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1 **Bronchoprotective tolerance with indacaterol is not modified by concomitant tiotropium**
2 **in persistent asthma**

3 **Running title:** Tiotropium and airway hyperresponsiveness
4

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38 **Abstract:**

39 Background: Tiotropium is a long acting antimuscarinic (LAMA), licenced as triple therapy
40 with inhaled corticosteroid and long acting beta-agonist (ICS/LABA). There may be a
41 synergistic benefit between LAMA and LABA as a consequence of receptor cross-talk, which
42 in turn could modify beta-2 receptor down-regulation and associated tolerance induced by
43 LABA.

44 Objective: We hypothesise this mechanism may result in a reduction of airway
45 hyperresponsiveness (AHR) when using triple therapy.

46 Methods: We evaluated 14 non-smoking asthmatics using an open-label, randomized crossover
47 design. ICS with Indacaterol and Tiotropium (IND/TIO) vs ICS with Indacaterol (IND) over 4
48 weeks with challenge performed after 1st and last doses at trough.

49 Results: We found no significant difference in mannitol sensitivity, expressed as the
50 provocative dose of mannitol required to reach a 15% drop in FEV₁, or mannitol reactivity,
51 expressed as the response dose ratio (RDR: max % fall in FEV₁ / cumulative dose) , when
52 comparing ICS/IND/TIO to ICS/IND. Geometric mean fold differences for RDR comparing
53 single and chronic dosing were 3.26 fold (95% CI 1.46-7.29) and 2.51 fold (95% CI 1.32-4.79)
54 for IND and IND/TIO respectively. Furthermore, salbutamol recovery post challenge was
55 significantly blunted after chronic compared to single dosing with either IND (P<0.005) or
56 IND/TIO (P<0.05).

57 Conclusion & Clinical Relevance: Our data suggests that concomitant tiotropium does not
58 modify the bronchoprotective tolerance induced by Indacaterol, in turn suggesting that cross-
59 talk may not be clinically relevant when using triple therapy. This study was registered on
60 clinicaltrials.gov as NCT02039011.

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73 **Abbreviations:**

74 AHR: Airway hyperresponsiveness

75 ACQ: Asthma control questionnaire

76 AX: Reactance area under the curve

77 FeNO: Exhaled nitric oxide

78 ICS: Inhaled corticosteroid

79 IND: Indacaterol

80 IOS: Impulse oscillometry

81 LABA: Long acting beta-2 agonist

82 LAMA: Long acting muscarinic antagonist

83 PD₁₅: Provocative dose of mannitol required to reduce FEV₁ by 15%

84 RDR: Response dose ratio

85 TIO: Tiotropium

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101 Tiotropium (TIO) is a long acting muscarinic antagonist (LAMA), which is functionally
102 selective for the post junctional M3 muscarinic receptor, found on airway smooth muscle [1].
103 TIO reduces asthma exacerbations by 21% in patients when used as add-on therapy in patients
104 receiving inhaled corticosteroids and long-acting beta-agonists (ICS/LABA)[2]. Whilst
105 blocking the M3 receptor inhibits acetylcholine induced bronchoconstriction, TIO exhibits only
106 modest improvements in FEV₁, which amounts to approximately 100ml at trough [2, 3], which
107 is less than the minimally important difference of 230ml [4]. It is therefore hard to explain the
108 protective effect on exacerbations on solely the basis of this small improvement in airway
109 calibre alone [5].

110

111 One mechanism by which TIO may exhibit its protective effects is by attenuating airway
112 hyperresponsiveness (AHR), via blockade of the post junctional M3 muscarinic receptor,
113 resulting in reduced response to cholinergic transmission [6]. M3, however, is not the only
114 muscarinic receptor to contribute to increased airway tone and AHR; asthma is also associated
115 with impaired pre-junctional M2 function [7] [8]. The pre-junctional M2 is an autoreceptor, as
116 it is stimulated by acetylcholine to reduce further acetylcholine secretion. In asthma, the loss of
117 this negative feedback mechanism results in increased AHR. Moreover, it has been postulated
118 that both pre-junctional beta-2 and M2 receptors are inhibitory to the release of acetylcholine
119 and that there is crosstalk between these receptor types [7, 8]. Hence it might be expected that
120 chronic dosing with LABA might remove the brake to acetylcholine release as a consequence
121 of down-regulation and subsensitivity of pre-junctional inhibitory beta-2 receptors, resulting in
122 augmented cholinergic transmission and bronchoconstriction [7]. In this regard, TIO rapidly
123 dissociates from M2 receptors, unlike its affinity for post junctional M3 receptors, thereby
124 facilitating additional inhibition of M2 receptors and reduced pre-junctional acetylcholine
125 release. This functional M3 selectivity may be a possible mechanism by which it reduces
126 exacerbations in asthma by attenuating AHR [1].

127

128 Another possible mechanism is that muscarinic M3 receptors promote beta-receptor
129 desensitization through protein kinase C-mediated phosphorylation [9], hence inhibition of this
130 effect by TIO may protect the beta-2 receptor from acetylcholine induced heterologous
131 desensitization by acetylcholine[10]. In this regard looking at the converse situation, tiotropium
132 has been shown to protect against propranolol induced bronchoconstriction [11].

133

134 TIO may also reduce exacerbations via a putative anti-inflammatory action by inhibiting the
135 paracrine effects of acetylcholine on inflammatory cells [12]. TIO has been shown to exhibit
136 inhibitory effects on the development of airway remodelling in the animal model of antigen
137 induced asthma[12, 13]. In vitro data have also suggested that there may be an anti-
138 inflammatory synergy between LABA and LAMA, via the cAMP pathway [9].

139

140 Pointedly no studies have looked at effects of TIO on AHR assessed by bronchial challenge
141 using non cholinergic agents. One study showed that as expected TIO produced prolonged
142 functional antagonism of M3 mediated smooth muscle constriction induced by the cholinergic
143 agonist methacholine [14]. As TIO is only currently indicated as add-on therapy to ICS/LABA
144 [15], the objective of this study was to evaluate the impact of adding TIO to ICS/LABA on
145 AHR, in patients with persistent asthma and whether TIO might also prevent against LABA
146 induced subsensitivity[7]. We chose an indirect bronchial challenge, namely mannitol, as this
147 is thought to more closely reflect physiological stimuli and acts by release of pro-inflammatory
148 mediators [16]. Moreover mannitol challenge has been shown to be related to an inflammatory
149 phenotype in asthma [17-19].

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151 **Patients and Methods:**

152 Non-smoking male or female patients, aged at least 18 years, with persistent asthma already
153 receiving ICS or ICS/LABA attended for a screening visit. Participants were recruited from the
154 National Health Service (NHS Scotland) boards of Tayside and Fife, and also our existing
155 database of asthma patients, at the Scottish Centre for Respiratory Research, in Ninewells
156 Hospital & Medical School, Dundee, Scotland. Participants had to have a minimum FEV₁ of
157 >50% predicted and be mannitol responsive i.e. provocative dose required to reduce FEV₁ by
158 15% (PD15) <635mg, to be enrolled. Participants were also required to have no history of
159 respiratory tract infection or oral corticosteroid use, in the last three months prior to screening.
160 After initial screening, any LABA therapy was first withdrawn for 2 weeks followed by halving
161 the ICS dose, to a minimum of 400µg/day (as beclometasone equivalent dose). If patients were
162 on secondary controllers such as leukotriene receptor antagonists, these were also stopped.
163 Participants then entered a 2 week run in on this dose of ICS, which was then continued
164 throughout the study.

165 The trial was a single centre, randomised open label cross-over design. Patients received either
166 4 weeks of indacaterol (Onbrez Breezhaler, Novartis, Calberley, UK) alone at a dose of 150µg
167 OD (IND), or combined with tiotropium (Spiriva Handihaler, Boehringer Ingelheim, Bracknell
168, UK) 18µg OD (IND/TIO) as add-on to pre-existing ICS. There was a 2 week washout in
169 between treatments while continuing to take the same dose of ICS. This washout was sufficient
170 to minimise the possibility of carry-over effects of both IND and TIO [20].

171 Including screening, there were 7 visits in total (figure 1.). Visits were performed, in the
172 mornings, at baseline after run-in and washout, and at 24 hours (i.e. trough) after the first and
173 last doses of each randomised treatment period. Patients were allowed short acting beta-2
174 agonists (SABAs) as a reliever during the study, but were asked to abstain from SABA use at
175 least 6 hours before each visit. The visits were conducted in the mornings (8am-10am).
176 Participants were asked to record study medication use on a diary, and compliance was checked
177 with returned empty capsule counts. This study was registered on clinicaltrials.gov as
178 NCT02039011. The study was approved the Tayside committee for medical ethics (reference:
179 13/ES/0072) and full informed consent was obtained from all patients.

180 The primary outcome was mannitol challenge. This was performed as previously described[21]
181 using a dry powder inhaler (Aridol Pharmaxis Ltd, Sydney, Australia) and increments up to a
182 maximum cumulative dose of 635 mg. Mannitol sensitivity was expressed as the provocative
183 dose of mannitol required to reach PD15, this was calculated by interpolation of the log-linear
184 dose–response curve. The data for PD15 were log transformed before analysis. Mannitol
185 reactivity was expressed as the response dose ratio (RDR) –i.e. maximum % fall in FEV₁
186 divided by the final mannitol dose. Impulse oscillometry, a secondary outcome, (Jaeger
187 Masterscreen IOS, Hochberg, Germany) was performed as previously described [22] in
188 triplicate, whilst subjects wore a nose clip, sealed lips tightly, and breathed quietly for 30
189 seconds, in accordance with manufacturer’s guidelines. Resistance at 5 Hz (R5) and 20Hz
190 (R20) is a measure of total and central airway resistance respectively, hence peripheral airway
191 resistance was ascertained by the difference between R5 and R20. Lung compliance as its
192 reciprocal reactance (X) and the area under the reactance curve (AX) was also measured. A
193 SuperSpiro spirometer (Micro Medical Ltd, Chatham, Kent, United Kingdom) was used to
194 perform spirometry in triplicate in accordance with European Respiratory Society
195 guidelines[23]. After mannitol challenge, salbutamol (400µg) was administered and 30 minute
196 recovery recorded. Exhaled nitric oxide (FeNO) was performed using an NIOX MINO analyser
197 (Aerocrine AB, Solna, Sweden), in accordance with the published guidelines [24]. Asthma
198 control questionnaire (ACQ-7) was measured using the standard 7 point paper

199 questionnaire[25] (Qoltech, UK) . Randomisation was done with a computer generated code
200 held by the Clinical Trials Pharmacy.

201 Data Analysis

202 The study was powered at 80% to detect a minimal important difference of one doubling dose
203 in mannitol PD 15 (the primary outcome), as change from baseline, comparing indacaterol
204 alone with indacaterol plus tiotropium, after single and chronic dosing, and a within-subject SD
205 of 1.3 doubling dose, requiring a sample size of 14 using a crossover design, with alpha error
206 of 0.05 (2 tailed). All data were first examined for normality and distribution. Repeated
207 measures analysis of variance (ANOVA) was carried out assessing for treatment and sequence
208 effects for the cross-over design. Where overall significance was found on ANOVA,
209 Bonferroni corrected pairwise comparisons were then carried out. Thus, pairwise comparisons
210 are reported as either significant ($p < 0.05$, two-tailed) or not. Statistical Analysis was done
211 using IBM SPSS (version 22, IBM analytics, New York).

212 Results:

213 The participant flow for the trial is shown in the consort diagram (Figure 2), of the 39 patients
214 screened 18 were randomised and 14 completed per protocol. Of the 14 ICS treated asthmatic
215 patients analysed, 12 had at least one positive skin prick test to common aeroallergens, mean
216 age was 46 years , mean FEV₁ 86% predicted , mean BMI 30kg/m², mean R5 160% predicted
217 , and mean ICS dose 693µg/day (beclometasone equivalent dose). No patients were current
218 smokers, two were ex-smokers with a mean pack year history of 2.6. Values comparing mean
219 ICS dose pre and post step down were 693 vs 429 µg/day ($P < 0.05$).

220 Data for all outcomes according to study visits are summarised in Table 1. All outcome
221 measures at first baseline and second baseline were assessed for carryover effect in order of
222 sequence. There was no statistical difference between baseline data justifying the use of a
223 pooled baseline value for comparison with randomised treatment arms. This confirmed an
224 adequate washout period. In particular, there was no significant difference between mean
225 baseline values for the primary outcome of mannitol PD15: 383mg vs 387 mg.

226 There were significant improvements ($P < 0.05$) in mannitol PD15 and RDR with IND or
227 IND/TIO vs baseline after single but not chronic dosing (Figure 3). There was a significant
228 difference ($P < 0.05$) in RDR between single and chronic dosing for both treatments: geometric
229 mean fold differences were 3.26 fold (95% CI 1.46-7.29) and 2.51 fold (95% CI 1.32-4.79) for
230 IND and IND/TIO respectively. Furthermore, salbutamol recovery post challenge was
231 significantly blunted after chronic compared to single dosing with either IND ($P < 0.005$) or
232 IND/TIO ($P < 0.05$) (Figure 4 and table 1).

233 IOS measures including R5, R5-R20, and AX were all significantly improved ($P < 0.05$) with
234 both treatments compared to baseline after single and chronic dosing. FEV₁ and FEF₂₅₋₇₅ were
235 significantly better after single dosing with both treatments ($P < 0.05$) but only after chronic
236 dosing with IND/TIO ($P < 0.05$). There were no significant differences between treatments after
237 chronic dosing for either mannitol AHR, spirometry or IOS outcomes. FeNO was unchanged
238 with either treatment compared to baseline. ACQ was also unchanged by either treatment.

239 Discussion:

240 Our results showed improvements in mannitol AHR with both treatments after single dosing
241 which were not maintained after repeated exposure, in addition to blunting of salbutamol
242 recovery. This is likely to be indicative of agonist induced down-regulation and uncoupling of
243 beta-2 receptors and associated tolerance of response .The loss of bronchoprotection induced

244 by indacaterol and associated cross tolerance seen as blunted salbutamol recovery has
245 previously been well documented with other twice daily LABA's in patients taking
246 concomitant ICS [26-28] [29] [30]. Indacaterol has a high degree of intrinsic efficacy at the
247 beta-2 receptor being 73% compared to the effect of isoprenaline in vitro.[31] In another study
248 using isolated human bronchi the maximal relaxant response was 77% for indacaterol versus
249 94% for formoterol [32]. In this regard prolonged stimulation with a high efficacy agonist like
250 indacaterol would be expected to result in marked down regulation and uncoupling of beta-2
251 receptors as has been previously shown with formoterol [28, 33-35].The loss of
252 bronchoprotection was seen with indacaterol at 24 hours after the last dose at trough , when the
253 airway might be particularly vulnerable to exogenous constrictor stimuli immediately prior to
254 the next dose. The degree of bronchoprotection loss was the same with both treatments while
255 chronic treatment with IND/TIO was no different compared to IND alone. Hence it can be
256 concluded that we did not see any clinical evidence of crosstalk between muscarinic and beta-
257 2 receptors ,at least in terms of bronchoprotective subsensitivity using indirect challenge with
258 mannitol [7]. The absence of any bronchoprotection seen with TIO is consistent with similar
259 findings with ipratropium using direct acting histamine challenge [36] . In terms of the choice
260 of challenge agent, mannitol was chosen as it is a well validated [37] indirect challenge and
261 hence better reflects other physiological stimuli than direct challenges such as methacholine or
262 histamine. Furthermore at the time of doing the study adenosine 5' monophosphate (AMP) for
263 human use was not commercially available. Whilst it is noted that response to mannitol is
264 influenced by ICS [38], our patients had to be mannitol responsive at the first visit whilst taking
265 a stable ICS dose, which remained constant throughout the study. Therefore we felt that any
266 changes in mannitol AHR would only reflect the impact of bronchodilator treatments.
267 Furthermore the PD₁₅ and RDR values were not statistically different between first and second
268 baseline, suggesting no carryover effects between randomised treatment arms.

269 For IOS and spirometry, both treatments conferred improvements which were maintained after
270 chronic dosing. As was the case with AHR, we found no significant differences in pulmonary
271 function outcomes after chronic dosing comparing between IND/TIO and IND alone. Previous
272 studies in more severe patients have shown that TIO in addition to ICS/LABA results in
273 approximately 100ml improvement in FEV₁ [2], in turn suggesting that improved airway
274 calibre per se is unlikely to be the explanation for reduced exacerbations[5] . We had originally
275 considered that IOS might be more sensitive than spirometry at picking up subtle differences
276 between double and triple therapy for bronchodilator effects measured at trough [39] [40].In
277 the presence of a raised baseline R5 value of 160 % predicted, one might expect there to plenty
278 of room for further improvement comparing double and triple therapy, which was not the case.
279 Further studies are indicated to look at whether IOS is more sensitive to effects of TIO in more
280 severe patients.

281 There was no improvement in ACQ score, this reflecting the mean baseline value of 0.72 which
282 is less than the 0.75 cut off value for optimal control[41] . However, the failure of add-on
283 therapy with LAMA to improve ACQ scores was also seen by Peters et al in a much larger and
284 more severe cohort [42]. FeNO was unchanged with either treatment, which could be explained
285 by levels being already suppressed by concomitant ICS. Nonetheless, one would still expect
286 the addition of TIO to have contributed to a modest further reduction from a mean baseline
287 value of 30ppb, as shown in another study in more severe patients looking at triple therapy [43].

288 The clinical relevance of our data is that when using triple therapy, at least in asthma, any
289 effects of LAMA on exacerbations is unlikely to be due to bronchoprotective effects. Moreover
290 concomitant LAMA does not mitigate tolerance induced by LABA or cross tolerance to
291 salbutamol. Hence for patients taking ICS/LABA who might experience reduced protection
292 against bronchoconstrictor stimuli, adding in a LAMA will not alleviate the situation, although
293 it might conceivably still produce fewer exacerbations. The caveat is that our patients only had
294 mild to moderate asthma and hence we did not see any significant additive bronchodilator

295 effects with LAMA. In other words if LAMA had produced altered airway geometry then
296 perhaps we might have seen some additional bronchoprotection. Against this is the previous
297 observation of Britton et al where ipratropium did produce a dose related bronchodilator
298 response which was disconnected from any effects on AHR to histamine challenge [36].

299
300 We accept that our study has limitations in that our patients were initially well controlled
301 Moreover, our sample size was not powered to detect additional bronchodilation with TIO. As
302 airway, geometry is an important determinant of bronchoprotection our negative findings with
303 TIO on mannitol challenge might simply reflect the lack of additional bronchodilator effect
304 with TIO. Although we did not have a comparator limb with TIO alone, one would have
305 expected to see additive effects on AHR after chronic dosing when the bronchoprotective effect
306 of LABA had diminished, in terms of there being room for potential further improvement after
307 the last dose. One could always argue that TIO is only indicated for use as add-on to
308 ICS/LABA, as was the case in the present study, and hence performing a study looking at TIO
309 alone or in conjunction with ICS would have no clinical resonance. Finally, we acknowledge
310 that we did not measure either sputum or blood eosinophils in the present study, although in
311 that respect our patients were selected a priori on the basis of AHR.

312
313 In conclusion, TIO did not modify the bronchoprotective tolerance induced by indacaterol or
314 the cross tolerance seen on blunting of salbutamol recovery. Further studies perhaps involving
315 bronchial biopsy might provide an insight into the putative anti-inflammatory action of TIO in
316 asthma to help further elucidate the mechanism by which it reduces exacerbations in patients
317 taking ICS/LABA.

318

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548 Table 1.

	INDACATEROL			INDACATEROL + TIOTROPIUM	
	Pooled baseline	Single dosing	Chronic dosing	Single dosing	Chronic dosing
FEV₁ (L)	2.56 (2.18-2.95)	2.69 (2.28-3.10)*	2.64 (2.26-3.02)	2.78 (2.38-3.19)*	2.71 (2.33-3.09)*
FEV1 percent predicted (%)	87 (78-97)	91 (82-100)*	90 (81-100)	95 (85-105)*	93 (83-102)*
FEF₂₅₋₇₅ (L)	1.79 (1.22-2.36)	2.02 (1.39-2.65)*	1.91 (1.25-2.57)	2.22 (1.49-2.95)*	1.94 (1.38-2.49)*
R5 (kPa/L.s)	0.54 (0.44-0.64)	0.45 (0.37-0.52)*	0.44 (0.37-0.50)*	0.39 (0.34-0.43)*	0.45(0.39-0.50)*
R5-R20 (kPa/L.s)	0.14 (0.07-0.22)	0.07 (0.03-0.11)*	0.07 (0.04-0.10)*	0.05 (0.03-0.07)*	0.08 (0.04-0.11)*
AX (kPa/l)	1.63 (0.58-2.68)	0.76 (0.34-1.19)*	0.68 (0.43-0.92)*	0.44 (0.25-0.63)*	0.78 (0.48-1.09)*
RDR (%/mg)	0.037 (0.025-0.055)	0.011 (0.005-0.026)*	0.037 (0.023-0.061)†	0.015 (0.008-0.029)*	0.035 (0.018-0.070)†
PD₁₅ (mg)	390 (291-521)	537 (438-619)*	455 (342-606)	487 (329-624)*	388 (255-593)
FENO (ppb)	30 (20-45)	30 (20-44)	30 (20-45)	32 (23-45)	29 (19-44)
Salbutamol Recovery (%.min)	47 (-79 - 172)	33 (-47 – 113)	259 (196 – 322)*	77 (19-136)	239 (177-300)*
ACQ7	0.72 (0.48-0.95)		0.44 (0.24-0.63)		0.50 (0.27-0.73)

Values are presented as mean (95% CI)

*Denotes significant (P<0.05) difference from pooled baseline.

†Denotes significant difference (P<0.05) between single and chronic dosing within treatment groups.

No statistically significant differences observed between Indacaterol vs Indacaterol + Tiotropium when comparing single vs chronic dosing at trough. Salbutamol recovery is expressed as the area under the curve (AUC) for 30 minutes.

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566 **Figure Legends**

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569 Figure 1.

570 Flowchart.

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572 Figure 2.

573 Consort diagram.

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575 Figure 3.

576 Effects of randomized treatments (as add on to ICS) compared to baseline on (a) mannitol
577 sensitivity and (b) reactivity. P value denotes significant difference for randomised treatments

578 compared to baseline. There was also a significant difference between single and chronic

579 dosing for reactivity with both treatments. There were no differences between treatments.

580 Values are geometric means and 95% CI.

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582 Figure 4.

583 Effects of single and chronic dosing with either (a) indacaterol alone or (b) indacaterol

584 +tiotropium (as add on to ICS) on salbutamol (400ug) recovery post challenge. P value

585 denotes significant overall blunting of the salbutamol recovery comparing chronic vs single

586 dosing. Values are means and SEM.

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