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2 **TITLE PAGE**

3 # Inhaled long-acting β_2-agonists enhance glucocorticoid receptor nuclear translocation and
4 efficacy in sputum macrophages in COPD

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ABSTRACT

Background: Combination inhaled therapy with long-acting β_2-agonist (LABA) and corticosteroid is beneficial in treating asthma and chronic obstructive pulmonary disease (COPD).

Objective: In asthma, LABAs enhance glucocorticoid receptor (GR) nuclear translocation in the presence of corticosteroids. Whether this biological mechanism occurs in COPD, a relatively corticosteroid-resistant disease, is uncertain.

Methods: Eight patients with mild/moderate COPD participated in a double-blind, placebo-controlled, crossover study and inhaled single-doses of; fluticasone propionate (FP)-100μg, FP-500μg, salmeterol(SLM)-50μg and combination FP-100μg+SLM-50μg. One hour post-inhalation sputum was induced, nuclear proteins isolated from purified macrophages and levels of activated nuclear GR quantified using a GR-GRE ELISA-based assay.

Results: Nuclear GR significantly increased after FP-500μg ($p<0.01$), but not after FP-100μg or SLM-50μg, compared to placebo. Interestingly, SLM in combination with FP-100μg increased nuclear GR levels equivalent to those of FP-500μg alone. This was significantly greater than either FP-100μg ($p<0.05$) or SLM-50μg ($p<0.01$) alone. *In vitro* in a human macrophage cell line, SLM (10^{-8}M) enhanced FP (10^{-9}M)-induced mitogen-activated protein kinase phosphatase-1 (MKP-1) mRNA (5.8 ± 0.6 vs. $8.4\pm1.1 \times 10^{-6}$ copies, $p<0.05$) and 2xGRE-luciferase reporter gene activity (250.1 ± 15.6 vs. 103.1 ± 23.6 fold induction, $p<0.001$). Addition of SLM (10^{-9}M) to FP (10^{-11}M) significantly enhanced FP-mediated suppression of IL-1β-induced CXCL8 ($p<0.05$)**.**

Conclusion: Addition of SLM-50μg to FP-100μg, enhanced GR nuclear translocation equivalent to that seen with a five-fold higher dose of FP in sputum macrophages from COPD patients. This may account for the superior clinical effects observed in COPD of combination LABA/corticosteroid treatment compared to either as monotherapy.

CLINICAL INFORMATION

Sputum macrophages from COPD patients are relatively corticosteroid insensitive. Inhaled salmeterol and fluticasone propionate greatly enhanced glucocorticoid receptor activation in sputum macrophages from COPD patients.

CAPSULE SUMMARY

Combination therapy with inhaled long-acting β_2-agonist and corticosteroid improves clinical outcomes in COPD and biologically, enhanced glucocorticoid receptor activation in sputum macrophages may partly explain this phenomenon.

KEYWORDS: long-acting β_2-agonist, corticosteroid, transcription factor, glucocorticoid receptor, sputum, COPD, macrophage

64 **ABBREVIATIONS**

65	COPD	Chronic obstructive pulmonary disease
66	FCS	Fetal calf serum
67	FP	Fluticasone propionate
68	FEV_1	Forced expiratory volume in one-second
69	GR	Glucocorticoid receptor
70	GRE	Glucocorticoid response element
71	HBSS	Hanks' buffered saline solution
72	ICS	Inhaled corticosteroids
73	LABAs	Long-acting β_2-agonists
74	MKP-1	Mitogen-activated protein kinase phosphatase-1
75	PBMCs	Peripheral blood mononuclear cells
76	SLM	Salmeterol xinafoate

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88 **INTRODUCTION**

89 It is well-established in asthmatic patients that the addition of long-acting β_2-agonist (LABA) to
90 inhaled corticosteroids (ICS) provides more effective disease control than monotherapy with ICS
91 [1-4]. Indeed, with recent concerns regarding the safety of LABA monotherapy in the treatment
92 of asthma, current guidance supports fixed dose combinations of LABA/ICS therapy from a
93 single inhaler [5-7]. Chronic obstructive pulmonary disease (COPD) is also characterized by
94 chronic airways inflammation, but unlike asthma, ICS treatment on its own has relatively little
95 effect on the accelerated decline in lung function seen in COPD patients [8,9]. However in
96 contrast, several large clinical trials of combination LABA/ICS therapy in patients with stable
97 COPD have shown better control of respiratory symptoms, lung function, quality of life, and
98 exacerbations with no greater risk of side-effects, compared to the use of either ICS alone or
99 LABA alone (10-13). Recent studies have demonstrated the effectiveness of LABA/ICS
100 combination therapy in reducing COPD morbidity and mortality compared with ICS treatment
101 alone (14, 15).

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103 The scientific rationale to explain the disproportionately superior clinical efficacy achieved by
104 the addition of LABAs to ICS in the treatment of asthma and COPD is being addressed, and
105 elucidating the underlying molecular mechanisms may help identify novel therapeutic targets
106 (16, 17). Combination therapy with SLM/FP reduces sputum differential cell counts, sputum
107 neutrophils and eosinophils, bronchial CD45+, CD8+ and CD4+ cells, and cells expressing genes
108 for tumor necrosis factor-α and interferon-γ in comparison to placebo (18). These anti-
109 inflammatory effects were also accompanied by improvements in the pre-bronchodilator forced
110 expiratory volume in one-second (FEV_1). Treatment with SLM/FP combination has also been

111 shown to reduce tissue CD8+ and CD68+ cells compared with placebo, whereas no effect was
112 observed with FP alone (19).
113
114 It has been proposed LABAs may have anti-inflammatory properties similar to ICS (20, 21).
115 Studies show LABAs enhance corticosteroid-dependent anti-inflammatory effects, and the
116 biological mechanism for this may be through ligand-independent priming of the glucocorticoid
117 receptor (GR) (22). The cellular actions of corticosteroids are mediated by intracellular GRs,
118 which after binding to corticosteroid, translocate from the cell cytoplasm into the nucleus
119 where they mediate corticosteroid-dependent effects (23). Enhanced GR activation by LABAs
120 has been demonstrated *in vitro* in human lung fibroblasts and smooth muscle cells (22, 24), in
121 human neutrophils (25), and *ex vivo* in the sputum of patients with asthma (17). However, it is
122 unknown whether LABAs cause GR translocation after ICS therapy in COPD patients, as this
123 disease is much less responsive to the effects of corticosteroids compared to asthma (26).
124 Indeed, macrophages from COPD patients have been shown to exhibit a poor anti-inflammatory
125 response to corticosteroids *in vitro* compared to cells from healthy smokers and non-smokers
126 (21).
127
128 In this study, we examined the effect of SLM/FP combination treatment on GR-glucocorticoid
129 response element (GRE) binding in the nucleus of macrophages isolated from the induced
130 sputum of patients with COPD. Importantly, we used inhaled drug doses used in the clinic. Our
131 aim was to determine whether GR activation in response to corticosteroid therapy was
132 enhanced by a LABA and whether this had any functional effects. As reduced nuclear
133 translocation of GR contributes to corticosteroid insensitivity (16, 26), we hypothesized that the

134 LABA, salmeterol, could contribute to improve corticosteroid insensitivity in cells from COPD
135 patients.
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158 **METHODS**

159 **Subjects**

160 Eight patients diagnosed with moderate COPD (GOLD stages 2 and 3 (27)), <12% reversibility in
161 FEV_1 and a smoking history of >10 pack-years, participated in a randomized, double-blind,
162 placebo-controlled cross-over study. All patients were aged between 40 – 80 years and were not
163 taking either ICS or LABAs (Table 1). At each study-visit, patients' FEV_1 was required to be within
164 15% of their screening FEV_1 value to control for differences in airway function and inflammation
165 between visits. Patients were allowed to take the bronchodilators tiotropium bromide and/or
166 albuterol during the study, but none of the subjects were treated with ICS or LABAs for at least
167 one month preceding the start of the study.

168

169 **Study design**

170 Single actuations of fluticasone propionate (FP)-100µg, FP-500µg, salmeterol xinafoate (SLM)-
171 50µg, combination therapy FP/SLM 100µg/50µg (Seretide™, GlaxoSmithKline, Stevenage, U.K.)
172 or placebo were delivered via a metered dose inhaler and spacer, separately at each study visit
173 Sputum was induced 60 minutes after drug inhalation and peripheral blood samples were also
174 taken at this time point. The study was approved by the Ethics Committee of the Royal
175 Brompton & Harefield Hospitals National Health Service Trust, and all subjects gave written
176 informed consent.

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178 There was a minimum washout period between treatments of seven days to prevent any
179 crossover effects. All patients were randomised (pre-assigned by picking balls out of a bag) to
180 determine the order of treatment. In this scenario, there were 24 possible crossover orders, and
181 with 8 subjects, there would have been possible treatment orders that were not received.

182 However, the sample size of 8 subjects was ethically chosen based on our previous study (17),
183 where 7 subjects received 5 treatments, which was sufficient to detect statistical differences
184 between ICS alone vs. LABA/ICS combination in asthmatic subjects.

185

186 **Sputum induction and processing**

187 Sputum induction was performed as previously described and processed within one hour of
188 collection (17). Briefly, sputum was filtered (70µm filter) and centrifuged (1600rpm for 5 min) to
189 obtain a cell pellet. Cells were resuspended in full RPMI-1640 media (Sigma-Aldrich),
190 supplemented with 1% L-glutamine, 10% fetal calf serum (FCS; Invitrogen Ltd, Carlsbad, CA), 100
191 U/ml penicillin and 100µg/ml streptomycin (Sigma-Aldrich). Total cell count (Kimura stain) and
192 viability (Trypan blue exclusion) were determined before cytospins were undertaken.

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194 **Ex-vivo stimulation of sputum macrophages**

195 Sputum macrophages were isolated from whole sputum using plastic adherence at a cell density
196 of $0.5x10^6$ cells/ml for 4 h in a 5% CO_2 humidified atmosphere at 37°C. Non-adherent cells were
197 removed and macrophages resuspended in charcoal-stripped RPMI-1640 minimal media (1% L-
198 glutamine and 0.5% FCS) overnight, before being exposed to SLM (10^{-7}M), FP (10^{-9}M), or a
199 combination of both, for 60 min. The same number of macrophage cells in culture were seeded
200 for each treatment visit and utilised for each of the experiments, standardised to a cell density
201 of $0.5x10^6$ cells/ml.

202

203 **Isolation of blood cells**

204 Venous blood (80 ml) was diluted 1:1 with Hanks' buffered saline solution (HBSS, Invitrogen) and
205 layered on Ficoll-Hypaque-Plus (Amersham plc, Amersham, UK). After centrifugation (30 min at

206 1,100 x *g* and 18°C), peripheral blood mononuclear cells (PBMCs) were collected, washed, and
207 centrifuged (250 x *g* for 10 min). PBMCs were resuspended in culture media and counted using
208 Kimura dye.
209
210 **Nuclear extraction**
211 Nuclear and cytoplasmic fractions were extracted using a Nuclear Extraction kit (Active Motif,
212 Carlsbad, CA). Briefly, cells were resuspended in hypotonic buffer, vortexed and incubated on
213 ice for 15 min to extract the cytoplasmic fraction. Thereafter, the remaining nuclear pellets were
214 resuspended in complete lysis buffer for 30 min on ice. The suspension was centrifuged
215 (14000rpm, 10 min, 4ºC) and the nuclear fraction obtained. The quality and purity of the nuclear
216 extract was determined by Western Blotting (Figure 1A).
217
218 **GRE binding assay**
219 GR activation was determined with a GR-GRE TransAM kit (Active Motif, Frixensart, Belgium)
220 according to the manufacturer's instructions and nuclear GR-GRE binding was determined.
221 Briefly, 5µg of nuclear extract of each sample was added to a well. Each well contained multiple
222 copies of a specific double-stranded oligonucleotide which activated GR binds to at its
223 consensus binding site. We confirmed using Western blotting in U937 cells that incubation with
224 the positive control, fluticasone propionate, led to an increase in nuclear GR, which correlated
225 to an induction in GR-GRE binding, relative to the negative control of untreated cells (Figure 1B
226 & C).
227
228 **Confocal microscopy**

229 Sputum macrophages were seeded onto glass coverslips in 6-well plates and allowed to attach
230 at 37°C. The cells were fixed with 4% paraformaldehye in phosphate buffered saline (PBS) for 10
231 min at 37°C and washed three times with PBS. Cells were then permeabilized with 0.2% Triton
232 X-100 (Sigma-Aldrich, Poole, UK) for 5min and then incubated with primary anti-GR antibody (H-
233 300, Santa Cruz Biotechnology, CA, USA) for 3 hours followed by 30 min with secondary
234 antibody Alexa 488 (Invitrogen). Coverslips were washed three times with PBS, rinsed in distilled
235 water and immediately dried. Prolong Antifade 4', 6-diamidino-2-phenylindole dihydrochloride
236 (DAPI) solution (Invitrogen) was added to delineate the nuclear boundary in each cell and help
237 define the distribution of GR protein (green) within the respective subcellular compartments
238 (Figure 2). Coverslips were mounted onto slides and allowed to dry for 4 hours before analysis
239 using confocal microscopy with imaging software (Leica Confocal Software Lite, Heidelberg,
240 Germany).
241
242 **Cell Culture**
243 U937 cells (ATCC, Rockville, MD) were maintained in RPMI-40 medium (Invitrogen),
244 supplemented with L-glutamine (1%, Invitrogen) and fetal calf serum (FCS, 10%, Invitrogen), and
245 were differentiated as previously described (28). After washing with HBSS, cells were allowed to
246 recover in medium supplemented with 1% L-glutamine and 0.1% charcoal-stripped FCS for 48
247 hours before experiments.
248
249 **MKP-1 mRNA expression**
250 Differentiated U937 cells were cultured in RPMI supplemented with 0.1% charcoal-stripped-FCS
251 and L-glutamine (1%) for 24 h before treatment. Total RNA was isolated from cells using an
252 RNeasy Kit (Qiagen, Hilden, Germany). cDNA was synthesised from 1μg of total RNA with the

253 Qiagen Quantitect Kit (Qiagen) according to the manufacturer's protocol. Expression of
254 mitogen-activated protein kinase phosphatase-1 (MKP-1) mRNA was quantified by RT-*q*PCR with
255 SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, US). Primers are shown in
256 Table 2. MKP-1 mRNA levels were normalized to an endogenous reference GAPDH and absolute
257 quantification was determined by using known standards of MKP-1. MKP-1 standards were
258 produced from PCR products containing the target sequence from U937 monocytes. The copy
259 number of the standards was determined by measuring the concentration using a
260 spectrophotometer.

261

262 **GR reporter gene assay**

263 Differentiated U937 cells were transfected with GRE-luciferase and β-galactosidase plasmids as
264 previously described and GR transactivation determined by luciferase assay as previously
265 described (17).

266

267 **CXCL8 enzyme-linked immunosorbent assay (ELISA)**

268 Serum-starved U937 cells were incubated with IL-1β for 30 min, followed by FP and SLM alone
269 and in combination for 16 hours. Subsequently, extracellular concentrations of CXCL8 were
270 measured with human CXCL8 Duoset ELISA kits (R&D Systems Europe, Abingdon, UK) according
271 to the manufacturer's instructions.

272

273 **Statistical analysis**

274 In the *in vivo* study, as sputum cells are not normally distributed, a non-Gaussian distribution
275 was adopted and data were analysed using non-parametric statistical analyses using the PC

276 analysis package Graph Pad Prism (Graph Pad Prism, San Diego, CA). Results were analyzed
277 using Friedman analysis of variance (ANOVA) including factors for period, treatment, and
278 multiple comparisons, and applying Bonferroni's post-test correction. Comparisons between
279 treatments were made using the Wilcoxon matched-pairs signed rank sum test. A p value of less
280 than 0.05 was considered statistically significant. For the difference between treatment groups
281 in the *in vitro* data, analyses were undertaken using the Mann-Whitney test for comparison of
282 two unpaired groups with Graph Pad Prism software. A p value of less than 0.05 was considered
283 statistically significant.

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295 **RESULTS**

296 **Patient characteristics**

297 The mean age of the eight patients (6 female) was 65 ± 8 years and the mean post-
298 bronchodilator FEV_1 was 72 ± 12% of predicted (Table 1). No patient showed >12% reversibility
299 to albuterol, was atopic, or had a previous history of asthma. Median % sputum cell counts are
300 shown in Table 1 averaged from different study visits across all patients, and most patients had
301 high levels of neutrophilia. The percentage of sputum macrophages obtained varied between
302 visits within each patient (range -6.7% to +12.2%) compared to the sample taken at visit 1.
303 However, the amount of protein extracted from the isolated macrophage nuclear extracts for
304 the GR-GRE assay was normalised to be the same for all samples from all patients for all visits,
305 irrespective of the variability in the sputum macrophages yielded following sputum induction,
306 and this was 5µg.

307

308 **Effect of LABA and corticosteroid on GR-GRE binding in sputum cells *in vivo***

309 GR-GRE binding levels in the nuclei of macrophages obtained from induced sputum were not
310 significantly altered at 60 minutes following inhalation of FP-100µg or SLM-50µg compared to
311 placebo (Figure 3A). In contrast, FP-500µg significantly increased GR-GRE binding levels 2.2-fold
312 compared to placebo (0.607 ± 0.089 vs. 0.274 ± 0.038 absorbance units, $p<0.01$) and induction
313 of GR-GRE binding was significantly greater with FP-500µg compared to that observed with FP-
314 100µg (0.607 ± 0.089 vs. 0.310 ± 0.028 absorbance units, $p<0.05$). Combination treatment of FP-
315 100µg with SLM-50µg increased nuclear GR-GRE levels to similar levels to those seen with FP-
316 500µg (respectively 0.598±0.061 vs. 0.607 ± 0.089, p = NS). This effect observed with the
317 combination treatment was significantly greater than that seen with either placebo ($p<0.01$), FP-
318 100µg alone ($p<0.05$) or SLM-50µg alone ($p<0.01$). The addition of SLM-50µg to FP-100µg as

319 combination treatment, achieved an enhancement of GR activation in sputum macrophages
320 from patients with COPD that was equivalent to that observed with a five-fold increase in the
321 dose of FP. That is, combination therapy allowed a five-fold reduction in the inhaled
322 corticosteroid dose, yet achieved the same biological effects. Confocal microscopy documented
323 the correlation between the glucocorticoid receptor GRE binding assay and glucocorticoid
324 receptor nuclear translocation in that, treatment with corticosteroid (FP-500μg) led to GR
325 nuclear translocation in sputum macrophage cells (Figure 2).
326
327 **Effect of LABA and corticosteroid on GR-GRE binding in PBMCs *in vivo***
328 The levels of GR-GRE binding in PBMCs were measured 60 minutes after inhalation of the drugs
329 (Figure 3B). None of the active treatments had an effect on GR-GRE binding levels and these
330 levels did not differ significantly from those obtained with placebo.
331
332 **Effect of LABA and corticosteroid on GR-GRE binding in sputum cells *ex vivo***
333 Sputum macrophages were isolated by adherence to plastic, allowed to recover for 24 h before
334 stimulation with SLM (10^{-7}M), FP (10^{-9}M) or a combination of both for 60 minutes. FP
335 significantly enhanced nuclear GR-GRE binding compared to control unstimulated cells (0.506 ±
336 0.042 vs. 0.252 ± 0.038 absorbance units, $p<0.01$). Salmeterol alone had no significant effect on
337 GR-GRE binding levels compared to control (Figure 4). However, the addition of SLM to FP
338 significantly enhanced the ability of FP alone (10^{-9}M) to stimulate GR-GRE binding (0.710 ± 0.073
339 vs. 0.506 ± 0.042 absorbance units, $p<0.05$).
340
341 **Induction of MKP-1 mRNA in macrophage-like U937 cells**

342 FP (10^{-9}M) alone significantly induced MKP-1 mRNA expression after 2 h compared to the levels
343 seen in control unstimulated cells ($5.8 \pm 0.6 \times 10^{-6}$ vs. $6.4 \pm 2.5 \times 10^{-8}$ copies, $p<0.001$), whilst SLM
344 (10^{-8}M) alone had no effect ($3.2 \pm 0.6 \times 10^{-8}$ copies, p=NS) (Figure 5A). However, upon addition
345 to FP, SLM significantly enhanced FP-induced MKP-1 mRNA (5.8 ± 0.6 vs. $8.4 \pm 1.1 \times 10^{-6}$ copies,
346 $p<0.05$).
347
348 **Induction of GRE-luciferase activity by LABA and corticosteroid in macrophage-like U937 cells**
349 Salmeterol (10^{-8}M) alone had no significant effect on a 2xGRE-luciferase reporter gene activity
350 compared to control unstimulated cells (1.0 ± 0.6 vs. 1.5 ± 0.4 fold induction, p=NS) (Figure 5B).
351 In contrast, FP (10^{-9}M) significantly enhanced GRE-luciferase activity compared to control (103.1
352 ± 23.6 fold induction, $p<0.05$). Yet, the addition of SLM to FP resulted in a highly significant
353 increase in GRE-luciferase activity compared to that seen with FP alone (250.1 ± 15.6 vs. 103.1 ±
354 23.6 fold induction, $p<0.001$).
355
356 **Effect of LABA and corticosteroid on IL-1β-induced CXCL8 in macrophage-like U937 cells**
357 FP (10^{-11}M) and (10^{-9}M) significantly suppressed IL-1β-induced CXCL8 (28.3 ± 3.2 and 71.2 ± 3.1
358 % respectively, $p<0.01$), whilst SLM (10^{-9}M) alone had no considerable effect (Figure 6).
359 However, the addition of SLM (10^{-9}M) to FP (10^{-11}M) significantly enhanced FP-mediated
360 suppression of IL-1β-induced CXCL8 (46.7 ± 4.8 vs. 28.3 ± 3.2 %, $p<0.05$).
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366 **DISCUSSION**

367 Our study shows that nuclear levels of activated GR in macrophages obtained from the induced
368 sputum of patients with COPD increased following single-dose inhalation treatment with high-
369 dose fluticasone propionate (FP)-500μg, but not after low-dose FP-100μg alone or salmeterol
370 (SLM)-50μg alone. Most importantly, the addition of SLM-50μg to low-dose FP-100μg increased
371 activated GR levels equivalent to those seen with high-dose FP-500μg. That is, in COPD patients,
372 addition of salmeterol to a low-dose of FP achieved an enhancement of GR nuclear
373 translocation equivalent to that observed with a five-fold higher corticosteroid dose. Consistent
374 with our *in vivo* patient data, we were able to demonstrate that salmeterol significantly
375 enhanced the ability of FP to induce GR activation in sputum macrophages *ex vivo* and enhanced
376 FP-induction of MKP-1 mRNA and 2xGRE reporter gene activity in a human cultured
377 macrophage cell line *in vitro*. Additionally, we were able to show a functional anti-inflammatory
378 effect of combination therapy in that salmeterol significantly enhanced FP-mediated
379 suppression of IL-1β-induced CXCL8 in macrophage-like U937 cells.

380

381 When LABAs were introduced as an add-on therapy to inhaled corticosteroids, the magnitude of
382 their clinical beneficial effect in the control of asthma, and in COPD, was not predicted. The role
383 of LABAs was seen purely as bronchodilatation without any appreciable effect on bronchial
384 inflammation. In asthma, LABAs combined with corticosteroids produced a better effect on
385 symptoms (1, 3), lung function (29), exacerbation rates (30) and health status (31) than
386 expected. Adding LABA to low-dose ICS was observed to be more effective than increasing the
387 dose of corticosteroids (1, 29, 30, 32) and asthma guidelines were updated to reflect these
388 findings (33).

389

390 The beneficial effects of the LABA/ICS combination have also been observed in COPD patients,
391 where they are now an established treatment. The TORCH (TOwards a Revolution in COPD
392 Health) study demonstrated reductions in exacerbation rates, improvements in health status
393 and lung function with SLM/FP combination compared to placebo in COPD patients (15). The
394 study observed a relative reduction in mortality of 17% over three years for patients receiving
395 SLM/FP, although this just failed to reach statistical significance. A subsequent Cochrane review
396 observed survival benefits with combination therapy compared both to placebo (34) and to ICS
397 alone (14). *In vivo* studies using bronchial biopsies have generated further supporting evidence
398 for complementary interactions between LABAs and corticosteroids on airway inflammation and
399 pathology in COPD patients (18, 19).

400

401 Our data reveal a complementary interaction between the LABA (salmeterol) and corticosteroid
402 (fluticasone propionate) in that the combination therapy has a greater anti-inflammatory effect
403 than either drug alone and was also steroid sparing; allowing a five-fold reduction in the inhaled
404 corticosteroid dose, yet achieving the same biological effects. Indeed our data support the
405 postulate that changes in the anti-inflammatory effects with combination therapy may account
406 for the improvements in the rate of decline in lung function seen in the TORCH study (35).

407

408 We confirm that sputum macrophages from COPD patients are less sensitive to corticosteroid
409 action compared to those obtained from asthmatic subjects (36, 37). In our previous study
410 examining the effect of corticosteroid (FP) on GR activation in sputum macrophages from
411 mild/moderate asthmatic subjects we demonstrated that 60 minutes post-treatment, FP-100μg
412 significantly enhanced GR nuclear translocation (17). Here we show that only the higher dose of
413 FP-500μg had an effect on GR-GRE binding. These data suggest that GR-GRE binding in sputum

414 macrophages may be a good biomarker for corticosteroid actions. As in our previous study (17),
415 we were able to detect a quantifiable degree of basal GR-GRE binding in sputum macrophages
416 in the placebo-treated patients (Figure 3) and this level of GR activation was maintained for at
417 least 24 hours in culture. Since none of the patients in this study were exposed to exogenous
418 inhaled or oral corticosteroids this activation must reflect the action of endogenous
419 corticosteroids.
420
421 Consistent with our *in vivo* patient data, our *in vitro* experiments, showed salmeterol enhanced
422 the ability of low concentrations of FP to induce GRE-luciferase activity and switch on MKP-1
423 expression in macrophage-like U937 cells. Similar data for enhanced induction of glucocorticoid-
424 responsive genes in the presence of a LABA such as MKP-1 and glucocorticoid inducible leucine
425 zipper (GILZ) has been reported in airway epithelial cells and smooth muscle cells (38). Our
426 results are consistent with literature that suggest the enhanced effects may result from the
427 ability of LABAs to promote GR nuclear translocation in human lung fibroblasts (22), airway
428 smooth muscle cells (24, 39, 40) and in sputum cells from asthmatic patients (17). This
429 enhancement of GR translocation achieved by LABAs when added to corticosteroids is likely to
430 underlie some of the clinical benefits seen with combination therapy. Additionally, we
431 demonstrated that the enhancement of GR translocation with combination therapy was
432 associated with a functional anti-inflammatory effect; in that, salmeterol significantly enhanced
433 FP-mediated suppression of IL-1β-induced CXCL8 in U937 cells.
434
435 We were unable to assess the anti-inflammatory properties of the combination treatment in
436 sputum macrophages *in vivo* due to limitations in cells numbers and ethical considerations
437 regarding repeated sputum induction in patients. Future experiments examining gene

438 expression profiles in sputum macrophages 4 hours after inhaled drugs will provide evidence for
439 additional genes that are regulated in a similar manner to those described here. A role for other
440 aspects of GR activation including GR post-translational modifications and the activity of GR
441 chaperone proteins is difficult to rule out as potential modulators of GR-GRE binding. An
442 experimental clinical patient study specifically designed to address these points would be
443 needed. In contrast to our observations in sputum macrophages, the levels of activated GR in
444 the nuclei of PBMCs did not change significantly after any treatment. This is likely to be due to
445 peripheral blood levels of the study medications being too low after a single-dose inhalation to
446 exert a measurable effect on the biological machinery of GR translocation. It would be
447 interesting to see if a measurable effect is achieved after a more prolonged course of treatment.
448
449 The marked effect of LABAs on GR translocation *in vitro* and *in vivo*, in asthmatics and in COPD
450 patients warrants further investigation, and elucidation of the precise molecular mechanisms of
451 how LABAs (which acting extracellularly via the β_2 receptor) are able to influence translocation
452 of cytoplasmic GRs. Altered phosphorylation, 'priming' of the GR increasing its tendency to
453 translocate without the need for its natural ligand has been proposed (41, 42). Recently we have
454 shown that LABA increase GR nuclear translocation through the activation of a phosphatase
455 PPA2, which dephosphorylates hyperphosphorylated GR (43).
456
457 In conclusion, we report here for the first-time that a LABA in combination with a low-dose of
458 ICS enhances GR-GRE binding *in vivo* in COPD patients as effectively as a five-fold higher dose of
459 ICS alone. This suggests LABAs may play an important role in the benefits clinically seen with
460 combination therapy. Our data has clinical relevance to the use of combination inhalers in view
461 of the reduced sensitivity to ICS in COPD. Further studies are needed to address whether these

462 differences are maintained following chronic treatment of LABAs with ICS in COPD patients, and
463 whether this results in an alteration in sputum cytokine profiles *in vivo*.
464
465
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584

585

586

587

588 **TABLES**

589 **TABLE 1.**

590 **Patient characteristics**

Age (years)	65 ± 8
Gender	2M/6F
GOLD stage	2 stage 2, 6 stage 3
FEV_1 (L)	1.8 ± 0.89
Post-bronchodilator FEV_1 (% predicted)	72.1 ± 11.8
Reversibility (%)	5.6 ± 4.2
FEV_1/FVC ratio	60.2±5.2
Smoking status	2 current, 6 ex
Smoking history (pack-years)	35.3 ± 16.2
Sputum Cell Counts	
Macrophages (% total) median % (range)	7 (4.8 – 33.4)
Neutrophils (% total) median % (range)	91.9 (39.7 – 93.1)
Lymphocytes (% total) median % (range)	1 (0 – 1.7)
Eosinophils (% total) median % (range)	0.6 (0 – 25.7)

591

592

593 **TABLE 2.**

594 **PCR primers for MKP-1 mRNA analysis**

595

	Forward	Reverse
MKP-1	5’-GTACATCAAGTCCATCTGAC	5’ GGTTCTTCTAGGAGTAGACA
GAPDH	5’- GAAGGTGAAGGTCGGAGTC	5’- GAAGATGGTGATGGGATTTC

596

597

598 **FIGURE LEGENDS**

599 **Figure 1. Validation of the quality of nuclear extract and GR-GRE binding.**

600 The quality of nuclear extracts (panel A) was determined by Western Blotting. Fluticasone
601 propionate (FP)-induced GR-GRE binding (panel B) and FP-induced GR nuclear localisation (panel
602 C) were assessed in U937 monocytes. Results represent the mean ± SEM of 3 independent
603 experiments. *$p<0.05$ denotes statistical significance *vs.* untreated cells.

604

605 **Figure 2. Confocal laser images of GR subcellular localisation in sputum macrophages**
606 **following corticosteroid (FP 500µg) treatment.** (panels A, D) 4', 6-diamidino-2-phenylindole
607 dihydrochloride (DAPI) staining (blue channel); (panels B, E) cytoplasmic and nuclear GR
608 immunostaining (green channel); (panels C, F) overlay of panels A and B.

609

610 **Figure 3. Effect of inhaled salmeterol (SLM) and fluticasone propionate (FP) on GR-GRE**
611 **binding in nuclear extracts from induced sputum macrophages (panel A) and PBMCs (panel B).**
612 Mean ± SEM absorbance values of GR-GRE binding are shown 60 minutes post-inhalation of
613 treatment, where numeric values are inhaled drug doses in micrograms. n = 8 subjects. .
614 *$p<0.05$ and **$p<0.01$ denote statistical significance between treatments.

615

616 **Figure 4. Fffect of fluticasone propionate (FP, 10^{-9}M) and salmeterol (SLM, 10^{-7}M) on GR-GRE**
617 **DNA binding in sputum macrophages from COPD patients *ex vivo*.** . Mean ± SEM absorbance
618 values of GR-GRE binding are shown. n = 5subjects. *$p<0.05$ denotes statistical significance
619 between treatments.

620

621 **Figure 5. Effect of fluticasone (FP, 10^{-9}M) and salmeterol (SLM, 10^{-8}M) on MKP-1 mRNA
622 induction and GRE-luciferase activity in macrophage-like PMA-treated U937 cells in vitro.** (A)
623 MKP-1 mRNA expression normalized with GAPDH (panel A) and the fold-increase in GR of
624 stimulated vs. non stimulated cells (panel B) are shown. Results represent the mean ± SEM of
625 three independent experiments. * $p<0.05$ and *** $p<0.001$ denote statistical significance
626 between treatments.
627
628 **Figure 6**. **Effect of fluticasone propionate (FP) and salmeterol (SLM) on IL-1β-induced CXCL8 in
629 macrophage-like U937 cells.** Results represent the mean ± SEM of 8 independent experiments.
630 *$p<0.05$ denotes statistical significance vs. FP (10^{-11}M) and #$p<0.01$ denotes statistical
631 significance vs. IL-1β stimulated.