, Department of Allergology; H.M. Boezen, D.F. Jansen, J.M. Vonk, Department of Epidemiology; M.D.W. Barentsen, W. Timens, M. Zeinstra-Smit, Department of Pathology; A.J. Luteijn, T. van der Molen, G. ter Veen, Department of General Practice; M.M.E. Gosman, N.H.T. ten Hacken, H.A.M. Kerstjens, M.S. van Maaren, D.S. Postma, C.A. Veltman, A. Verbokkem, I. Verhage, H.K. Klooster, Department of Pulmonology; Groningen University Medical Center, Groningen, The Netherlands; J.B. Snoeck-Stroband, H. Thiadens, Department. of General Practice; J.K. Sont, Department of Medical Decision Making; I. Bajema, Department of Pathology; J. Gast-Strookman, P.S. Hiemstra, K. Janssen, T.S. Lapperre, K.F. Rabe, A. van Schadewijk, J. Smit-Bakker, J. Stolk, A.C.J.A. Tiré, H. van der Veen, M.M.E. Wijffels and L.N.A. Willems, Department of Pulmonology; Leiden University Medical Center, Leiden, The Netherlands; P.J. Sterk, Department of Medical Centre, Amsterdam, The Netherlands, T. Mauad, University of Sao Paulo, Sao Paulo, Brazil.

## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ejphar.2012.08.015.

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