Biomarkers in Adult Asthma: a Systematic Review of 8-Isoprostane in Exhaled Breath Condensate

Authors: Adam M Peel[1], Christina-Jane Crossman-Barnes[1], Jonathan Tang[1], Stephen J Fowler[2], Gwyneth A Davies[3], Andrew M Wilson[1], Yoon K Loke[1].

Author affiliation: [1]Norwich Medical School, University of East Anglia, Norwich Research Park, Norwich, NR4 7TJ. [2]Education and Research Centre, University Hospital South Manchester, Wythenshawe, Manchester, M23 9LT. [3]Asthma & Allergy Group, Swansea University, Singleton Park, Swansea, SA2 8PP.

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Abstract

Objectives: We aimed to assess the evidence for the use of 8-isoprostane in exhaled breath condensate (EBC) as a biomarker in adult asthma. **Design:** A systematic review and meta-analysis of EBC 8-isoprostane. **Methods:** We searched a number of online databases (including PubMed, Embase and Scopus) in January 2016. We included studies of adult non-smokers with EBC collection and asthma diagnosis conducted according to recognised guidelines. We aimed to pool data using random effects meta-analysis and assess heterogeneity using I^2. **Results:** We included twenty studies, the findings from which were inconsistent. Seven studies (n = 329) reported 8-isoprostane levels in asthma to be significantly higher than that of control groups, whilst six studies (n = 403) did not. Only four studies were appropriate for inclusion in a random effects meta-analysis of mean difference. This found a statistically significant between-groups difference of 22pg/ml. Confidence in the result is limited by the small number of studies and by substantial statistical heterogeneity (I^2 = 94). **Conclusion:** The clinical value of EBC 8-isoprostane as a quantitative assessment of oxidative stress in asthma remains unclear due to variability in results and methodological heterogeneity. It is essential to develop a robust and standardised methodology if the use of EBC 8-isoprostane in asthma is to be properly evaluated.

Introduction

With the ascendance of personalised medicine and recognition of the heterogeneity within asthma there has been a drive to develop non-invasive measures of disease activity. Collecting and analysing the condensate from exhaled breath (EBC) is one such method, studied since the early 1980's (1). Several different commercial devices are available and this methodology has been adopted in a number of studies looking at an ever growing number of potential biomarkers.

Oxidative stress is thought to play an important role in asthma, as both a causative factor and a result of inflammation (2, 3). It occurs where there is a failure of homeostasis - due either to an excess of reactive oxygen species (ROS) or to a lack of antioxidants - and can cause cellular damage, proinflammatory mediator release, mucous secretion, remodelling of extracellular matrix, smooth muscle contraction and bronchoconstriction (3-5).

The reaction of ROS with other molecules is so rapid that their direct measurement is difficult; however, end products of ROS 'attack' are more stable and may be useful as surrogate markers for oxidative stress. 8-isoprostane is one such marker; specific to oxidative stress, stable, and measurable in EBC (6-8). Paediatric studies of EBC 8-isoprostane have been the subject of a systematic review (9) which found the majority of studies reported a significant association between 8-isoprostane and asthma, however, as biomarker thresholds vary with age (10), there is a need to review the adult literature. We aimed to assess the evidence regarding the efficacy of EBC 8-isoprostane as a biomarker – its ability to identify disease, disease severity and response to treatment. We chose to conduct a comprehensive systematic review because this enables us to view the evidence as whole, and to identify common themes as well as inconsistencies that may only become apparent through evaluation of the entire dataset.

Methods

Study design

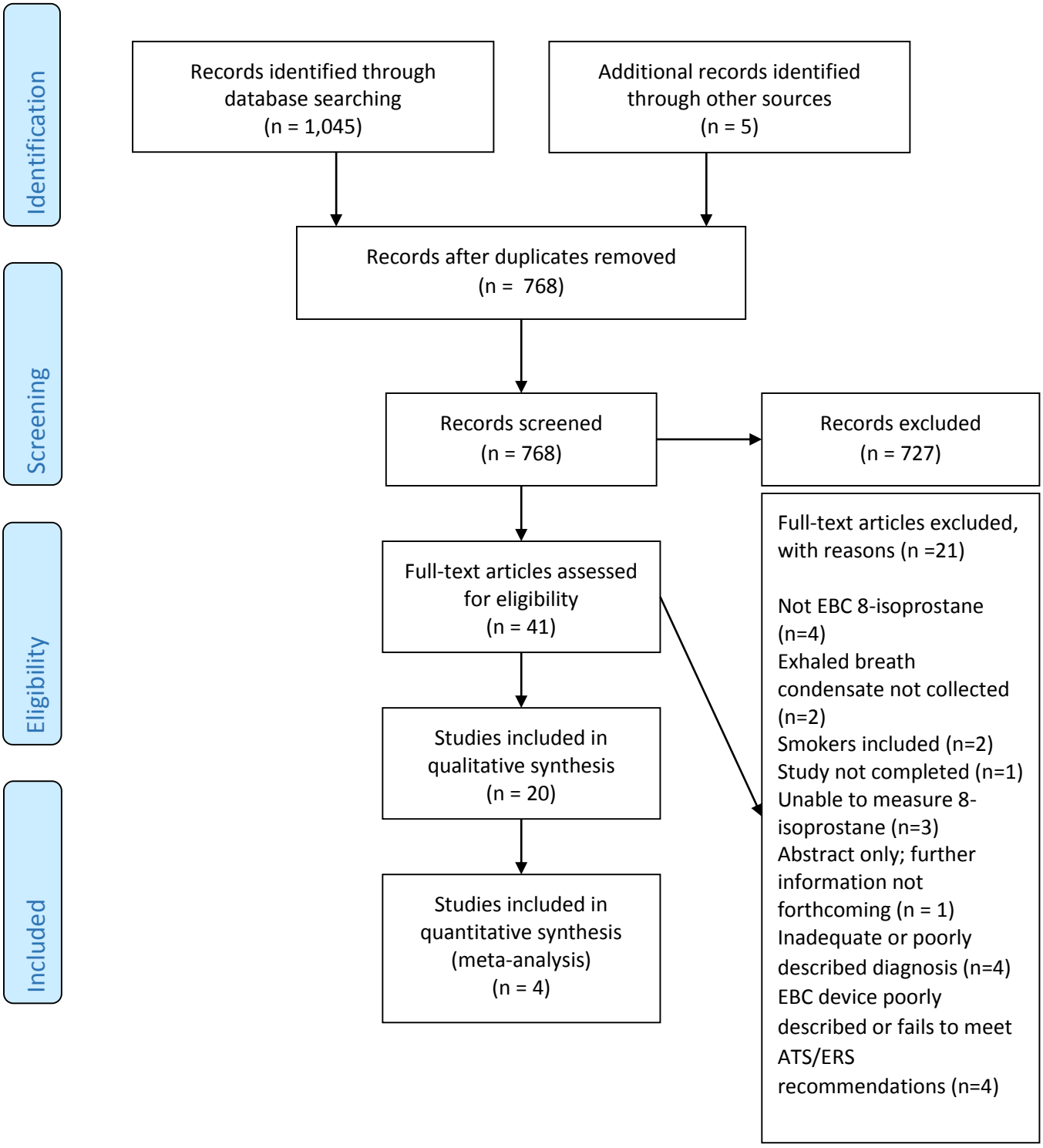
The study protocol was registered with and is available from the International Prospective Register of Systematic Reviews (PROSPERO) (registration number CRD42016027312). The primary objective of the review was to assess the ability of 8-isoprostane to identify and distinguish between a) those with asthma and healthy controls b) levels of asthma severity, and c) response to treatment. A secondary objective was to determine possible thresholds appropriate to a diagnosis of asthma or classification of severity.

Search Strategy

A search strategy was developed using terms relating to asthma, exhaled breath condensate and 8-isoprostane (see appendix table 1).

Two reviewers (AMP & CJCB) screened titles and abstracts for inclusion, resolving discrepancies through discussion with a third reviewer (YKL). The screening and selection process is described in a PRISMA flow chart (see figure 1).

Fig. 1 – PRISMA Diagram



Eligibility criteria

Inclusion / exclusion criteria are described in table 1 below.

Table 1 – Inclusion & Exclusion Criteria

Inclusion criteria	Exclusion criteria
Abstract in English	Review articles
Primary data	Studies including paediatric patients
Quantitative data	Studies of occupational asthma
Diagnosis of asthma according to recognised guidelines	Studies of current smokers
EBC 8-isoprostane measured	In vitro studies
Human subjects	Use of a custom EBC device with insufficient description or which fails to meet ATS/ERS guideline recommendations (11).
Adult participants (aged 18+)	Studies published as comment / letters will have a request for further information made; they will be excluded if further detail is not forthcoming.

Studies were excluded if the EBC collection device failed to meet ATS/ERS construction guidelines (12) (or was described insufficiently to determine this), or if the method of asthma diagnosis failed to meet recognised guidelines or was incompletely described. An exception to this was the use of nose-clips; although this was recommended, the guidelines state that there were no data underpinning this recommendation. A study by Vass et al (13) published since the guidelines found no significant difference between samples collected with or without nose-clips (although 8-isoprostane was not one of the mediators studied).

During the initial screening process several conference abstracts were found. On contacting the authors it was confirmed that the results had not been published more fully elsewhere but insufficient information was forthcoming to determine suitability for inclusion. In order to avoid selective dissemination bias an analysis of these papers was included.

Data Extraction & Quality Assessment

Data extraction and quality assessment was conducted by two reviewers independently (AMP and CJCB). Data were extracted directly into SPSS (14); papers were assessed for quality and risk of bias (15); and the overall strength of evidence was assessed (16, 17). Discrepancies between the two reviewers were resolved through discussion with a third (YKL).

Statistical methods

We aimed to produce a quantitative synthesis using methods appropriate to the data extracted and to assess statistical heterogeneity using the I^2 statistic. We used Open-Meta Analyst to conduct a random effects meta-analysis of mean difference (between asthma and control groups) for those studies reporting continuous data with a mean and standard deviation (SD). Where the SD was not reported we calculated it from confidence intervals or standard error (except where data had been transformed). In studies with multiple arms we combined data. We were not able to include papers which presented their results as a median and range.

Results

We identified 1,045 papers through the database search and a further five through reference searches (see PRISMA diagram, appendix figure 1). This was reduced to 768 on removal of duplicates. Title and abstract screening resulted in 41 papers which was reduced to 20 after screening full texts. Study characteristics are summarised in table 2.

Table 2. Study characteristics and results

Author	Publication type	Country	N =	EBC device	Method of analysis	Study focus	Se a po
Battaglia et al (2005)	Journal	Netherlands	31	EcoScreen	ELISA	Small airway function	Mild
Brussino et al (2010)	Journal	Italy	32	RTube	ELISA	Allergen challenge	Mild
Carpagnano et al (2006)	Journal	Italy	26	EcoScreen	ELISA + GC-MS	GORD	Mild
Fritscher et al (2012)	Journal	Canada	67	RTube	LC-MSMS	COPD & asthma	Mild
Gratziou et al (2008)	Journal	Greece	28	EcoScreen	ELISA	Seasonal allergic rhinitis & asthma	Mild untre
Head & Mickleborough (2013)	Journal	USA	7	EcoScreen	LC-MS	Supplements	Mild- mode
Komakula et al (2007)	Journal	USA	114	RTube	ELISA	BMI	Mode seve
Kostikas et al (2002)	Journal	Greece	50	Custom device	ELISA	pH	Mild mode
Mastalerz et al (2011)	Journal	Poland	21	EcoScreen	GC-MS	Aspirin sensitivity	Mild- mode
Mastalerz et al (2015)	Journal	Poland	53	EcoScreen	GC-MS	Aspirin sensitivity	Mode
Mickleborough et al (2013)	Journal	USA	20	EcoScreen	ELISA	Supplements	Mild- mode
Piotrowski et al (2011)	Journal	Poland	52	EcoScreen	ELISA	Asthma severity	Seve treat

Samitas et al (2009)	Journal	Greece	62	EcoScreen	ELISA	Asthma severity	Mild mod sever
Shimizu et al (2007)	Journal	Japan	62	EcoScreen	ELISA	GORD	Mod
Sood et al (2013)	Journal	USA	14	RTube	ELISA	Allergen	Mild
Zhao et al (2008)	Journal	Japan	64	EcoScreen	ELISA	GORD	Mild

Potentially eligible studies (conference abstracts)

Gemicioglu et al (2014)	Conference abstract	Turkey	19	No info	No info given	Smokers & non-smokers	Newly
Holguin & Fitzpatrick (2009)	Excerpt in review article	USA	125	RTube	No info given	BMI	Moder severe
Sedlak et al (2013)	Conference abstract	Czech Republic	61	EcoScreen	LC-MS	Inflammatory phenotype	Severe
Sedlak et al (2012)	Conference abstract	Czech Republic	20	No info	LC-MS	Oral steroids	Difficu contro

p< = a significant relationship reported

NS = a non-significant relationship reported

\- = not analysed or not-reported

N = number of participants in asthma and healthy control groups eligible for inclusion

ELISA = Enzyme-lin

GC-MS = Gas chrom

LC-MS = Liquid chro

GORD = Gastro-oeso

QUALITY ASSESSMENT

Results of the Quadas-2 quality assessment can be found in appendix table 3. It was not possible to assess the risk of bias arising from patient selection methods or from the conduction of the index test (EBC collection); in all but one paper description of patient sampling and/or recruitment methods was absent, and in only one paper was it clear whether the laboratory analysis of EBC was conducted by someone blinded to the participants' asthma status.

The time between reference and index standards was not clearly stated in five of the papers. The larger the interval the greater the risk of a change in condition between the two assessments and potential misclassification of asthma severity; we deemed asthma assessment within 1 week of EBC collection to be acceptable. Participant drop-out occurred in very few studies.

Variability: Pre-analytical

One study (Samitas et al (18)) coated the condenser surface of their EBC collection device in Tween-20 (a non-ionic surfactant) to reduce eicosanoid adherence. They report 8-isoprostane concentrations which are towards the higher end of results within this review. The extent to which this was due to the use of Tween-20 is unclear; Sood et al (19) examined this method and found no significant difference in 8-isoprostane between samples collected with or without Tween.

Three studies (Battaglia et al (20), Fritscher et al (21) and Sood et al (19)) undertook or cited 8-isoprostane recovery rates obtained from spiking tests; all were over 90%. Sood et al found that concentrating their samples by lyophilisation had no effect on recovery rates, whereas Battaglia et al found lower rates when they used an immunoaffinity sorbent and lyophilisation.

Kostikas et al (22) cooled their condensing surface to minus 10^{o}C whereas other studies used minus 20^{o}C. We included this study as it does not contravene ATS/ERS recommendations and evidence on the effect of temperature on EBC 8-isoprostane collection is conflicting (23-25).

Not mentioned in the ATS/ERS guidelines but specified by Cayman in their enzyme-linked immunosorbent assay (ELISA) information (26) is the use of an anti-oxidant - butylated hydroxytoluene (BHT) - for EBC samples which are being frozen and stored for later analysis. This is to prevent further (in vitro) oxidative formation of 8-isoprostane. The majority of studies using ELISA kits stored their samples for later analysis but none reported the use of BHT.

Relatively few studies reported the length of time samples were stored for but Samitas et al evaluated the stability of 8-isoprostane at minus 80^{o}C and found no significant difference in samples tested at one, four and eight weeks (although an upward trend could be noted).

Variability: Analytical

For their ELISA, Cayman cited a sensitivity of 3pg/ml and inter-assay variation rates of 10-24% however this validation was not undertaken in EBC. Sood et al (19) found the intra-assay CV in EBC to be 37.7% compared to 6% in buffer diluent. They concluded that interference from the EBC matrix was possible; the extent to which this might be a confounder in other studies is unclear as Sood et al's analysis was conducted on a lyophilised, concentrated EBC sample. The majority of studies in this review cite intra-assay and inter-assay CV <10%.

Several studies utilised mass spectrometry techniques as their method of analysis – GCMS and LC-MS/MS methods offer improved sensitivity and selectivity over immunoassays, hence they are often regarded as the superior method for measurement of isoprostanes (27-29). Fritscher et al (21) report the limit of detection with LC-MS to be 0.05-0.1pg; while Mastalerz et al (30) report that of GC-MS to be between 0.17 and 0.89pg/ml. The results found by studies using mass-spectrometry frequently fell below the lower detection limit of immunoassays. Two papers compared the results produced by ELISA methods with a) GC-MS (Carpagnano et al) and b) radioimmunoassay (Sood et al). Sood et al report discordance between methods while Carpagnano do not.

The absence of prime certified standard reference materials (SRM) produced by accredited bodies (such as NIST) for the production of calibration curves is a further source of potential inaccuracy and inter-laboratory variation.

Grade Assessment

A GRADE assessment was completed (using GradePro GDT (31)) for the twelve studies reporting on both asthma and control groups (see appendix table 4). The strength of the evidence pertaining to the differentiation of disease status was judged to be very low due to the inconsistency and imprecision of results.

Summary

For the majority of included papers there are no concerns over applicability to the review question but the risk of bias in the studies is largely unclear and there are unresolved methodological questions. Overall assessment of the evidence grade is very low.

QUANTITATIVE SYNTHESIS

Prediction of asthma attack or treatment response

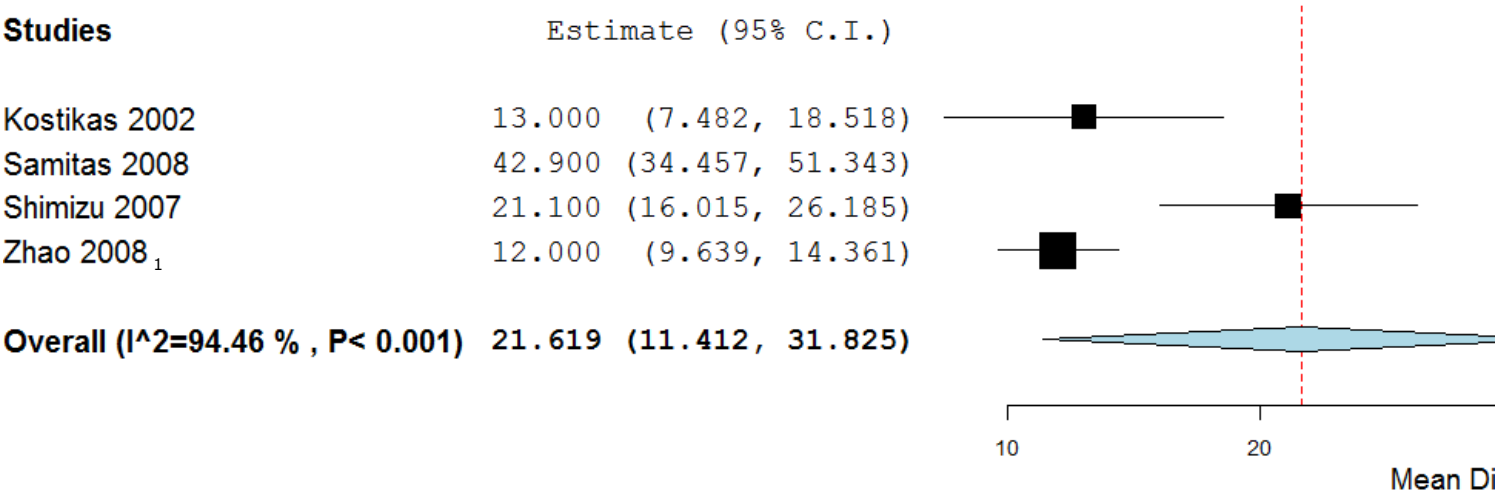
There were no studies examining the strength of association between 8-isoprostane concentration and frequency of asthma attack, nor studies examining the ability of 8-isoprostane to predict the risk of attack or response to treatment.

Differentiation of disease status

There was a large degree of clinical heterogeneity; studies examined different asthma phenotypes and severities, and utilised different interventions (including provocation tests and treatments). Given the broad study question we were addressing we considered the studies sufficiently homogenous for meta-analysis despite these differences.

Using Open Meta Analyst (32) we conducted a random effects meta-analysis of mean difference between groups (see figure 2). The estimated mean difference was +21.62 pg/ml in those with asthma (standard error 5.21). The p-value of <0.001 suggests statistical significance, and the lower bound of the meta-analytical point estimate -11.4pg/ml – is above the detection limit for the ELISA (2.8 to 7pg/ml). However, the I^2 test result - 94 - suggests a considerable degree of statistical heterogeneity, and the estimated mean difference (21.62pg/ml) should be viewed in light of the overall range of averages for EBC 8-isoprostane which varied from 0.25pg/ml to 78.10pg/ml.

Figure 2 - Random Effects Meta-Analysis of Mean Between-Group Difference (asthma vs controls)



Study weights: Kostikas 25%, Samitas 23%, Shimizu 25%, Zhao 27%.

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[1] Zhao et al (33) cited a median and IQR but also gave a mean. There was little difference between the mean and median (calculated SD from the IQR and included this study in the meta-analysis.

QUALITATIVE SYNTHESIS

Ten papers (n = 419) reported average 8-isoprostane levels to be higher in asthma than in healthy controls, while five papers (n = 389) reported averages to be the same or higher in controls.

Of the ten studies reporting higher concentrations in asthma, only seven (n=329) reported the difference to be statistically significant. However, of the three which were excluded, two (22, 34) simply omitted to report the significance level, while the third study – Sood et al (19) - was not powered to detect a between-group difference in 8-isoprostane concentration.

With the exclusion of conference abstracts, five papers (n=248) report a significant difference, and five papers (n=278) report either no significant difference or higher concentrations amongst controls. All papers scored similarly in their quality assessment. A full list of results can be found in appendix table 5.

Results from those papers reporting a median (figure 3) and those reporting a mean (figure 4) are displayed below. Even when looking only at those studies reporting a significant between-groups difference, there is a considerable overlap of results between studies - the range of values for controls in one study being similar to those for asthma in another. This degree of statistical heterogeneity precludes the determination of threshold values.

Figure 3 – Median 8-isoprostane and Range: Asthma groups versus controls

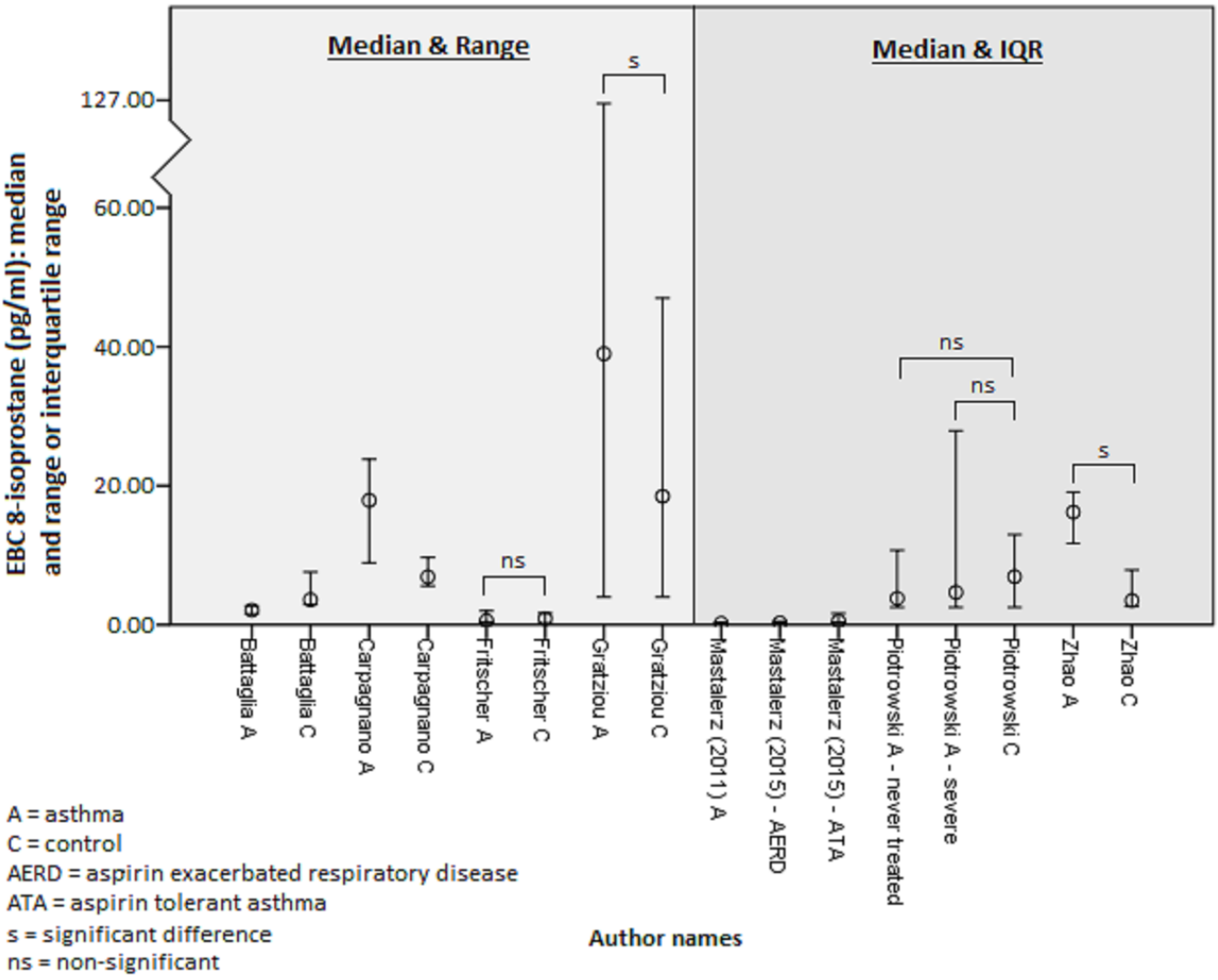
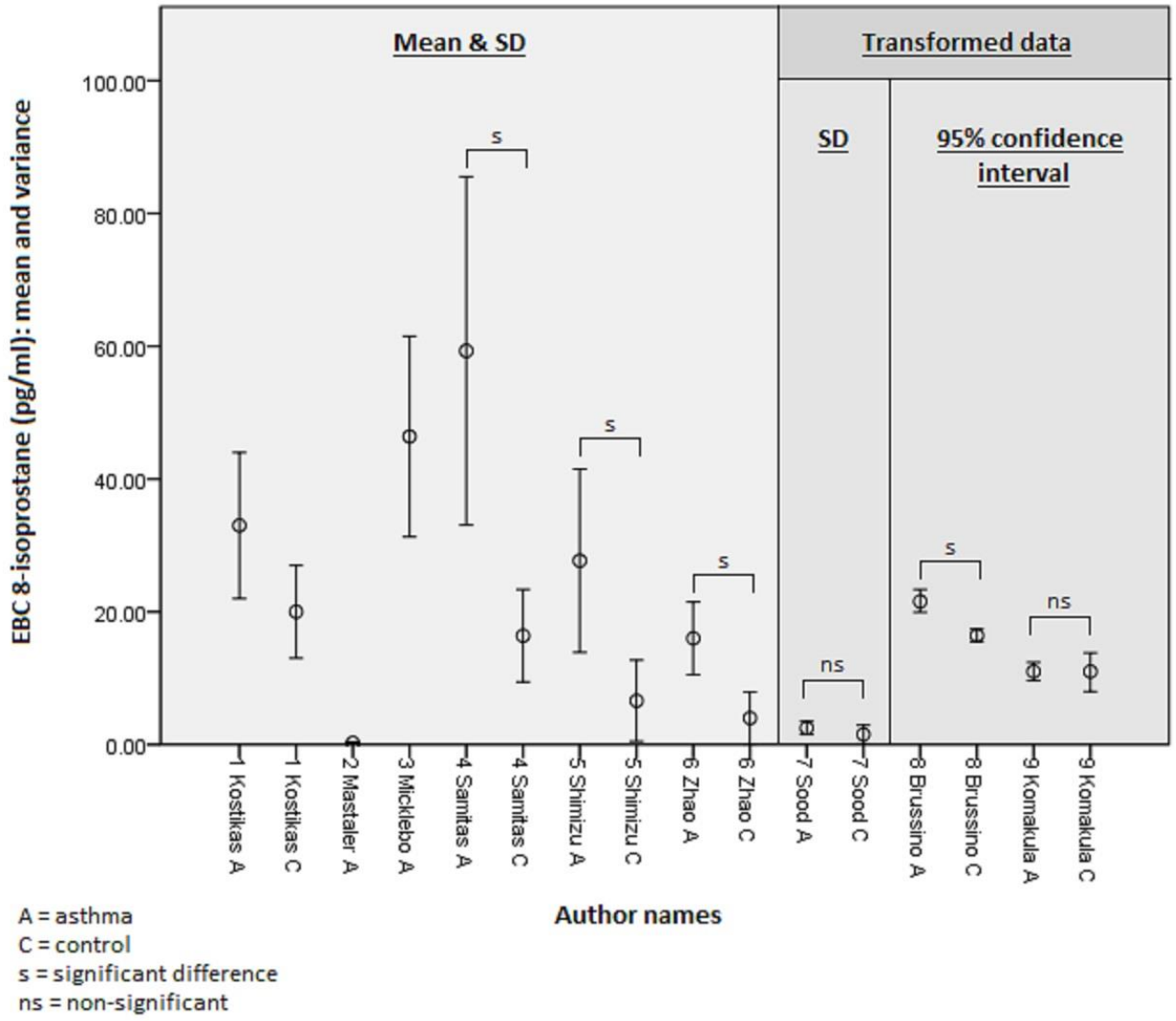


Figure 4 – Mean 8-isoprostane and Standard Deviation: Asthma groups versus controls



There was a large degree of overlap in 8-isoprostane concentration between severities of asthma. This may be attributable to between-study methodological differences, however, three studies (18, 21, 35) made within-study comparisons of severity. Samitas et al (18) report a significant difference between the severe and milder asthma groups, whereas Piotrowski et al (35) report a small, non-significant difference (0.87 pg/ml). Kostikas et al (36) report a difference of 15 pg/ml but do not comment on its statistical significance.

Both Brussino et al (37) and Sood et al (19) investigated the effect of allergen challenge on EBC 8-isoprostane concentration. Brussino et al reported a statistically significant increase while Sood et al reported no such change.

Gratziou (38) studied patients with seasonal allergic rhinitis and concurrent asthma, reporting significantly higher levels of 8-isoprostane during pollen season, and a significant decrease after treatment with nasal corticosteroids. Mastalerz et al (30, 39) conducted a pair of studies in which patients with aspirin intolerant asthma (AIA) or aspirin exacerbated respiratory disease (AERD) were subjected to an aspirin challenge; they found no significant difference in 8-isoprostane after challenge.

Baseline measures of pulmonary function (spirometry) were commonly reported in order to characterise study populations; their relation to EBC 8-isoprostane was less commonly examined. Eight studies conducted such an analysis, of which only two reported a significant (negative) correlation. Similarly, baseline blood eosinophil count was reported by five papers but analysed in relation to 8-isoprostane by only one (reporting no correlation). FeNO was measured by six studies; four assessed the degree of correlation with EBC 8-isoprostane only one of which yielded a statistically significant (positive) association. Two papers undertook sputum analysis; one reported on the relationship with EBC 8-isoprostane - no significant association was found.

SUBGROUP ANALYSIS

Methodological heterogeneity has been suggested as one of the factors inhibiting clinical use of EBC (12). Those papers included in the meta-analysis all used ELISA as their method of analysis but represent a mixture of asthma severities and EBC collection devices. A sub-group analysis of EBC collection and analytical methods was used as a means of exploring this heterogeneity.

Five of the seven studies using the EcoScreen reported a positive difference between asthma and control groups; four were statistically significant, the fifth was not reported upon. Of the four studies using the RTube, two reported a positive difference between groups of which one was statistically significant. The condensing surface of the RTube is polypropylene while on the EcoScreen it is teflon. Several papers have looked at the possible impact of device and condensing surface upon EBC results (23-25, 40-44). Czebe et al (24) compared the RTube and EcoScreen and concluded that both temperature and condenser surface had an impact on biomarker levels. Soyer et al (23) found similar results although neither study examined 8-isoprostane. Rosias et al (25) did study 8-isoprostane and concluded that condenser surface did have an effect but that there was no difference between polypropylene and teflon. Moreover they concluded that temperature difference between the two did not appear to have a significant effect on 8-isoprostane collection. Based on current studies of methodology we cannot be certain that choice of device explains any of the heterogeneity in the results.

Regarding analytic method, if the outlier generated by the inclusion of conference abstracts (Sedlak et al (45)) is excluded, the results from mass spectrometry exhibit a smaller range and are considerably lower than the majority of results from ELISA. However, Carpagnano et al (34) – the only study to confirm their ELISA results using gas chromatograph-mass spectrometry - report no discrepancy between the two measures; this is in line with previous studies (46). That analytical method is responsible for a degree of heterogeneity in the results is plausible but cannot be stated with certainty.

The inclusion of papers studying mild or intermittent asthma - in which there may be little or no oxidative stress – might explain the lack of consistently observed difference between asthma and control groups.

A sub-group analysis comparing moderate-to-severe asthma with controls was conducted to explore this possibility. Results were inconclusive; of the eight included papers (18, 22, 35, 45, 47-50) five reported a between-groups difference (four of which were statistically significant) while three reported no difference (see appendix table 6).

Discussion

This review highlights a lack of comparability between studies, as well as evidence gaps which create difficulties in determining 8-isoprostane thresholds for diagnosis or severity classification of asthma. The clinical value of EBC 8-isoprostane as a quantitative assessment of oxidative stress in asthma remains unclear due to variability in results and inadequate standardization.

The previously published paediatric review (9) reported more consistent findings - five of the six identified studies found a significant difference between asthma and healthy control groups. However, the studies exhibited a similarly large degree of variance in their results (ranging between 4.2 – 56.4pg/ml for asthma and 2.6 – 34.2pg/ml for control groups).

The ATS / ERS taskforce of 2005 (11) was set-up to address variability in EBC results and lack of standardisation in methods. They suggested two likely contributors to variability - varying EBC dilution levels and biomarkers being at the lower end of assay sensitivity. That there exists a large degree of variance in 8-isoprostane concentration levels even where studies have used the same EBC collection method would support these assertions.

Ahmadzai et al in 2013 (51) discuss three possible methods of calculating a dilution factor, none of which has established itself as a gold standard and none of which were used in the studies comprising this review. Only one study (30) used a dilution factor, giving their results in both pg/ml and parts per million of palmitic acid. It remains to be seen whether this improves reproducibility.

It has been suggested that lyophilisation may be useful for reducing variability by concentrating samples thereby raising biomarkers away from the lower end of assay sensitivity. There are a lack of studies examining the reliability and reproducibility of this method (51). Unfortunately the only studies in this review to have used this approach (Battaglia et al (20) and Sood et al (19)) concentrated their samples to differing levels (threefold and fourteenfold respectively). Furthermore, Sood et al reported an intra-assay CV of 37.7% and an inter-day CV of 71.6% when using this method.

The validity of any assessment of diagnostic test accuracy rests upon the accuracy of the reference standard to which it is compared; we included studies where diagnosis was conducted according to recognised guidelines.

A large number of exclusions were due to lack of diagnostic clarity; many undertook spirometry as a study measure rather than a diagnostic assessment and - unless reviewed by a physician and judged against a clearly described standard - can't be accepted as diagnostic confirmation. Furthermore, guidelines stress the importance of variable airflow obstruction to diagnosis; this cannot be assessed by a single spirometry measurement thereby complicating the process for any study wishing to have a rigorous diagnosis as the basis for inclusion.

Of concern were studies where it was neither explicitly stated that smokers were excluded, nor was smoking status featured in the participant description. There were six studies in which this occurred and over which there must be some concern that data might include that from smoking participants. This would be a potential confounder; there is evidence that EBC 8-isoprostane is significantly higher in smokers compared to healthy controls (52) and may increase in an acute smoking response (53).

Another potential confounder is the effect of food and drink; sixteen of the studies did not mention fasting prior to tests. The ATS/ERS guidelines (12) state that eating and drinking do not affect the non-volatile components of EBC as far as is known, but Ahmadzai (51) point out that food & drink may elevate levels of oxidants in body fluids and has the potential to influence oxidant concentrations in EBC (although they identify no studies describing any such effect on 8-isoprostane). The extent to which this might constitute a confounder is unknown.

Several authors confirmed they were unable to measure 8-isoprostane in a majority of their samples (54-56). Of those studies in this review which reported undetectable samples the percentage ranged from 16% (Komakula et al) to 50% (Piotrowski et al). Not all papers made clear the cause of missing data (whether an inability to obtain EBC samples or an inability to detect 8-isoprostane) nor how this was handled in the analysis. Gratziou et al (38) gave non-detectable levels of 8-isoprostane a value of 3.9pg/ml (the lower limit of

assay detection) while Sood et al ascribed undetectable levels a value half the lower detection limit; neither state how many cases this applied to. If these samples came predominantly from healthy controls, raising them might obfuscate any difference between asthma and controls.

The absence of oxidative stress is a potential explanation of inability to detect 8-isoprostane. This might be the case for studies of mild or intermittent asthma. The use of provocation tests or the study of moderate-to-severe asthma is one potential approach to this problem but the results of such studies were no less conflicted.

Although not one of our primary objectives we examined those factors for which an association with 8-isoprostane was reported. The majority of studies which assessed GORD and BMI reported a significant association with 8-isoprostane. It is possible that these are important confounders which may need to be controlled for in future studies.

Limitations

By employing rigorous inclusion criteria for asthma diagnosis and EBC methodology several 'key' papers were excluded, including that of Montuschi et al (46) frequently cited by others as justification for their methodology. We believe these exclusions were justified; the use of rigorous inclusion criteria are crucial for a review of diagnostic test accuracy.

Inability to assess the risk of bias in key domains of the QUADAS-2 quality assessment tool makes any conclusions from this review necessarily tentative. Furthermore, we were able to conduct meta-analysis of only four studies due to the frequent use of median, range, and log-transformed data.

The increasing ability to examine several biomarkers - for example Sedlak et al (45, 47) – creates a risk that non-significant findings may go unreported unless high reporting standards are adhered to. Hussain et al mention EBC 8-isoprostane in the methods section of a conference abstract (57) but not in the results, nor anywhere in the full published paper (58); suggesting that 8-isoprostane was either undetectable or the results were non-significant. Although these may constitute a publication bias, the under-representation of negative findings makes the lack of positive findings in this review more robust.

Conclusion

There is a trend towards higher EBC 8-isoprostane concentrations in subjects with asthma compared to controls. Twice as many studies reported higher levels amongst those with asthma than did not. However the strength of this evidence is weak and the number of studies reporting a significant difference was the same as that reporting none. A random

effects meta-analysis found a significant difference between groups however its rigour is compromised by the small number of studies and substantial statistical heterogeneity.

Concentrating EBC samples may address some of the variability and difficulty arising from the use of ELISA. However, the central issue of calculating EBC dilution cuts across analytical methods and a gold standard is still to be determined. It will be essential to develop accurate, reliable and standardised methods of both EBC collection and 8-isoprostane analysis if its use as a biomarker in asthma is to be properly evaluated.

Appendix

Table 1 - Search Terms

Terms relating to the condition of interest - asthma	**Asthma* OR "Bronch* hyperreactivity"**
Terms relating to the collection method - exhaled breath condensate.	"Exhaled breath condensate" OR "Breath test*" OR "Lung function test*" OR "Expired air"
Terms relating to the biomarker of interest - 8-isoprostane	*isoprostane* OR Dinoprost* OR *prost* OR "Lipid peroxid*" OR *prostaglandin*
Master search string (adapted for use in individual databases as required)	(Asthma* OR "Bronch* hyperreactivity") AND ("Exhaled breath condensate" OR "Breath test*" OR "Lung function test*" OR "Expired air") AND (*isoprostane* OR Dinoprost* OR *prost* OR "Lipid peroxid*" OR *prostaglandin*)

The strategy was modified as required for individual databases and the implemented in the following online databases: Cochrane, Embase, PubMed, Lilacs, Scopus, ClinicalTrials.gov, Open Grey and ProQuest.

Table 2 - Data Extraction Table

Data extraction table
Study ID number, Authors, Year of publication, Country of study, Source / type of publication
Study design, Diagnostic criteria used, Time horizon,
Sample size (total), number in control group, number in asthma group
Average age in asthma group, Percentage female
Average age of controls, Percentage female
Character of asthma group (severity)
Atopic status, Ethnicity, Intervention (e.g. allergen challenge, steroid therapy),
EBC collection Device, Methodological omissions, Method of EBC analysis, Units used for 8-isoprostane
Average 8-isoprostane in control group (mean or median), Baseline 8-isoprostane in asthma group (mean or median)
Average 8-isoprostane after any intervention, average 8-isoprostane in severity groups (e.g. mild, moderate, severe), average 8-isoprostane in steroid treated group (baseline & post-treatment), average 8-isoprostane in steroid naïve patients with asthma.
8-isoprostane correlations with - FeNO, CO, pulmonary function (e.g. FEV1 or FVC (% predicted), sputum 8-isoprostane, plasma 8-isoprostane, serum inflammatory markers (e.g. ESR, CRP).
8-isoprostane correlations with - age, BMI, Cys-LTs, PGE2, results of methacholine provocation test, interleukins (e.g. IL4, IL6), pH, sputum cell count, LTB4, urinary markers, any other associations reported on (present or absent, positive or negative)
Reported difference between controls and asthmatics; Significance of difference.

Table 3 - QUADAS-2 Assessment

Study	RISK OF BIAS				APPLICABILITY CONCERNS		
	PATIENT SELECTION	INDEX TEST	REFERENCE STANDARD	FLOW AND TIMING	PATIENT SELECTION	INDEX TEST	REFERENCE STANDARD
1. Battaglia 2005	?	?	☺	☹	☺	☺	☺
2. Brussino 2010	?	?	☺	☺	☺	☺	☺
3. Carpagnano 2006	☺	?	☺	☺	☺	☺	☺
4. Fritscher 2012	?	?	☺	?	☺	☺	☺
5. Gratziou 2008	?	?	☺	☺	☺	☺	☺
6. Head 2013	?	?	☺	☹	?	☺	☺
7. Komakula 2007	?	?	☺	☺	☺	☺	☺
8. Kostikas 2002	?	?	☺	☺	☺	☺	☺
9. Mastalerz 2011	?	?	☺	☺	☺	☺	☺
10. Mastalerz 2015	?	?	☺	☺	?	☺	☺
11. Mickelborough 2013	?	?	☺	?	?	☺	☺
12. Piotrowski 2011	?	?	☺	☺	☺	☺	☺
13. Samitas 2009	?	?	☺	☺	?	☺	☺
14. Shimizu 2007	?	?	☺	?	☺	☺	☺
15. Sood 2013	?	☺	☺	?	☺	☺	☺
16. Zhao 2008	?	?	☺	?	☺	☺	☺
Potentially eligible studies (conference abstracts)							
a. Gemicioglu 2014 [conf. abstract]	?	?	☺	☺	☺	?	☺
b. Holguin 2009 [review excerpt]	?	?	?	?	☺	?	?
c. Sedlak 2013 [conf. abstract]	?	?	?	?	?	☺	?
d. Sedlak 2012 [conf. abstract]	?	?	?	?	?	?	?

☺ = low risk / low level of concern regarding applicability

? = unclear risk / unclear level of concern regarding applicability

☹ = high risk / high level of concern regarding applicability

Table 4 – GRADE Evidence Profile

Setting: Adult non-smokers in any clinical setting.

Bibliography: Battaglia, Hertog, Timmers et al (2005); Brussino, Badiu, Sciascia et al (2010); Carpagnano, Resta, Ventura et al (
Gratziou, Rovina, Makris et al (2008); Komakula, Khatri, Mermis et al (2007); Kostikas, Papatheodorou, Ganas (2002); Piotrow
Chorianopoulos, et al (2009); Shimizu, Dobashi, Zhao et al (2007); Sood, Qualls, Seagrave et al (2013); Zhao, Shimizu, Dobashi

Quality assessment							Impa
№ of studies	**Study design**	**Risk of bias**	**Inconsistency**	**Indirectness**	**Imprecision**	**Other considerations**	
Is exhaled breath condensate 8-isoprostane capable of differentiating between healthy controls and those with asthma?							
12	Mixture of observational and experimental studies	Not serious	Very serious [1]	Not serious	Very serious [2]	Publication bias strongly suspected; all plausible residual confounding would reduce the demonstrated effect [3]	Cases (asthma) 353; c

1. Significant unexplained variability in results; I-squared test for statistical heterogeneity = 94
2. Large variance in study data
3. Probable publication bias

GRADE Working Group grades of evidence
High quality: We are very confident that the true effect lies close to that of the estimate of the effect
Moderate quality: We are moderately confident in the effect estimate: The true effect is likely to be close to the estimate of substantially different
Low quality: Our confidence in the effect estimate is limited: The true effect may be substantially different from the estimat
Very low quality: We have very little confidence in the effect estimate: The true effect is likely to be substantially different fr

Table 5 - Study Results: Averages and Variance

Author			Average EBC 8-isoprostane level	
	Units (measure of central tendency)	**Measure of variance**	**Asthma (variance)**	**Control (variance)**
Battaglia, Hertog, Timmers et al	pg/ml (median)	Range	2.10 (1.6 - 2.7)	3.6 (2.9 - 7.6)
Brussino, Badiu, Sciascia et al*	pg/ml (geometrical mean)	95% confidence interval	21.56 (19.92 - 23.35)	16.43 (15.50 - 17.41)
Carpagnano, Resta, Ventura et al	pg/ml (median)	Range	17.90 (8.9 - 23.8)	6.9 (5.6 – 9.7)
Fritscher, Post, Rodrigues et al	pg/ml (median)	Range	0.60 (0.4 - 2.0)	0.9 (0.2 - 1.7)
Gratziou, Rovina, Makris et al	pg/ml (median)	Interquartile range	39.0 (4.0 - 125)	18.5 (4 - 37)
Head & Mickleborough	pg/µl (mean)	Standard error	3.08 (+/- 1.5)	_
Komakula, Khatri, Mermis et al*	pg/ml (mean)	95% CI	11.0 (9.6 - 12.4)	11.0 (8.0 – 13.8)
Kostikas, Papatheodorou, Ganas	pg/ml (mean)	Standard deviation	33.0 (11)	20.0 (7)
Mastalerz, Sanak, Kumik et al	pg/ml (mean)	Standard deviation	0.25 (+/- 0.12)	_
Mastalerz, Januszek, Kaszuba et al AERD & ATA (two asthma groups within study)	pg/ml (median)	Interquartile range	0.28 (0.19 - 0.49) 0.54 (0.35 – 1.65)	_ _
Mickleborough, Vaughn, Shei et al	pg/ml (mean)	Standard deviation	46.40 (+/- 15.1)	_
Piotrowski, Majewski, Marczak et al Severe asthma & Never treated asthma (two groups within study)	pg/ml (median)	Interquartile range	3.8 (2.5 – 10.73) 4.67 (2.5 – 27.92)	6.93 (2.5 - 12.98)
Samitas, Chorianopoulos, et al	pg/ml (mean)	Standard error	59.30 (+/- 4)	16.4 (+/- 1.6)
Shimizu, Dobashi, Zhao et al	pg/ml (mean)	Standard error	27.70 (+/- 2.3)	6.6 (+/- 1.2)
Sood, Qualls, Seagrave et al*	pg/ml (mean)	Standard deviation	2.50 (+/- 0.99)	1.54 (+/- 1.39)
Zhao, Shimizu, Dobashi et al	pg/ml (median)	Interquartile range	16.20 (11.7 – 19.1)	3.5 (2.6 – 7.9)

* Log transformed data

Potentially eligible studies (conference abstracts)				
Gemicioglu, Duman, Akdeniz et al	No units given (mean)	Standard deviation	135.72 (+/- 38.85)	
Holguin & Fitzpatrick	pg/ml (mean)	95% confidence interval	Unable to extract data	_
Sedlak, Cap, Kacer et al	No units given (?)	No measure of variance given	Result not directly cited	_
Sedlak, Cap, Kacer et al	pg/ml (?)	No measure of variance given	78.10	_

Table 6 – Sub-Group Analysis: Moderate or Severe asthma

	8-isoprostane concentration levels	
	Asthma > controls	**Controls ≥ asthma**
All papers	5 studies (n = 253)	3 studies (n = 273)
Conference abstracts removed	3 studies (n=174)	2 studies (n =166)

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