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Almqvist C et al

STOPPA twin study

## **Cohort profile: Swedish Twin study On Prediction and Prevention of Asthma (STOPPA)**

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**Key words**

asthma, allergic disease, concordance, discordance, epidemiology, epigenetic, genetic, lung function, twin

**Abbreviations**

|        |   |
|--------|---|
| ACC    | asthma concordant   |
| ADC    | asthma concordant   |
| BMI    | body mass index   |
| CATSS  | Child and Adolescent Twin Study in Sweden                 |
| CBC    | complete cell count                                       |
| DZ     | dizygotic   |
| FeNO   | fractional exhaled NO                                     |
| HCC    | healthy concordant  |
| MZ     | monozygotic   |
| STOPPA | Swedish Twin study On Prediction and Prevention of Asthma |

**ABSTRACT**

Asthma is a common childhood disease and several risk factors have been identified, however the impact of genes and environment is not fully understood. The aim of the Swedish Twin study On Prediction and Prevention of Asthma (STOPPA) is to identify environmental (birth characteristics and early life) and genetic (including epigenetic) factors as determinants for asthmatic disease.

Based on the Child and Adolescent Twin Study in Sweden (parental interview at 9 or 12 years, N~23,900) and an asthma and/or wheezing algorithm, we identified a sample of monozygotic (MZ) and dizygotic (DZ) same-sexed twin pairs. The twin pairs were identified as asthma concordant (ACC), asthma discordant (ADC) and healthy concordant (HCC). A sample of 9- to 14-year-old twins and their parents were invited to participate in a clinical examination. Background characteristics were collected in questionnaires and obtained from the National Health Registers. A clinical examination was performed to test lung function and capacity (spirometry with reversibility test and exhaled nitric oxide) and collect blood (serology and DNA), urine (metabolites), feces (microbiota) and saliva (cortisol).

In total, 376 twin pairs (752 individual twins) completed the study, response rate 52%. All participating twins answered the questionnaire and >90% participated in lung function testing, blood and saliva sampling.

This article describes the design, recruitment, data collection, measures, background characteristics as well as ongoing and planned analyses in STOPPA. Potential gains of the study include the identification of biomarkers, the emergence of candidates for drug development and new leads for prevention of asthma and allergic disease.

## INTRODUCTION

Asthma is an inflammatory and obstructive disease of the airways, with increasing prevalence around the world (Asher, Montefort et al. 2006). Observed marked variation in prevalence between genetically similar populations strongly implies that a substantial proportion is attributable to environmental aspects (von Mutius, Martinez et al. 1994). Heritability estimates specific to populations and given environments have indicated genetic factors to be responsible for 60-80% of liability to asthma and respiratory measures, and the remaining 20-40% due to environmental influence (Lichtenstein and Svartengren 1997; Los, Postmus et al. 2001; Wu, Boezen et al. 2010). There has been an upsurge in observational birth cohort studies and randomised controlled trials such as BAMSE in Sweden (Wickman, Kull et al. 2002), the German Multicentre Allergy Study (Lau, Nickel et al. 2002), PIAMA Holland (Wijga, Smit et al. 2001), the Childhood Asthma Prevention Study in Sydney (Mihirshahi, Peat et al. 2001) and the Manchester cohort in UK (Custovic and Simpson 2004), along with large consortia to better understand the impact of environmental risk factors of asthma and allergic disease. Several risk factors have been identified, but there are still controversies over many exposures.

We have recently shown that there is an association between birth weight and subsequent asthma (Ortqvist, Lundholm et al. 2009) and eczema (Lundholm, Ortqvist et al. 2010) in twins. The observed association was independent of gestational age, familial environment and genetic factors, implying that altered foetal growth affects early metabolic, immunologic or physiologic mechanisms in utero, leading to subsequent disease. The foetal origins hypothesis (Barker 1995) states that foetal malnutrition in utero may result in disproportionate neonatal growth that may lead to catch-up growth, causing an increased risk of subsequent obesity (Stettler, Zemel et al. 2002). Both asthma and obesity seem to have their commencement in early childhood (Snethen, Hewitt et al. 2007; Drever, Saade et al. 2010). It is possible that

other unshared environmental exposures such as breast feeding and diet, physical activity and body mass index (BMI) may predispose individuals to both these conditions and may explain how they are associated. However, just as asthma, most exposures are influenced by genetic effects, and therefore genetic confounding cannot be excluded. This is one area where twin studies may provide an alternative and very powerful approach.

There is also accumulating epidemiologic (Braun-Fahrlander, Gassner et al. 1999; Ball, Castro-Rodriguez et al. 2000; Karmaus and Botezan 2002), clinical (Matricardi 2002), and experimental (Blaser, Chen et al. 2008) data supporting the “hygiene hypothesis” (Strachan 1989), and it would not be far-fetched to postulate that perturbations in the gastrointestinal microflora could disrupt the normal mechanisms of immunological tolerance in the mucosa (Noverr and Huffnagle 2005), leading to an increased risk of several childhood-onset allergic disorders (Sjogren, Jenmalm et al. 2009). The quantification of eicosanoid metabolites in human urine has been demonstrated to provide insight into the inflammatory and oxidative stress status of the individual, which might play a role in the pathobiology and severity of asthma (Balgoma, Larsson et al. 2013). There is also an association between early measures of stress and asthma (Fang, Hoglund et al. 2011) and although findings are inconsistent (Vink, Boezen et al. 2013), measures of stress in saliva cortisol may affect allergic symptoms (Stenius, Borres et al. 2011). Thus, if the effects are causal it is expected that the associations between objective measures such as spirometry, serology, gut microflora, urinary metabolites as well as saliva cortisol and parent-reported asthma remain also within twin pairs.

Twin siblings share genes (half if DZ and all if MZ), some intrauterine exposures, maternal factors and early environment. These renowned differences in genetic similarity, together with the assumption that twins within a pair are exposed equally to environments, irrespective of zygosity, create the foundation for the twin design, exploring the effects of genetic and environmental variance on a phenotype. Differences between MZ twins may either be an

effect of purely environmental factors or genetic variation that arises after fragmentation of the fertilized embryo, and MZ twin pairs discordant for asthma are especially powerful to study the effect of early environment such as breast feeding and diet, BMI, physical activity and personality on disease development. It has been demonstrated that genetic variation can occur after birth, either through somatic mutations or epigenetic changes (Wong, Caspi et al. 2010; Martino, Loke et al. 2013). It has also been hypothesised that epigenetics may be a molecular link between environmental factors and phenotypic changes (Lund, Kongerud et al. 2007; Bruder, Piotrowski et al. 2008; Prescott and Clifton 2009; Zhang, Li et al. 2012). A few epigenetic mechanisms have been proposed to be involved in the development of asthma (Hollingsworth, Maruoka et al. 2008; Liu, Ballaney et al. 2008). However, more direct mechanistic links to asthma pathogenesis needs to be determined.

The overarching purpose of this research program is to identify environmental (birth characteristics and early life), genetic and epigenetic factors as determinants for asthmatic disease. We will use the powerful approach of questionnaires and objective measures based on clinical examinations in discordant and concordant MZ and DZ twins.

## **MATERIAL and METHODS**

### *Study design and population*

In 2004, the Swedish Twin Registry initiated the Child and Adolescent Twin Study in Sweden (CATSS) including all twins born from July 1992 onwards (N~23,900 twins in March 2014) whose parents answered a telephone interview on birth characteristics, zygosity, parental background factors as well as outcomes such as asthma and wheeze at 9 or 12 years of age (Lichtenstein, Sullivan et al. 2006; Anckarsater, Lundstrom et al. 2011). The questions on zygosity have been validated with DNA markers in previous studies and proved correct in 95% (Lichtenstein, De Faire et al. 2002).

Based on the CATSS interview ascertainment, the Swedish Twin study On Prediction and Prevention of Asthma (STOPPA) was initiated in May 2011. Validated questions on asthma ever (yes/no) and wheezing (current or after three years of age/no) from the International Study of Asthma and Allergies in Childhood, ISAAC (Asher, Keil et al. 1995) were used to create an algorithm to identify twins discordant and concordant for asthma, *Figure 1*. Three groups of monozygotic (MZ) and dizygotic (DZ) same-sexed twin pairs were identified; 1) both twins with asthma or wheezing (asthma concordant - ACC), 2) one twin with asthma or wheezing and the other without (asthma discordant - ADC), and 3) none of the twins with asthma or wheezing (healthy concordant - HCC).

### **Figure 1 about here**

From each group (ACC, ADC and HCC), 9-14 year old twin pairs and their parents were invited to participate in a clinical examination including questionnaires and objective measures. Twins already recruited to other CATSS substudies were excluded. Eligible twins were invited in rounds related to examination site and date, age groups and the purpose to obtain equal sizes of the sub-groups. The families were contacted through an invitation letter,

followed by a telephone call to inform about the study. A clinical examination including questionnaires and objective measures were performed at Karolinska Institutet (Stockholm), Queen Silvia Children's Hospital (Göteborg), Linköping's University hospital, Centralsjukhuset in Karlstad, Norrland's University hospital (Umeå), Lund Children's Hospital, Lomma Primary Health Care Centre and Centrallasarettet in Växjö. The protocol between the pilot and the full scale study were identical apart from minor revisions of the questionnaire and collection of urinary samples during the last year of the study. After a successful pilot study including 15 MZ twin pairs in Stockholm and Uppsala, the full scale study started in November 2011 and was finalised in June 2014.

### *Questionnaires*

At the test centre, the parents were invited to answer a questionnaire divided into eight main themes; parental background, lifestyle and medical history along with questions on each twin's life style, general health status, medical history, puberty and home environment. The questionnaire was designed to collect information on factors that may be associated with childhood asthma, allergies and comorbidities with previously validated questions (Asher, Keil et al. 1995; Wickman, Kull et al. 2002; Danell, Bergstrom et al. 2013; Ekstrom, Magnusson et al. 2014). Questions on twin's medical history included respiratory diagnoses in infancy, wheezing and asthma including doctor's diagnosis, age at onset and frequency (Asher, Keil et al. 1995; Wickman, Kull et al. 2002; Danell, Bergstrom et al. 2013; Ekstrom, Magnusson et al. 2014). For life style, general health and home environment, established questionnaires were used (Almqvist, Adami et al. 2011; Anckarsater, Lundstrom et al. 2011). Each twin was invited to answer a questionnaire, which was divided into four themes; life style, medical history, puberty and stress. Questions on puberty were those in the Pubertal Development Scale developed and validated by Petersen et al (Petersen, Crockett et al. 1988). The Perceived Stress Scale was used for the pilot (Cohen, Postma et al. 2011) and replaced by

the 21 item questionnaire “Stress in Children” (SiC) which has been validated against Beck Youth Inventory scales and objective measures of stress (Osika, Friberg et al. 2007) in the full scale study.

The Asthma Control Test (ACT), a trademark of QualityMetric Incorporated including five items on symptoms and medication during the last four weeks, was used for twins >12 years of age and the Childhood Asthma Control Test (C-ACT) answered by twins <12 years (four items) and parents (three items) with permission from GlaxoSmithKline (Nathan, Sorkness et al. 2004; Liu, Zeiger et al. 2010).

The parental and twin questionnaires were slightly revised between the pilot phase and full scale study and a few questions on early life respiratory diagnoses and exposure to antibiotics, physical activity, pet exposure and stress were added or modified. A research nurse contacted and completed missing information for the majority of pilot participants in retrospect.

#### *Clinical examination*

Spirometry. Lung function tests to measure how an individual inhales or exhales volumes (litres) of air as a function of time (seconds), was carried out with SpiroStar (Pro version, Medikro, Kuopio, Finland) in the majority of study participants. Approximately 100 twins were tested using the Jaeger MasterScreen-IOS system (Carefusion Technologies, San Diego, California) at one of the test centers in Stockholm. The procedure was performed in accordance with American Thoracic Society/European Respiratory Society recommendations (Miller, Hankinson et al. 2005). Both systems were calibrated using a 3L syringe. A reversibility test was included when the procedure was repeated at least three times before and 15 min after inhalation of terbutalin 0.5 mg.

Fractional exhaled NO (FeNO). Levels of FeNO, a non-invasive marker of airway inflammation (Dweik, Boggs et al. 2011) were measured with a hand-held electrochemical

analyzer (NIOX Mino, Aerocrine, Solna, Sweden) with an expiration time of 10 seconds in the majority of study participants. Approximately 100 twins were tested with the CLD 88 FeNO test analyser (Ecomedics, Duernten, Switzerland) at one of the test centers in Stockholm. Each subject performed the test at least twice; if there was >5% difference between the first two measurements a third attempt was performed.

### *Biosamples*

Blood. At the test centers EDTA whole blood for analyses of DNA (4 ml), Complete Blood Count (CBC; 4ml) and plasma aliquots (from EDTA 10 ml) were collected, along with tubes of lithium heparin with gel for analyses of proteins and PAXgene Blood RNA Tube (PreAnalytiX GmbH, Hombrechtikon, Switzerland) for analyses of RNA.

Gut microflora. To investigate differences in the twin's gut microflora, fecal samples were taken at home and brought to the test center. The fecal samples were collected in a container with screw cap and spoon (length 101 mm/ ø 16.5 mm), and the twins were asked to fill at least 2/3 of the container. The samples were frozen at home in -18°C in a pre-frozen cool transport container. The container with the samples was transported to the test center in Styrofoam casing to ensure that the samples remained frozen.

Saliva. Samples of morning- and night saliva for analysis of cortisol levels, a marker of stress, were collected at home in a commercial Salivette tube containing a cotton wool swab. The swab was rotated in the mouth for at least 1 min and then inserted back into the tube and kept in +5°C before transportation to the test center. At the test center the cortisol samples were centrifuged and thereafter primarily stored at -20°C.

Urinary metabolites. Urinary samples were collected at two time points at the test center; on arrival and before leaving the center. The subjects were asked to pass all urine directly into a

measuring container and the total volume at each collection was noted. The urine was thereafter transferred to five 8 mL test tubes with caps before primary storage at -20°C.

All test tubes and material used to collect fecal samples, saliva and urinary samples were provided from SARSTEDT AG & Co, Nümbrecht, Germany.

At the end of each data collection, all samples, except those that were analysed directly at the test site for the full scale study (CBC), were transported to and stored at Karolinska Institutet's Biobank at -80°C, until analysed for its specific cause.

### *National registers*

The universal use of the Personal Identity Number, a unique identifier for each resident in Sweden, enables unambiguous linkage between the registers held by the National Board of Health and Welfare and the Swedish Twin Registry. Information on pre- and perinatal factors such as birth weight, birth length, gestational age, head circumference and mode of delivery from the Medical Birth Register can be linked to each twin. Information on the twins' asthma medications from the Swedish Prescribed Drug Register and diagnosis in the National Patient Register provides additional information on asthma status. We have recently shown that asthma medication is a suitable proxy for asthmatic disease in children in this age group (Ortqvist, Lundholm et al. 2013).

### *Child- and school health records*

To investigate how growth over time affects the risk of subsequent asthma, consent to collect the twins' child- and school health records was collected.

### *Statistical analyses*

For future studies and dependent on the specific research question, the collected data from questionnaires, objective measures and samples will be analysed with twin design such as structural equation models (SEM) or through co-twin control analyses where discordant twin

pairs are thought of as matched case-control pairs. Twin pairs who are discordant for the outcome will be analysed with the non-affected co-twin as a matched control to the affected twin with the exposure being the individual background characteristic. The outcome-concordant twins and healthy concordant twins will be used as control groups. Analyses will be weighted, in order to account for the difference in sampling fractions in the six sampling groups (MZ ACC, MZ ADC, MZ HCC, DZ ACC, DZ ADC and DZ HCC).

Descriptive statistics on numbers of examined twins, collected information from questionnaire and clinical measures presented here was calculated by Stata statistical software, version 13.

The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional committees on human experimentation and with the Helsinki Declaration of 1975, as revised in 2008. The study was approved by the regional ethical review board in Stockholm, Sweden. Consent

## RESULTS

In total, 6174 twins were eligible, 1448 contacted, 870 accepted participation and 752 came to the clinical examination, response rate 52%, *Figure 2*. During recruitment, we invited twin pairs to obtain six groups of fairly similar size and our aim was to recruit as many ADC as possible. Among those eligible, the proportion of HCC twin pairs (65%) was higher than ADC (22%) and ACC (13%), and the proportion of MZ twin pairs was lower in the ADC (27%) than the ACC group (55%). In the final study population 38% of the twin pairs were ADC (49% MZ), 31% ACC (61% MZ) and 31% HCC (55% MZ). Altogether, 410 twins (205 twin pairs) were MZ (54%) and 342 (171 pairs) DZ.

### **Figure 2 about here**

The proportion of males (53%) was slightly higher than females in the final study population, the majority of twins were born 1999-2000 (46%) or 2001-2002 (34%) and had their clinical examination in Stockholm (60%), Göteborg (19%) or Lund/Lomma (8%), *Table 1*. The distribution of males and females, year of birth and site for clinical examination for the separate MZ and DZ subgroups was similar to the total study population, except that the proportion of males was slightly lower in MZ ADC (47%), MZ HCC (45%) and DZ HCC (44%).

### **Table 1 about here**

In the final sample, all twins and their parents answered the questionnaires, 100% of the twins participated in measures of height and weight and the majority of twins took part in lung function measures (spirometry 98%, FeNO 94%), blood sampling (>90%) and saliva (97%), *Figure 3*. Fecal samples were collected in 47% and urinary samples in 19%.

### **Figure 3 about here**

## DISCUSSION

This is the first twin study on asthma concordant, discordant and healthy concordant twin children examined with questionnaires and a thorough clinical examination. The study design has a powerful approach with invitation of a large sample of 9-14 year old Swedish MZ and DZ twins based on the total population. There are a few previous similar studies on adult populations and of smaller size (Lund, Kongerud et al. 2007; Wu, Boezen et al. 2010). This article describes the design, recruitment, data collection, measures, background characteristics as well as ongoing and planned analyses in STOPPA.

We collected questionnaires and objective data from lung functions tests and biological samples. Well validated ISAAC questions on asthma and allergy including queries on current asthma were incorporated into the questionnaires to update information from the CATSS questionnaire which defined the asthma dis- and concordant groups ACC, ADC and HCC. We also obtained information on asthma severity from the ACT survey, and additional phenotypic information will be obtained from lung functions tests and exhaled NO. In addition, we will have the possibility to link data to the national health registers (diagnoses and prescribed drugs) for further phenotypic information.

Questionnaires were completed by both parents and twins at the clinical test center, which ensured a high response rate. The majority of children also participated in lung function tests and consented to biosamples being taken. We had the possibility to travel to several sites and thus ensure that twins throughout Sweden were examined in a standardized way using mostly identical equipment and a tight team of study nurses. There are also some inherent limitations in the study. Twins were examined throughout the year including during pollen seasons and autumn with higher risk of upper respiratory tract infections, which may have affected some of their responses. Also, although we aimed to invite all ADC and ACC twins, we had to

perform examinations related to site, date and age group and thus made a great effort to obtain equal sizes of the sub-groups and a response rate of >50%.

MZ twins discordant for asthma have interested clinicians and researchers over the years. Following developments in biotechnology, with the new possibilities to perform systematic large-scale investigations of DNA sequencing, DNA modifications, RNA, metabolites, and proteins levels, the interest in using twins for molecular epidemiology has increased, the rationale being that identified molecular differences may reflect causal mechanisms involving environmental exposures and/or constitute a direct cause of the phenotypic difference. DZ twins also give additional information. Mutations occurring after the split of the initial intact embryo and epigenetic (DNA methylation, histone acetylation) differences have been put forward as promising candidate mechanisms.

This article describes the design, recruitment, data collection, measures, background characteristics as well as ongoing and planned analyses in STOPPA. We will make comparisons on the effect of environmental factors on outcomes across discordant and concordant twin pairs and initiate novel analyses on genome-wide methylation in extracted DNA. Potential gains of the study include the identification of biomarkers, the emergence of candidates for drug development, translational modeling, and new leads for prevention of asthma and allergic disease.

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Figure 1

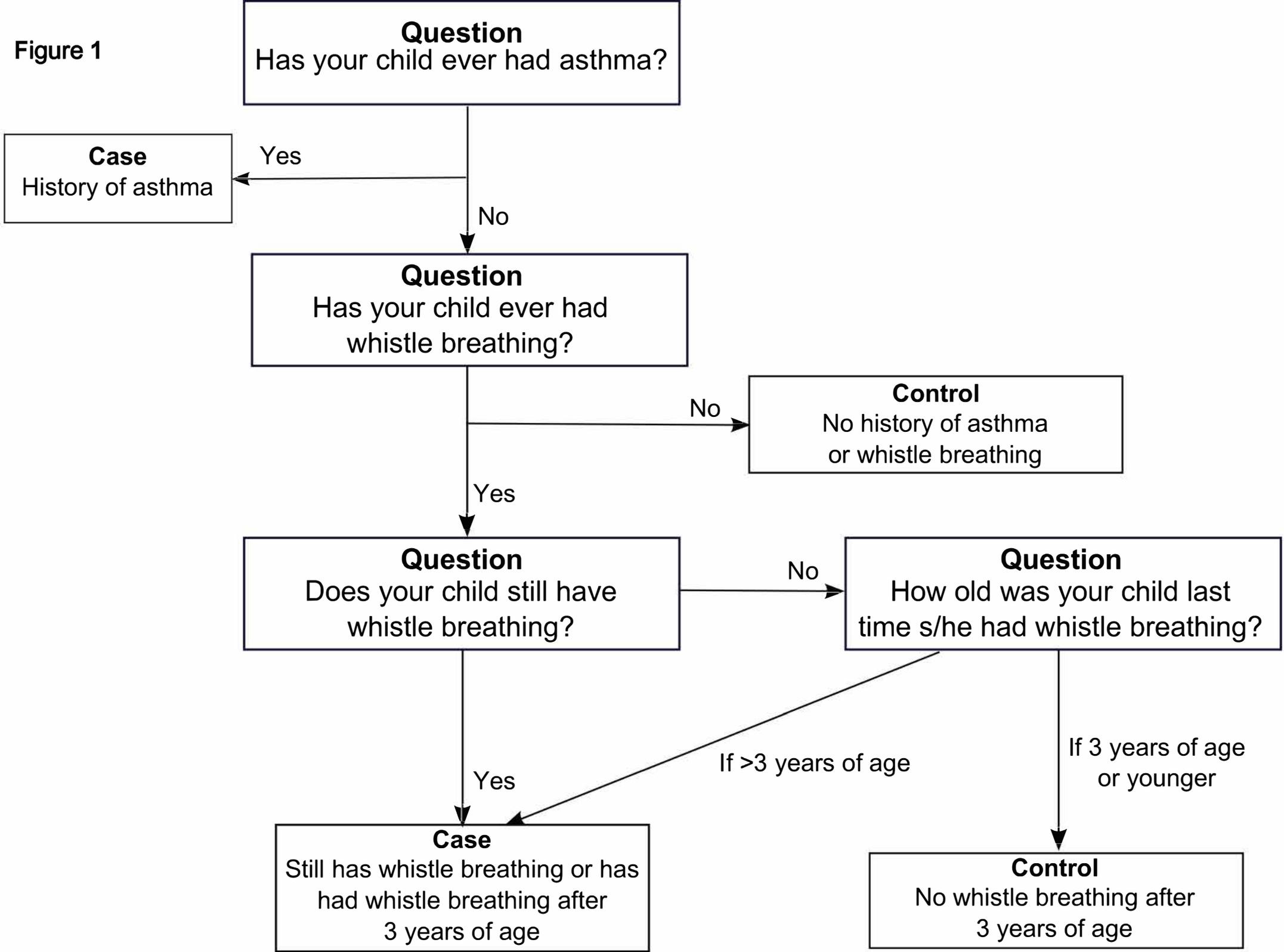


Figure 2

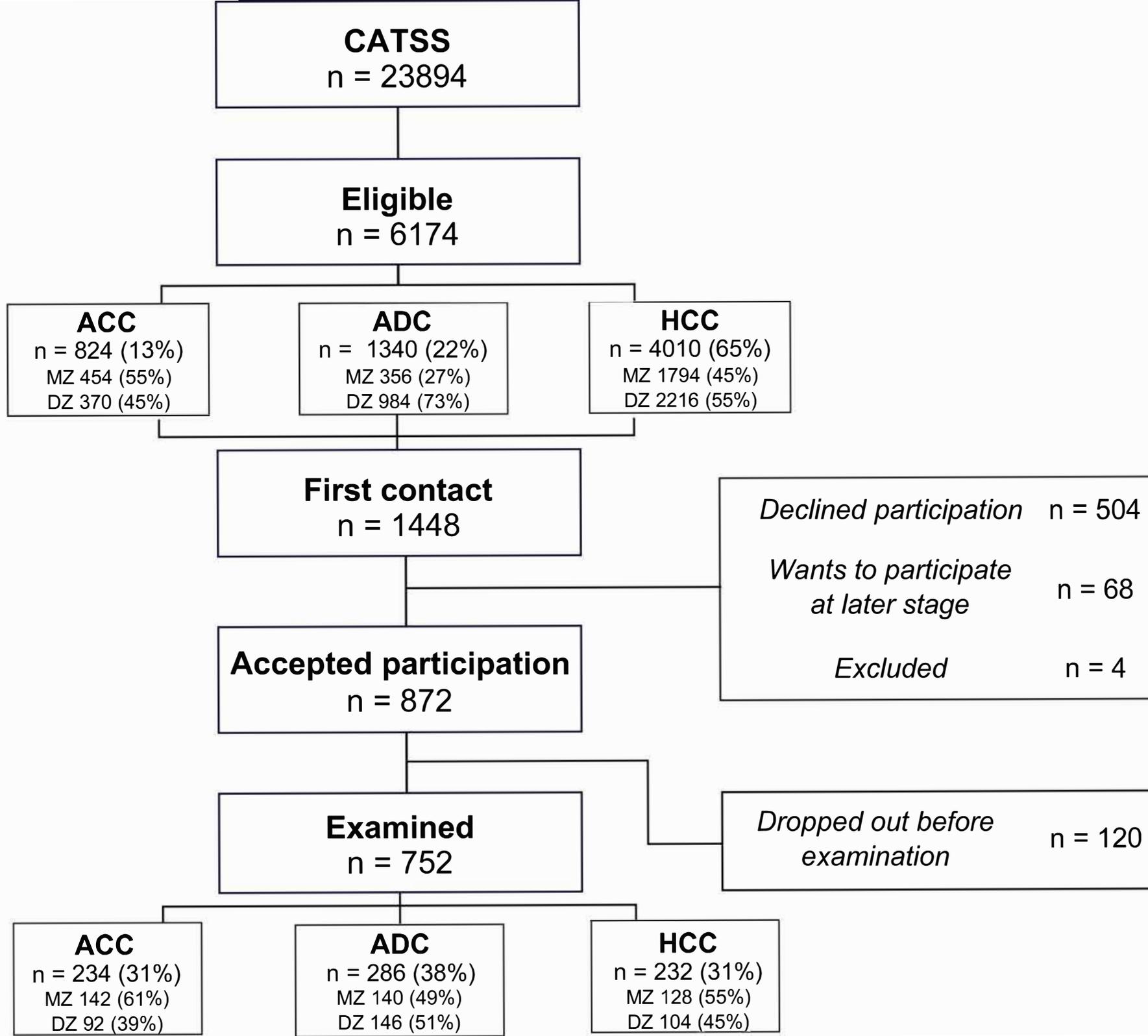


Figure 3

