

Evaluation of interrupter resistance in methacholine challenge testing in children

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34 35	SUMMARY
36	Bronchial hyperresponsiveness is a key feature of asthma and is assessed using bronchial
37	provocation tests. The primary outcome in such tests (a 20% decrease in forced expiratory
38	volume in 1 second (FEV ₁)) is difficult to measure in young patients. This study evaluated the
39	sensitivity and specificity of the interrupter resistance (R_{int}) technique, which does not require
40	active patient participation, by comparing it to the primary outcome measure.
41	Methacholine challenge tests were performed in children with a history of moderate
42	asthma and bronchial hyperresponsiveness. Mean and individual changes in R_{int} and FEV_1
43	were studied. A receiver operating characteristic (ROC) curve was used to describe sensitivity
44	and specificity of R _{int} .
45	Seventy-three children (median age: 9.2 yrs; range: 6.3-13.4 yrs) participated. There
46	was a significant (p< 0.01) increase in mean R_{int} with increasing methacholine doses.
47	However, individual changes of R_{int} showed large fluctuations. There was great overlap in
48	change of R_{int} between children who did and did not reach the FEV ₁ endpoint. A ROC-curve
49	showed an area under the curve of 0.65.
50	Because of low sensitivity and specificity, the use of R_{int} to diagnose bronchial
51	hyperresponsiveness in individual patients seems limited.
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57	specificity.
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59 INTRODUCTION

According to GINA guidelines bronchial hyperresponsiveness (BHR) is a key feature of asthma (1). To objectify BHR, forced flow volume manoeuvres are required to measure the forced expiratory volume in one second (FEV₁), as primary outcome measure in bronchial challenge testing (2). Because of the difficulty of obtaining forced lung function measurements in early childhood the diagnosis of asthma in this age group is challenging and complicated. Several studies have advocated measurement of the interrupter resistance (R_{int}) as an alternative to quantify airway obstruction in preschool children and in children with developmental delay (3;4). The interrupter technique is non-invasive and can be obtained during tidal breathing (5). R_{int} can be measured in children from two years of age as it requires minimal cooperation (6). The R_{int} technique has been compared to body plethysmography (7) and transcutaneous partial pressure of oxygen measurements (7-10) during methacholine challenge tests, but most studies made no comparison with the outcome measure FEV₁. The one study that compared R_{int} with FEV₁ during methacholine challenge testing reported a mean increase in R_{int} with increasing doses of methacholine (11). However, this study did not present the changes in R_{int} for individual patients nor how these related to individual changes in FEV₁. The ability of R_{int} to detect methacholine induced bronchoconstriction in individual patients, as measured by a 20% decrease in FEV₁, has never been studied.

The latest ATS/ERS statement on pulmonary function testing in preschool children recommended the need to determine the role of R_{int} in bronchial challenge tests (5). The current study evaluated the sensitivity and specificity of R_{int} to detect airflow obstruction during methacholine challenge tests in asthmatic children by comparing measurements with FEV₁ as the gold standard.

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83 MATERIALS AND METHODS

This study was a substudy of the multicentre COMBO-study in children between 6 and 16 years with moderate asthma (see online supplement) (12). Nine out of 19 hospitals performed additional R_{int} measurements during methacholine challenge tests. The children from these centres were included in this substudy. It was approved by the local medical ethical committees and written informed consent was obtained from the parents (and the child if ≥ 12 years) before start of the study. For additional information see online supplement.

91 Study protocol

Methacholine provocation tests were performed twice (at start of study and after 26 weeks) measuring R_{int} and FEV₁ after each methacholine dose. All children abstained from long-acting $\beta 2$ agonists and inhaled steroids for 12 hours before the first methacholine provocation test (and 36 hours before the second methacholine provocation test), and from short acting $\beta 2$ agonists for 6 hours before provocation testing. To prevent possible, unwanted physiologic effects of deep inhalation on Rint, Rint was measured prior to FEV1. Pulmonary function tests were performed to conform with latest ATS/ERS guidelines (2;5;13) and briefly described below. The complete methodology is described in the online supplement.

101 Methacholine challenge testing

Methacholine challenge tests were performed using the dosimeter method (2;14) with a
nebulizer (model 646, DeVilbiss Healthcare, Somerset, USA) attached to a dosimeter
(Rosenthal French dosimeter, PDS Instrumentation, Louisville CO, USA). Methacholine
chloride was administered in doubling doses and provocation was continued after at least five
minutes and until the dose at which at least 20% fall in FEV₁ (PD₂₀) or the maximum dose
was reached.

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$\begin{array}{c} 5 \\ 6 \\ 7 \\ 8 \\ 9 \\ 10 \\ 11 \\ 12 \\ 13 \\ 14 \\ 15 \\ 16 \\ 17 \\ 18 \\ 19 \\ 21 \\ 22 \\ 22 \\ 22 \\ 22 \\ 22 \\ 22$	109	Interrupter resistance
	110	Airway resistance measurements were carried out using the MicroRint (Micro Medical
	111	Limited, Kent, UK). A bacterial filter was used (Spirosafe bacterial filter, Micro Medical,
	112	Kent, UK). Reference values of Merkus et al. (15) were used.
	113	
	114	Spirometry
	115	Maximal expiratory flow-volume measurements were performed with standardized
	116	equipment, using reference values of Stanojevic (16).
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	118	Analysis
	119	Changes in FEV_1 and R_{int} were analyzed as percentages change from baseline. Change in R_{int}
	120	was also assessed as change in percentage predicted and change in z-score, computed from
	121	the latest R _{int} reference values (15). Wilcoxon Signed Ranks test was used to investigate
	122	whether there was a significant increase in mean change from baseline of R_{int} at each dose
	123	step. To quantify the variability of FEV_1 and R_{int} within individuals between the various
	124	dosesteps, coefficients of variation (CV) were calculated (see online supplement for
	125	calculation). The resulting individual paired CV's of FEV_1 and R_{int} were compared using
45 46 47	126	Wilcoxon's test. Comparison of mean CV's between centers was done using the Kruskal-
48 49	127	Wallis test. The association between changes in FEV_1 and changes in R_{int} at PD_{20} was
50 51	128	analysed using Spearman's correlation (r_s). At each separate dose step Mann Whitney tests
5∠ 53 54	129	were computed to compare the difference in changes from baseline between subjects who did
55 56	130	and who did not reach the PD_{20} endpoint at the dose step. Cox regression, with change from
57 58 50	131	baseline of R_{int} as time-dependent variable, was used to analyse whether these changes were
60	132	predictive for having reached the PD_{20} endpoint at the subsequent dose steps. The sensitivity

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2 3 4	133	and specificity of R_{int} in relation to having reached FEV ₁ -based PD ₂₀ was described by
5 6 7 8 9 10 11 12	134	receiver operating characteristic (ROC) curves. The area under the curve (AUC) shows
	135	whether the change in R_{int} can correctly classify those who do and do not reach the PD_{20}
	136	endpoint. We used the coefficient of repeatability calculated by Beydon et al. (a 32.1%
12 13 14	137	increase in R_{int}) as a cut-off value to calculate the sensitivity and specificity of R_{int} to predict a
15 16 17	138	20% fall in FEV ₁ .
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RESULTS

143 At the first COMBO-study visit 73 children with moderate asthma (60 % boys) with a median 144 age of 9.2 years (range: 6.3 - 13.4 years) performed both R_{int} and FEV₁ measurements during 145 methacholine challenge. Baseline characteristics are shown in Table 1. Fifty-two children 146 reached the PD₂₀ endpoint and were defined as responders (Table 2).

There was a significant increase from baseline of R_{int} starting at the sixth methacholine dose step and all dose steps thereafter (all p<0.01). From the first dose step mean FEV₁ was significantly different from baseline value (all p< 0.01), except for dose step 2. Visual inspection of individual R_{int} profiles during the challenge test showed large fluctuations with rises and falls at subsequent dose steps. In contrast, much smoother profiles with gradual falls were found for FEV_1 (see E-figure 1 in the online supplement). To express the variability in individual curves, the CV of measured values was calculated per patient. For R_{int} the mean CV of all patients was 10.6% ($10^{\text{th}} - 90^{\text{th}}$ percentile: 5.5% – 16.3%). The mean CV for FEV₁ was significantly smaller: 2.9% (1.7% - 4.3%, p<0.001). Further analysis of CV R_{int} showed that there was no correlation with age ($r_s = -0.13$, p = 0.26). Also centres did not significantly differ from each other (Kruskal-Wallis test: p=0.17).

Analysing the 52 children who reached the PD_{20} endpoint, there was no significant correlation between changes from baseline of FEV_1 and changes from baseline of R_{int} at the last dose step at which the PD_{20} endpoint was reached ($r_s = 0.11$, p = 0.44).

Figure 1 demonstrates the mean changes from baseline of FEV_1 and R_{int} at each dose step, while at each dose step patients are grouped according to whether or not the PD₂₀ endpoint was reached at the dose step. At none of the dose steps there was a significant difference in the mean change of R_{int} from baseline between those who reached the PD₂₀ endpoint at that dose step versus those who did not, except at dose step 6 (p=0.014) (figure 1B). A combined analysis of all dose steps comparing responders and non-responders, using Page 9 of 68

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167	Cox regression analysis, showed that a greater change of R_{int} was associated with a higher
168	probability of reaching the PD_{20} endpoint (p= 0.038). However, when inspecting the
169	individual changes from baseline, there is large overlap of children who did and who did not
170	reach the PD_{20} endpoint at the various dose steps (Figure 2). A ROC curve of changes from
171	baseline of R_{int} as a measure to detect a 20% decrease in FEV ₁ is shown in Figure 3, resulting
172	in a small AUC of 0.65.
173	When using a 32.1% increase in R_{int} as cut-off value to calculate the sensitivity and
174	specificity of R_{int} to predict a 20% fall in FEV ₁ , we found a sensitivity of 50% and a
175	specificity of 43%. This means that R_{int} was able to detect half of the responders as
176	determined by PD20 and that in 12 out of 21 children R_{int} increased with more than 32%
177	without a concomitant fall of FEV_1 with 20%.
178	Repeating all analyses with change in R _{int} expressed as change in percentage predicted
179	and change in z-score led to similar results (data not shown). AUC's of ROC curves for
180	change in percentage predicted and for change in z-score were both 0.66.
181	In 64 children the methacholine challenge test was repeated after 26 weeks. The
182	analyses to compare R_{int} with FEV ₁ (as described above) showed similar results (data not
183	shown). The AUC of the ROC curve was 0.61.
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DISCUSSION

This study showed that, although there was a significant increase of mean R_{int} values with higher doses of methacholine, individual changes in R_{int} are highly variable during methacholine challenge. The sensitivity and specificity of R_{int} to detect methacholine induced bronchoconstriction is low, which might limit the use of R_{int} in individual patients. Several studies evaluated lung function measures requiring less patient effort than FEV_1 , which could possibly be used as alternative measures of changes in bronchoconstriction. Klug et al showed that R_{int} was significantly less sensitive than specific airway resistance in detecting changes in airway function (7). Phagoo et al found that R_{int} was also less sensitive and showed more variability than transcutaneous partial pressure of oxygen measurements (9;10). In a study of Beydon et al. the majority of children with a 20% fall in transcutaneous partial pressure of oxygen $(PD_{20}P_{tc}O_2)$ showed a significant change of R_{int} during methacholine challenge. However, all children who did not reach the $PD_{20}P_{tc}O_2$ had at least once a significant change from baseline (8), resulting in many false positive as well as false negative outcomes. Despite these data R_{int} is still advocated as an alternative measure for airway obstruction in those who are not able to perform forced breathing manoeuvres. Several studies showed significant correlation between gold standard FEV₁ and R_{int} in groups of patients (4;6), and reference values for R_{int} measurement in healthy controls have been published (15;17-20). Although there is convincing evidence on the value of R_{int} measurement as a measure of airway obstruction in groups of patients, the sensitivity of R_{int} to detect changes in airway obstruction in individual patients has been little researched. Only one previous study compared R_{int} with FEV₁ during methacholine challenge testing (11) and found that on average R_{int} increased during methacholine challenge comparable to changes in FEV₁ measurements, but individual changes in Rint were not shown. Kannisto et al. compared Rint

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with FEV_1 during exercise challenge to diagnose BHR (21). However, in this study only 11 (22%) children reached the R_{int} endpoint (15% increase from baseline) and only 6 of them also reached the FEV₁ endpoint (10% decrease from baseline). The children who showed a 15% rise in R_{int} but who did not have a 10% decrease in FEV₁ could be misclassified by R_{int} as having BHR. Davies et al. investigated the correlation between R_{int} and FEV_1 in bronchodilator response, and despite a correlation between FEV1 and Rint in a group of patients, they found no significant correlation in individual children (22). To evaluate the sensitivity and specificity of the R_{int} technique to detect changes in

219 airway obstruction in individual children we performed simultaneous measurements of R_{int} 220 and gold standard FEV₁ in a large group of children with asthma. We show that in individual 221 cases the variability of R_{int} measurements is high (mean intra-patient CV 10.6%), precluding 222 proper prediction of changes in FEV₁. In the present study the ROC curve, describing the 223 sensitivity and specificity of R_{int} in detecting BHR as determined by the primary outcome 224 FEV₁, had a small AUC of 0.65. This predictive capacity is lower compared to impulse 225 oscillometry (AUC 0.68 to 0.75 (23)).

Large individual fluctuations in R_{int} might be due to large intrapatient short-term variation in airway resistance during tidal breathing (11;17;20;24). This variability, resulting in low sensitivity and specificity, could limit the ability to detect thresholds in individual airway obstruction as required in clinical practice. Furthermore the sensitivity might be lower compared to FEV₁ because during (severe) airflow obstruction equilibration of alveolar pressure might take longer than is realised during the shutter closure time during R_{int} measurement (25). This may explain the lower R_{int} values in case of (severe) peripheral airway obstruction and hence lead to misclassifications during methacholine challenge testing. A third explanation of high variability of R_{int} might be the variability of upper airway

resistance. Proper positioning of the head and neck and adequate support of the cheeks ishighly warranted and might lead to intra-individual variability.

The findings of our study imply that on group level BHR can be documented, since we showed on average a significant rise in R_{int} with increasing methacholine doses. This may be useful in epidemiologic research. However, R_{int} seems to have insufficient capacity to diagnose BHR in individual patients and therefore it is of limited value in BHR measurements in clinical practice.

A major strength of our study is the fact that the methacholine challenge test was repeated after 26 weeks and resulted in a similarly low sensitivity and specificity of R_{int}, which validated the results. Beside this, the present study may have some limitations. Firstly, the study was performed in children with a mean age of 9.3 years, because preschool children are seldom able to perform FEV₁ measurements. It could be questioned if the found results are applicable to younger children. Since R_{int} is a measure of total airway resistance, an induced bronchoconstriction will lead to a relative larger increase in R_{int} in young children compared to older children, because in young children lower airways resistance contributes more to total airway resistance (26). Nevertheless, the fluctuations in R_{int} are expected to be similar as the short term repeatability of R_{int} that has been found to be independent of age (20:27). Additionally we used a 20% fall as 'gold standard' in detecting methacholine induced bronchoconstriction. When we used a 32% increase in R_{int} (the coefficient of repeatability of R_{int} measurements as described by Beydon et al.) as a cut-off value to determine BHR, there were 12 children with a significant change in R_{int} but without a 20% fall in FEV₁. The pathophysiology of a fall in FEV₁ during a forced manoeuvre might be different from an increase of R_{int} during tidal breathing. Therefore, there is a possibility that FEV₁ might not be the gold standard for bronchial challenges especially in young children.

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259 Therefore, there could have been children with BHR, who did not have a 20% fall in 260 FEV₁ but who did have a significant increase in R_{int}. This might have resulted in too low 261 sensitivity because of too many false positives. However, FEV₁ is the primary outcome in 262 methacholine challenge test, and no better 'gold standard' is available. 263 The current study showed on average an increase of R_{int} with subsequent methacholine 264 doses during methacholine challenge tests and therefore it might be useful as a measure of 265 airflow obstruction in groups of patients, e.g. in large epidemiological studies. However, we 266 conclude that R_{int} seems to have insufficient capacity to diagnose BHR in individual patients. 267 268

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Global strategy for asthma management and prevention: GINA executive summary.

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3 4	373	FIGURE LEGENDS
5 6 7	374	
8 9	375	Figure 1. Mean changes from baseline of $FEV_1 (1A)$ and $R_{int} (1B).$ At each dose step
10 11	376	patients are grouped according to whether (closed symbols) or not (open symbols) the
12 13 14	377	PD_{20} endpoint (20% fall in FEV_1) was reached at that dose step. Error bars represent
15 16	378	SEM.
17 18 19	379	
20 21 22	380	Figure 2. Individual changes from baseline of R_{int} at each dose step, divided according to
23 24	381	whether (closed symbols) or not (open symbols) the PD_{20} endpoint (20% fall in FEV_1)
25 26 27	382	was reached. At each dose step there is large overlap in changes from baseline of R_{int}
28 29	383	between both groups.
30 31 32	384	
33 34 35	385	Figure 3. Receiver operator characteristic (ROC) curve showing the sensitivity and false
36 37	386	positive rate of the change from baseline of R_{int} as a measure to detect a 20% fall in
38 39 40 41 42 43	387	FEV ₁ (PD ₂₀). Area under the curve (AUC) = 0.65.
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Table 1: Baseline characteristics of children with moderate asthma (n=73) who performed a methacholine provocation test with both R_{int} and FEV_1 measurement at first visit.

		Median (interquartile range)
Age (years)		9.2 (7.6 – 10.6)
Height (cm)		135.6 (128.5 – 146.0)
Sex (number	(%) males)	43 (58.9%)
FEV ₁ ^a	z-score	-0.26 (-0.74 – 0.32)
	% predicted	96.8 (90.8 - 104.2)
FVC ^b	z-score	0.10 (-0.37 – 0.67)
	% predicted	101.4 (95.7 – 108.3)
FEV ₁ /FVC	z-score	-0.81 (-1.340.21)
	% predicted	94.1 (89.7 – 98.6)
R_{int} ^c	z-score	0.78 (0.05 – 1.57)
	% predicted	123.4 (107.0 – 147.2)

a) FEV ₁ = Forced expiratory volume in 1 second;	b) FVC = Forced expiratory volume;
c) R _{int} = Interrupter resistance.	

Table 2: Number of children with moderate asthma (n = 73) who reached the PD₂₀

endpoint ^{a)} at subsequent methacholine dose steps.

Dose step	Number
1 = 0.625 μg	0
$2 = 1.25 \ \mu g$	0
$3 = 2.5 \ \mu g$	0
$4 = 5.0 \ \mu g$	2
$5 = 10.0 \ \mu g$	6
$6 = 20 \ \mu g$	10
$7 = 40 \ \mu g$	10
8 = 80 μg	10
9 = 160 μg	9
10 = 320 μg	5
No threshold	21

a) PD_{20} endpoint = dose step at which a fall of more than 20% in FEV₁ was reached.













Figure 2. Individual changes from baseline of R_{int} at each dose step, divided according to whether (closed symbols) or not (open symbols) the PD₂₀ endpoint (20% fall in FEV₁) was reached. At each dose step there is large overlap in changes from baseline of R_{int} between both groups. 191x190mm (96 x 96 DPI)



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1	ONLINE DATA SUPPLEMENT
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3	EVALUATION OF INTERRUPTER RESISTANCE IN METHACHOLINE CHALLENGE
4	TESTING IN CHILDREN
5	
6	Authors: Marije Koopman, MD ¹ ; Hein J.L. Brackel, MD, PhD ² ; Anja A.P.H. Vaessen-
7	Verberne, MD, PhD ³ ; Wim C. Hop, PhD ⁴ ; Cornelis K. van der Ent, MD, PhD ¹ ; on behalf of
8	the COMBO-Rint research group.
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15	4. Department of Biostatistics, Erasmus MC – University Medical
16	Centre Rotterdam, Rotterdam, the Netherlands
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18	COMBO-Rint research group consists of: H.G.M. Arets, N.J. van den Berg, P.L.P. Brand,
19	E.J. Duiverman, H.J. Hendriks, J.W.C.M. Heynens, J.C. van Nierop, M. Nuysink.
20	
21	Financial support: This study was supported by an unconditional grant from
22	GlaxoSmithKline Pharma Europe.

23 MATERIALS AND METHODS

This study was a substudy performed in some centres participating in the multicentre COMBO-study. The COMBO-study is a blinded study comparing the clinical effects of fluticasone propionate 200 mcg BD versus salmeterol/fluticasone propionate 50/100 mcg BD on number of symptoms free days in children between 6 and 16 years of age (trial registration number NCT00197106; GSK study number SAM101667) (1). It was performed in 158 patients in 19 hospitals in the Netherlands, between June 2005 and October 2008. All children had a clinical history of moderate asthma (according to NAEPP guidelines (2)) and bronchial hyperresponsiveness (BHR) (methacholine dose at which at least 20% fall in FEV₁ (PD₂₀) \leq 150 µg). Nine hospitals performed additional Rint measurements during methacholine challenge tests, so children from these centres were included in this comparative lung function study. The study was approved by the local medical ethical committees of the different centres and written informed consent was obtained from the parents (and also from the child if ≥ 12 years old) before the start of the study.

38 Measurements

39 Methacholine challenge testing

Methacholine challenge tests were performed using the dosimeter method (3;4) with a nebulizer (model 646, DeVilbiss Healthcare, Somerset, USA) attached to a dosimeter (Rosenthal French dosimeter, PDS Instrumentation, Louisville CO, USA). After baseline R_{int} and FEV1 measurements, 0.9% NaCl was inhaled to rule out non-specific reactions and additionally methacholine chloride was administered in doubling doses (0.625 μ g, 1.25 μ g, 2.5 µg, 5.0 µg, 10.0 µg, 20.0 µg, 40.0 µg, 80 µg, 160 µg, 320 µg). The procedure was performed according to the latest ATS/ERS guidelines (4) as follows: after manually triggering of the dosimeter children were instructed to inhale slowly from forced residual

48 capacity (FRC) to total lung capacity (TLC) and then hold their breath for 2 seconds. Two 49 minutes after each inhalation, R_{int} and FEV_1 were measured. Provocation was continued after 50 at least five minutes and until the PD₂₀ or the maximum dose was reached.

52 Interrupter resistance

Airway resistance measurements were carried out using the MicroRint (Micro Medical Limited, Kent, UK) that contains a pneumotachometer, a flow interruption valve and a pressure transducer to measure mouth pressure post-occlusion. A bacterial filter was used (Spirosafe bacterial filter, Micro Medical, Kent, UK). Measurements were performed conform the latest ATS/ERS guideline (5). The child was seated and diverted to ensure quiet tidal breathing. Children were breathing through a mouth piece while the technicians supported the cheeks to reduce the change in upper airway compliance. Ten interruptions of 100 ms were made at peak expiratory flow with a random frequency, with the valve closing within 10 ms. The MicroRint software automatically rejected measurements that showed an artefact on the pressure curve. Additionally measurements that showed tachypnoea or irregular breathing, vocalisation, or hyperextension or flexion of the neck were manually discarded as well as were tracings with a horizontal or declining pressure signal suggesting air leakage around the mouthpiece. R_{int} values were calculated using the two-point linear fit back extrapolation technique to t=15ms (6). A minimum of five successful interruptions was required to calculate the median R_{int} value. Reference values of Merkus et al. (7) were used to convert R_{int} outcomes into percentage predicted values.

70 Spirometry

71 Maximal expiratory flow-volume measurements were performed with standardized

9 72 equipment. Seven centres used a Jaeger pneumotachometer (Viasys Healthcare, Hochberg,

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Germany), 1 centre used the Microloop (Micro Medical, Kent, UK) and 1 centre used a Zan spirometer (nSpire Health GmbH, Oberthulba, Germany). Measurements were performed according to the latest ATS/ERS guidelines (8) with the child sitting and wearing a nose clip, without using a bacterial filter. Equipment calibration was performed daily and all measurements were BTPS corrected. After a full inspiration children performed a maximally forced and full expiration. At least three technician-accepted flow-volume curves were obtained and the largest FEV₁ was selected. Reference values of Stanojevic (9) were used.

81 Analysis; Calculation of intrapatient coefficients of variation (CV):

To quantify the variability of FEV_1 and R_{int} measurements between the various dose steps during the methacholine provocation test, a coefficient of variation (CV) was calculated for each separate patient. This was done by fitting smooth third degree polynomials of each parameter separately versus log-methacholine dose for each individual patient to allow a gradual change over the subsequent dose steps. The resulting standard deviation of residuals for each patient was subsequently divided by the same patients mean value to obtain an individual CV of the measurements. The resulting individual paired CV's of FEV₁ and R_{int} were compared using Wilcoxon's test.

RESULTS

92 Visual inspection of individual R_{int} profiles during the challenge test showed large

93 fluctuations with rises and falls at subsequent dose steps. In contrast, much smoother profiles

94 with gradual falls were found for FEV_1 (E-figure 1).

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135 E-FIGURE LEGEND

136 E-figure 1. Individual fluctuations of absolute values of FEV₁ (E1A) and R_{int} (E1B)

137 plotted against dose steps of methacholine in 73 children with moderate asthma. Dose

138 step 0.5 denotes Saline.

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dose step

E-figure 1. Individual fluctuations of absolute values of FEV₁ (E1A) and R_{int} (E1B) plotted against dose steps of methacholine in 73 children with moderate asthma. Dose step 0.5 denotes Saline. 190x196mm (96 x 96 DPI)



E-figure 1. Individual fluctuations of absolute values of FEV₁ (E1A) and R_{int} (E1B) plotted against dose steps of methacholine in 73 children with moderate asthma. Dose step 0.5 denotes Saline. 190x199mm (96 x 96 DPI)

Pediatric Pulmonology

1 2				
3 4	1	Title: Evaluation of interrupter resistance in methacholine challenge testing in children		
5 6 7	2			
7 8 9	3	Authors: Marije Koopman, MD ¹ ; Hein J.L. Brackel, MD, PhD ² ; Anja A.P.H. Vaessen-		
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31 This article has supplementary material.

, l challenge 33 **Running head:** R_{int} in bronchial challenge testing

Pediatric Pulmonology

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34 35	SUMMARY
36	Bronchial hyperresponsiveness is a key feature of asthma and is assessed using bronchial
37	provocation tests. The primary outcome in such tests (a 20% decrease in forced expiratory
38	volume in 1 second (FEV ₁)) is difficult to measure in young patients. This study evaluated the
39	sensitivity and specificity of the interrupter resistance (R_{int}) technique, which does not require
40	active patient participation, by comparing it to the primary outcome measure.
41	Methacholine challenge tests were performed in children with a history of moderate
42	asthma and bronchial hyperresponsiveness. Mean and individual changes in R_{int} and FEV_1
43	were studied. A receiver operating characteristic (ROC) curve was used to describe sensitivity
44	and specificity of R _{int} .
45	Seventy-three children (median age: 9.2 yrs; range: 6.3-13.4 yrs) participated. There
46	was a significant (p< 0.01) increase in mean R_{int} with increasing methacholine doses.
47	However, individual changes of R_{int} showed large fluctuations. There was great overlap in
48	change of R_{int} between children who did and did not reach the FEV ₁ endpoint. A ROC-curve
49	showed an area under the curve of 0.65.
50	Because of low sensitivity and specificity, the use of R_{int} to diagnose bronchial
51	hyperresponsiveness in individual patients seems limited.
52	
53	Word count abstract: 190
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55	Key words: Bronchial hyperresponsiveness, bronchial provocation test, forced expiratory
56	volume in 1 second, interrupter resistance, methacholine challenge test, sensitivity,
57	specificity.
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59 INTRODUCTION

According to GINA guidelines bronchial hyperresponsiveness (BHR) is a key feature of asthma (1). To objectify BHR, forced flow volume manoeuvres are required to measure the forced expiratory volume in one second (FEV₁), as primary outcome measure in bronchial challenge testing (2). Because of the difficulty of obtaining forced lung function measurements in early childhood the diagnosis of asthma in this age group is challenging and complicated. Several studies have advocated measurement of the interrupter resistance (R_{int}) as an alternative to quantify airway obstruction in preschool children and in children with developmental delay (3;4). The interrupter technique is non-invasive and can be obtained during tidal breathing (5). R_{int} can be measured in children from two years of age as it requires minimal cooperation (6). The R_{int} technique has been compared to body plethysmography (7) and transcutaneous partial pressure of oxygen measurements (7-10) during methacholine challenge tests, but most studies made no comparison with the outcome measure FEV₁. The one study that compared R_{int} with FEV₁ during methacholine challenge testing reported a mean increase in R_{int} with increasing doses of methacholine (11). However, this study did not present the changes in R_{int} for individual patients nor how these related to individual changes in FEV₁. The ability of R_{int} to detect methacholine induced bronchoconstriction in individual patients, as measured by a 20% decrease in FEV₁, has never been studied.

The latest ATS/ERS statement on pulmonary function testing in preschool children recommended the need to determine the role of R_{int} in bronchial challenge tests (5). The current study evaluated the sensitivity and specificity of R_{int} to detect airflow obstruction during methacholine challenge tests in asthmatic children by comparing measurements with FEV₁ as the gold standard.

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83 MATERIALS AND METHODS

This study was a substudy of the multicentre COMBO-study in children between 6 and 16 years with moderate asthma (see online supplement) (12). Nine out of 19 hospitals performed additional R_{int} measurements during methacholine challenge tests. The children from these centres were included in this substudy. It was approved by the local medical ethical committees and written informed consent was obtained from the parents (and the child if ≥ 12 years) before start of the study. For additional information see online supplement.

91 Study protocol

Methacholine provocation tests were performed twice (at start of study and after 26 weeks) measuring R_{int} and FEV₁ after each methacholine dose. All children abstained from long-acting $\beta 2$ agonists and inhaled steroids for 12 hours before the first methacholine provocation test (and 36 hours before the second methacholine provocation test), and from short acting $\beta 2$ agonists for 6 hours before provocation testing. To prevent possible, unwanted physiologic effects of deep inhalation on Rint, Rint was measured prior to FEV1. Pulmonary function tests were performed to conform with latest ATS/ERS guidelines (2;5;13) and briefly described below. The complete methodology is described in the online supplement.

101 Methacholine challenge testing

Methacholine challenge tests were performed using the dosimeter method (2;14) with a nebulizer (model 646, DeVilbiss Healthcare, Somerset, USA) attached to a dosimeter (Rosenthal French dosimeter, PDS Instrumentation, Louisville CO, USA). Methacholine chloride was administered in doubling doses and provocation was continued after at least five minutes and until the dose at which at least 20% fall in FEV₁ (PD₂₀) or the maximum dose was reached.

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2 3 4	108	
5 6 7 8 9	109	Interrupter resistance
	110	Airway resistance measurements were carried out using the MicroRint (Micro Medical
10 11	111	Limited, Kent, UK). A bacterial filter was used (Spirosafe bacterial filter, Micro Medical,
12 13	112	Kent, UK). Reference values of Merkus et al. (15) were used.
14 15 16	113	
17 18	114	Spirometry
19 20 21	115	Maximal expiratory flow-volume measurements were performed with standardized
21 22 23	116	equipment, using reference values of Stanojevic (16).
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26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42	118	Analysis
	119	Changes in FEV_1 and R_{int} were analyzed as percentages change from baseline. Change in R_{int}
	120	was also assessed as change in percentage predicted and change in z-score, computed from
	121	the latest R _{int} reference values (15). Wilcoxon Signed Ranks test was used to investigate
	122	whether there was a significant increase in mean change from baseline of R_{int} at each dose
	123	step. To quantify the variability of FEV_1 and R_{int} within individuals between the various
	124	dosesteps, coefficients of variation (CV) were calculated (see online supplement for
43 44	125	calculation). The resulting individual paired CV's of FEV_1 and R_{int} were compared using
45 46	126	Wilcoxon's test. Comparison of mean CV's between centers was done using the Kruskal-
47 48 49	127	Wallis test. The association between changes in FEV_1 and changes in R_{int} at PD_{20} was
50 51	128	analysed using Spearman's correlation (r _S). At each separate dose step Mann Whitney tests
52 53	129	were computed to compare the difference in changes from baseline between subjects who did
54 55 56	130	and who did not reach the PD_{20} endpoint at the dose step. Cox regression, with change from
57 58	131	baseline of R_{int} as time-dependent variable, was used to analyse whether these changes were
59 60	132	predictive for having reached the PD_{20} endpoint at the subsequent dose steps. The sensitivity

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2 3 4	133	and specificity of R_{int} in relation to having reached FEV ₁ -based PD ₂₀ was described by
5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25	134	receiver operating characteristic (ROC) curves. The area under the curve (AUC) shows
	135	whether the change in R_{int} can correctly classify those who do and do not reach the PD_{20}
	136	endpoint. We used the coefficient of repeatability calculated by Beydon et al. (a 32.1%
	137	increase in R_{int}) as a cut-off value to calculate the sensitivity and specificity of R_{int} to predict a
	138	20% fall in FEV ₁ .
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RESULTS

143 At the first COMBO-study visit 73 children with moderate asthma (60 % boys) with a median 144 age of 9.2 years (range: 6.3 - 13.4 years) performed both R_{int} and FEV₁ measurements during 145 methacholine challenge. Baseline characteristics are shown in Table 1. Fifty-two children 146 reached the PD₂₀ endpoint and were defined as responders (Table 2).

There was a significant increase from baseline of R_{int} starting at the sixth methacholine dose step and all dose steps thereafter (all p<0.01). From the first dose step mean FEV₁ was significantly different from baseline value (all p< 0.01), except for dose step 2. Visual inspection of individual R_{int} profiles during the challenge test showed large fluctuations with rises and falls at subsequent dose steps. In contrast, much smoother profiles with gradual falls were found for FEV_1 (see E-figure 1 in the online supplement). To express the variability in individual curves, the CV of measured values was calculated per patient. For R_{int} the mean CV of all patients was 10.6% ($10^{\text{th}} - 90^{\text{th}}$ percentile: 5.5% – 16.3%). The mean CV for FEV₁ was significantly smaller: 2.9% (1.7% - 4.3%, p<0.001). Further analysis of CV R_{int} showed that there was no correlation with age ($r_s = -0.13$, p = 0.26). Also centres did not significantly differ from each other (Kruskal-Wallis test: p=0.17).

158 Analysing the 52 children who reached the PD_{20} endpoint, there was no significant 159 correlation between changes from baseline of FEV_1 and changes from baseline of R_{int} at the 160 last dose step at which the PD_{20} endpoint was reached ($r_s = 0.11$, p = 0.44).

Figure 1 demonstrates the mean changes from baseline of FEV_1 and R_{int} at each dose step, while at each dose step patients are grouped according to whether or not the PD₂₀ endpoint was reached at the dose step. At none of the dose steps there was a significant difference in the mean change of R_{int} from baseline between those who reached the PD₂₀ endpoint at that dose step versus those who did not, except at dose step 6 (p=0.014) (figure 1B). A combined analysis of all dose steps comparing responders and non-responders, using Page 43 of 68

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167	Cox regression analysis, showed that a greater change of R_{int} was associated with a higher
168	probability of reaching the PD_{20} endpoint (p= 0.038). However, when inspecting the
169	individual changes from baseline, there is large overlap of children who did and who did not
170	reach the PD_{20} endpoint at the various dose steps (Figure 2). A ROC curve of changes from
171	baseline of R_{int} as a measure to detect a 20% decrease in FEV ₁ is shown in Figure 3, resulting
172	in a small AUC of 0.65.
173	When using a 32.1% increase in R_{int} as cut-off value to calculate the sensitivity and
174	specificity of R_{int} to predict a 20% fall in FEV ₁ , we found a sensitivity of 50% and a
175	specificity of 43%. This means that R_{int} was able to detect half of the responders as
176	determined by PD20 and that in 12 out of 21 children R_{int} increased with more than 32%
177	without a concomitant fall of FEV_1 with 20%.
178	Repeating all analyses with change in R _{int} expressed as change in percentage predicted
179	and change in z-score led to similar results (data not shown). AUC's of ROC curves for
180	change in percentage predicted and for change in z-score were both 0.66.
181	In 64 children the methacholine challenge test was repeated after 26 weeks. The
182	analyses to compare R_{int} with FEV ₁ (as described above) showed similar results (data not
183	shown). The AUC of the ROC curve was 0.61.
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DISCUSSION

This study showed that, although there was a significant increase of mean R_{int} values with higher doses of methacholine, individual changes in R_{int} are highly variable during methacholine challenge. The sensitivity and specificity of R_{int} to detect methacholine induced bronchoconstriction is low, which might limit the use of R_{int} in individual patients. Several studies evaluated lung function measures requiring less patient effort than FEV_1 , which could possibly be used as alternative measures of changes in bronchoconstriction. Klug et al showed that R_{int} was significantly less sensitive than specific airway resistance in detecting changes in airway function (7). Phagoo et al found that R_{int} was also less sensitive and showed more variability than transcutaneous partial pressure of oxygen measurements (9;10). In a study of Beydon et al. the majority of children with a 20% fall in transcutaneous partial pressure of oxygen $(PD_{20}P_{tc}O_2)$ showed a significant change of R_{int} during methacholine challenge. However, all children who did not reach the $PD_{20}P_{tc}O_2$ had at least once a significant change from baseline (8), resulting in many false positive as well as false negative outcomes. Despite these data R_{int} is still advocated as an alternative measure for airway obstruction in those who are not able to perform forced breathing manoeuvres. Several studies showed significant correlation between gold standard FEV₁ and R_{int} in groups of patients (4;6), and reference values for R_{int} measurement in healthy controls have been published (15;17-20). Although there is convincing evidence on the value of R_{int} measurement as a measure of airway obstruction in groups of patients, the sensitivity of R_{int} to detect changes in airway obstruction in individual patients has been little researched. Only one previous study compared R_{int} with FEV₁ during methacholine challenge testing (11) and found that on average R_{int} increased during methacholine challenge comparable to changes in FEV₁ measurements, but individual changes in Rint were not shown. Kannisto et al. compared Rint

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with FEV_1 during exercise challenge to diagnose BHR (21). However, in this study only 11 (22%) children reached the R_{int} endpoint (15% increase from baseline) and only 6 of them also reached the FEV₁ endpoint (10% decrease from baseline). The children who showed a 15% rise in R_{int} but who did not have a 10% decrease in FEV₁ could be misclassified by R_{int} as having BHR. Davies et al. investigated the correlation between R_{int} and FEV_1 in bronchodilator response, and despite a correlation between FEV₁ and R_{int} in a group of patients, they found no significant correlation in individual children (22). To evaluate the sensitivity and specificity of the R_{int} technique to detect changes in airway obstruction in individual children we performed simultaneous measurements of Rint and gold standard FEV_1 in a large group of children with asthma. We show that in individual cases the variability of R_{int} measurements is high (mean intra-patient CV 10.6%), precluding proper prediction of changes in FEV_1 . In the present study the ROC curve, describing the sensitivity and specificity of R_{int} in detecting BHR as determined by the primary outcome FEV₁, had a small AUC of 0.65. This predictive capacity is lower compared to impulse oscillometry (AUC 0.68 to 0.75 (23)). Large individual fluctuations in R_{int} might be due to large intrapatient short-term variation in airway resistance during tidal breathing (11;17;20;24). This variability, resulting in low sensitivity and specificity, could limit the ability to detect thresholds in individual

airway obstruction as required in clinical practice. Furthermore the sensitivity might be lower
compared to FEV₁ because during (severe) airflow obstruction equilibration of alveolar
pressure might take longer than is realised during the shutter closure time during R_{int}
measurement (25). This may explain the lower R_{int} values in case of (severe) peripheral
airway obstruction and hence lead to misclassifications during methacholine challenge
testing. A third explanation of high variability of R_{int} might be the variability of upper airway

resistance. Proper positioning of the head and neck and adequate support of the cheeks ishighly warranted and might lead to intra-individual variability.

The findings of our study imply that on group level BHR can be documented, since we showed on average a significant rise in R_{int} with increasing methacholine doses. This may be useful in epidemiologic research. However, R_{int} seems to have insufficient capacity to diagnose BHR in individual patients and therefore it is of limited value in BHR measurements in clinical practice.

A major strength of our study is the fact that the methacholine challenge test was repeated after 26 weeks and resulted in a similarly low sensitivity and specificity of R_{int}, which validated the results. Beside this, the present study may have some limitations. Firstly, the study was performed in children with a mean age of 9.3 years, because preschool children are seldom able to perform FEV₁ measurements. It could be questioned if the found results are applicable to younger children. Since R_{int} is a measure of total airway resistance, an induced bronchoconstriction will lead to a relative larger increase in R_{int} in young children compared to older children, because in young children lower airways resistance contributes more to total airway resistance (26). Nevertheless, the fluctuations in R_{int} are expected to be similar as the short term repeatability of R_{int} that has been found to be independent of age (20:27). Additionally we used a 20% fall as 'gold standard' in detecting methacholine induced bronchoconstriction. When we used a 32% increase in R_{int} (the coefficient of repeatability of R_{int} measurements as described by Beydon et al.) as a cut-off value to determine BHR, there were 12 children with a significant change in R_{int} but without a 20% fall in FEV₁. The pathophysiology of a fall in FEV₁ during a forced manoeuvre might be different from an increase of R_{int} during tidal breathing. Therefore, there is a possibility that FEV₁ might not be the gold standard for bronchial challenges especially in young children.

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The current study showed on average an increase of R_{int} with subsequent methacholine doses during methacholine challenge tests and therefore it might be useful as a measure of airflow obstruction in groups of patients, e.g. in large epidemiological studies. However, we 262 conclude that R_{int} seems to have insufficient capacity to diagnose BHR in individual patients. 263 264

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FIGURE LEGENDS

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871	Figure 1. Mean changes from baseline of $FEV_1(1A)$ and $R_{int}(1B).$ At each dose step
372	patients are grouped according to whether (closed symbols) or not (open symbols) the
373	PD_{20} endpoint (20% fall in FEV_1) was reached at that dose step. Error bars represent
374	SEM.
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876	Figure 2. Individual changes from baseline of R_{int} at each dose step, divided according to
877	whether (closed symbols) or not (open symbols) the PD_{20} endpoint (20% fall in FEV_1)
378	was reached. At each dose step there is large overlap in changes from baseline of R_{int}
379 380	between both groups.
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381	Figure 3. Receiver operator characteristic (ROC) curve showing the sensitivity and false
382	positive rate of the change from baseline of R_{int} as a measure to detect a 20% fall in
383	FEV ₁ (PD ₂₀). Area under the curve (AUC) = 0.65.

Table 1: Baseline characteristics of children with moderate asthma (n=73) who performed a methacholine provocation test with both R_{int} and FEV_1 measurement at first visit.

		Median (interquartile range)
Age (years)		9.2 (7.6 – 10.6)
Height (cm)		135.6 (128.5 – 146.0)
Sex (number (%) males)		43 (58.9%)
FEV ₁ ^a	z-score	-0.26 (-0.74 – 0.32)
	% predicted	96.8 (90.8 - 104.2)
FVC ^b	z-score	0.10 (-0.37 – 0.67)
	% predicted	101.4 (95.7 – 108.3)
FEV ₁ /FVC	z-score	-0.81 (-1.340.21)
	% predicted	94.1 (89.7 – 98.6)
R_{int} ^c	z-score	0.78 (0.05 – 1.57)
	% predicted	123.4 (107.0 – 147.2)

a) FEV ₁ = Forced expiratory volume in 1 second;	b) FVC = Forced expiratory volume;
c) R _{int} = Interrupter resistance.	

Table 2: Number of children with moderate asthma (n = 73) who reached the PD₂₀

endpoint ^{a)} at subsequent methacholine dose steps.

Dose step	Number
$1 = 0.625 \ \mu g$	0
$2 = 1.25 \ \mu g$	0
$3 = 2.5 \ \mu g$	0
4 = 5.0 μg	2
$5 = 10.0 \ \mu g$	6
$6 = 20 \ \mu g$	10
$7 = 40 \ \mu g$	10
8 = 80 µg	10
9 = 160 μg	9
10 = 320 μg	5
No threshold	21

a) PD_{20} endpoint = dose step at which a fall of more than 20% in FEV₁ was reached.









Figure 1. Mean changes from baseline of FEV_1 (1A) and R_{int} (1B). At each dose step patients are grouped according to whether (closed symbols) or not (open symbols) the PD_{20} endpoint (20% fall in FEV_1) was reached at that dose step. Error bars represent SEM. 173x190mm (96 x 96 DPI)



Figure 2. Individual changes from baseline of Rint at each dose step, divided according to whether (closed symbols) or not (open symbols) the PD_{20} endpoint (20% fall in FEV₁) was reached. At each dose step there is large overlap in changes from baseline of R_{int} between both groups. 191x190mm (96 x 96 DPI)



All sepositive rate

Figure 3. Receiver operator characteristic (ROC) curve showing the sensitivity and false positive rate of the change from baseline of R_{int} as a measure to detect a 20% fall in FEV₁ (PD₂₀). Area under the curve (AUC) = 0.65. 186x161mm (96 x 96 DPI)

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ONLINE DATA SUPPLEMENT

2 3 EVALUATION OF INTERRUPTER RESISTANCE IN METHACHOLINE CHALLENGE 4 **TESTING IN CHILDREN** 5 Authors: Marije Koopman, MD¹; Hein J.L. Brackel, MD, PhD²; Anja A.P.H. Vaessen-6 Verberne, MD, PhD³; Wim C. Hop, PhD⁴; Cornelis K. van der Ent, MD, PhD¹; on behalf of 7 8 the COMBO-Rint research group. 9 10 **Institutions:** 1. Department of Paediatric Pulmonology, Wilhelmina Children's Hospital, University 11 12 Medical Centre Utrecht, Utrecht, the Netherlands. 13 2. Department of Paediatrics, Catharina Hospital, Eindhoven, the Netherlands. 3. Department of Paediatrics, Amphia Hospital, Breda, the Netherlands 14 15 4. Department of Biostatistics, Erasmus MC – University Medical 16 Centre Rotterdam, Rotterdam, the Netherlands 17 COMBO-Rint research group consists of: H.G.M. Arets, N.J. van den Berg, P.L.P. Brand, 18 19 E.J. Duiverman, H.J. Hendriks, J.W.C.M. Heynens, J.C. van Nierop, M. Nuysink. 20 21 **Financial support:** This study was supported by an unconditional grant from 22 GlaxoSmithKline Pharma Europe.

23 MATERIALS AND METHODS

This study was a substudy performed in some centres participating in the multicentre COMBO-study. The COMBO-study is a blinded study comparing the clinical effects of fluticasone propionate 200 mcg BD versus salmeterol/fluticasone propionate 50/100 mcg BD on number of symptoms free days in children between 6 and 16 years of age (trial registration number NCT00197106; GSK study number SAM101667) (1). It was performed in 158 patients in 19 hospitals in the Netherlands, between June 2005 and October 2008. All children had a clinical history of moderate asthma (according to NAEPP guidelines (2)) and bronchial hyperresponsiveness (BHR) (methacholine dose at which at least 20% fall in FEV₁ (PD₂₀) \leq 150 µg). Nine hospitals performed additional Rint measurements during methacholine challenge tests, so children from these centres were included in this comparative lung function study. The study was approved by the local medical ethical committees of the different centres and written informed consent was obtained from the parents (and also from the child if ≥ 12 years old) before the start of the study.

38 Measurements

39 Methacholine challenge testing

Methacholine challenge tests were performed using the dosimeter method (3;4) with a nebulizer (model 646, DeVilbiss Healthcare, Somerset, USA) attached to a dosimeter (Rosenthal French dosimeter, PDS Instrumentation, Louisville CO, USA). After baseline R_{int} and FEV1 measurements, 0.9% NaCl was inhaled to rule out non-specific reactions and additionally methacholine chloride was administered in doubling doses (0.625 μ g, 1.25 μ g, 2.5 µg, 5.0 µg, 10.0 µg, 20.0 µg, 40.0 µg, 80 µg, 160 µg, 320 µg). The procedure was performed according to the latest ATS/ERS guidelines (4) as follows: after manually triggering of the dosimeter children were instructed to inhale slowly from forced residual

48 capacity (FRC) to total lung capacity (TLC) and then hold their breath for 2 seconds. Two 49 minutes after each inhalation, R_{int} and FEV_1 were measured. Provocation was continued after 50 at least five minutes and until the PD₂₀ or the maximum dose was reached.

52 Interrupter resistance

Airway resistance measurements were carried out using the MicroRint (Micro Medical Limited, Kent, UK) that contains a pneumotachometer, a flow interruption valve and a pressure transducer to measure mouth pressure post-occlusion. A bacterial filter was used (Spirosafe bacterial filter, Micro Medical, Kent, UK). Measurements were performed conform the latest ATS/ERS guideline (5). The child was seated and diverted to ensure quiet tidal breathing. Children were breathing through a mouth piece while the technicians supported the cheeks to reduce the change in upper airway compliance. Ten interruptions of 100 ms were made at peak expiratory flow with a random frequency, with the valve closing within 10 ms. The MicroRint software automatically rejected measurements that showed an artefact on the pressure curve. Additionally measurements that showed tachypnoea or irregular breathing, vocalisation, or hyperextension or flexion of the neck were manually discarded as well as were tracings with a horizontal or declining pressure signal suggesting air leakage around the mouthpiece. R_{int} values were calculated using the two-point linear fit back extrapolation technique to t=15ms (6). A minimum of five successful interruptions was required to calculate the median R_{int} value. Reference values of Merkus et al. (7) were used to convert R_{int} outcomes into percentage predicted values.

70 Spirometry

71 Maximal expiratory flow-volume measurements were performed with standardized

9 72 equipment. Seven centres used a Jaeger pneumotachometer (Viasys Healthcare, Hochberg,

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Germany), 1 centre used the Microloop (Micro Medical, Kent, UK) and 1 centre used a Zan spirometer (nSpire Health GmbH, Oberthulba, Germany). Measurements were performed according to the latest ATS/ERS guidelines (8) with the child sitting and wearing a nose clip, without using a bacterial filter. Equipment calibration was performed daily and all measurements were BTPS corrected. After a full inspiration children performed a maximally forced and full expiration. At least three technician-accepted flow-volume curves were obtained and the largest FEV₁ was selected. Reference values of Stanojevic (9) were used.

81 Analysis; Calculation of intrapatient coefficients of variation (CV):

To quantify the variability of FEV_1 and R_{int} measurements between the various dose steps during the methacholine provocation test, a coefficient of variation (CV) was calculated for each separate patient. This was done by fitting smooth third degree polynomials of each parameter separately versus log-methacholine dose for each individual patient to allow a gradual change over the subsequent dose steps. The resulting standard deviation of residuals for each patient was subsequently divided by the same patients mean value to obtain an individual CV of the measurements. The resulting individual paired CV's of FEV₁ and R_{int} were compared using Wilcoxon's test.

RESULTS

92 Visual inspection of individual R_{int} profiles during the challenge test showed large

93 fluctuations with rises and falls at subsequent dose steps. In contrast, much smoother profiles

94 with gradual falls were found for FEV_1 (E-figure 1).

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135 E-FIGURE LEGEND

136 E-figure 1. Individual fluctuations of absolute values of FEV₁ (E1A) and R_{int} (E1B)

137 plotted against dose steps of methacholine in 73 children with moderate asthma. Dose

138 step 0.5 denotes Saline.

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dose step

E-figure 1. Individual fluctuations of absolute values of FEV₁ (E1A) and R_{int} (E1B) plotted against dose steps of methacholine in 73 children with moderate asthma. Dose step 0.5 denotes Saline. 190x196mm (96 x 96 DPI)



E-figure 1. Individual fluctuations of absolute values of FEV₁ (E1A) and R_{int} (E1B) plotted against dose steps of methacholine in 73 children with moderate asthma. Dose step 0.5 denotes Saline. 190x199mm (96 x 96 DPI)