



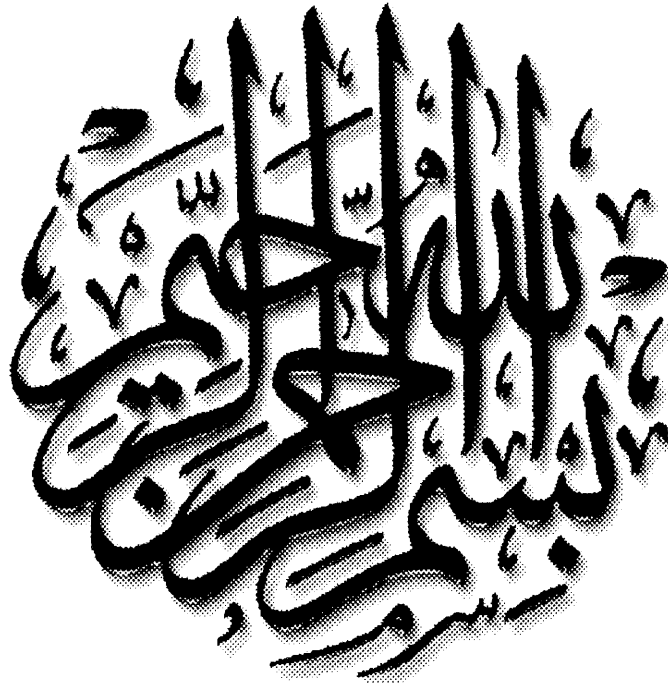
**INHALED NITRIC OXIDE FOR ACUTE CHEST SYNDROME IN PEOPLE
WITH SICKLE CELL DISEASE AND FUTURE GENETIC IMPLICATIONS:
A SYSTEMATIC REVIEW OF RANDOMISED CONTROLLED TRIALS AND
A PROPOSAL FOR A POTENTIAL GENETIC THERAPY**

BY

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A report submitted in partial fulfilment of the requirements
for the degree of Master of Medical Genetics University of Glasgow

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August 2007



In the name of Allah, Most Gracious, Most Merciful

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Abstract

Objective

To perform a systematic review of the literature on the effectiveness of inhaled nitric oxide in the treatment of acute chest syndrome in patients with sickle cell disease and to explore the genetic implications of this review.

Methods

Electronic databases were searched from their initiation to 2007. Additional searches were carried out of abstracts of conference proceedings. These comprehensive searches yielded 9 citations all of them are randomized controlled trials. None of the trials examined the use of inhaled nitric oxide in acute chest syndrome.

Results

No relevant trials matching the inclusion criteria of this systematic review were identified. This reflects the lack of clinical trials on patients with acute chest syndrome despite the high prevalence of the disease. A descriptive summary was presented and a new therapeutic modality in the form gene therapy is proposed.

Conclusion

Although no trials were identified to answer the research question in this review, there is evidence that inhaled nitric oxide may be effective in the treatment of acute chest syndrome, but to support this, well-designed randomised controlled trials are needed.

Nitric oxide synthase gene transfer may hold promise as a future therapeutic strategy for acute chest syndrome.

Acknowledgements

I would like to thank Dr. Douglas Wilcox who has great faith in me, Dr Shaikha Al Arrayed for facilitating my study at the University of Glasgow and Dr Maria Jackson for her encouragement.

Special thanks to Dr Zbys Fedorowicz for supporting and guiding me and to my colleagues in the Cochrane Collaboration.

I am most indebted to my fiancé Mr. Nasser Saleh for his patience and encouragement throughout the course of my MSc and my mother “Shaikha Abdulla” without whose support and prayers I could not have accomplished this project.

Nitric oxide is a vasodilator which has as its main mechanism of action the stimulation of soluble guanylate cyclase and the production of cyclic guanosine monophosphate. It also inhibits platelet and leukocyte activation and adhesion and their production of active intermediates. It has also been demonstrated that the local effects of inhaled NO (inhNO) on pulmonary hypertension and consequently right ventricular dysfunction, oxygenation, inflammation, or pulmonary oedema and Acute Respiratory Distress Syndrome (ARDS) may support its use in (ACS) in sickle cell disease. All of these effects are likely to lead to a decrease in ACS mortality (Troncy *et al.* 1998).

There has been extensive research on the pathophysiology of (ACS) by Rossaint *et al.* (1993), and on the use of NO in ARDS by Gerlach *et al.* (1993), but to the best of my knowledge this is the first systematic review of the use of inhaled NO in patients with sickle cell disease who are suffering from (ACS).

Systematic reviews provide the best clinical evidence in medical research by applying scientific strategies to the critical appraisal of relevant trials. Therefore, a systematic review has been conducted to explore possible future genetic therapeutics.

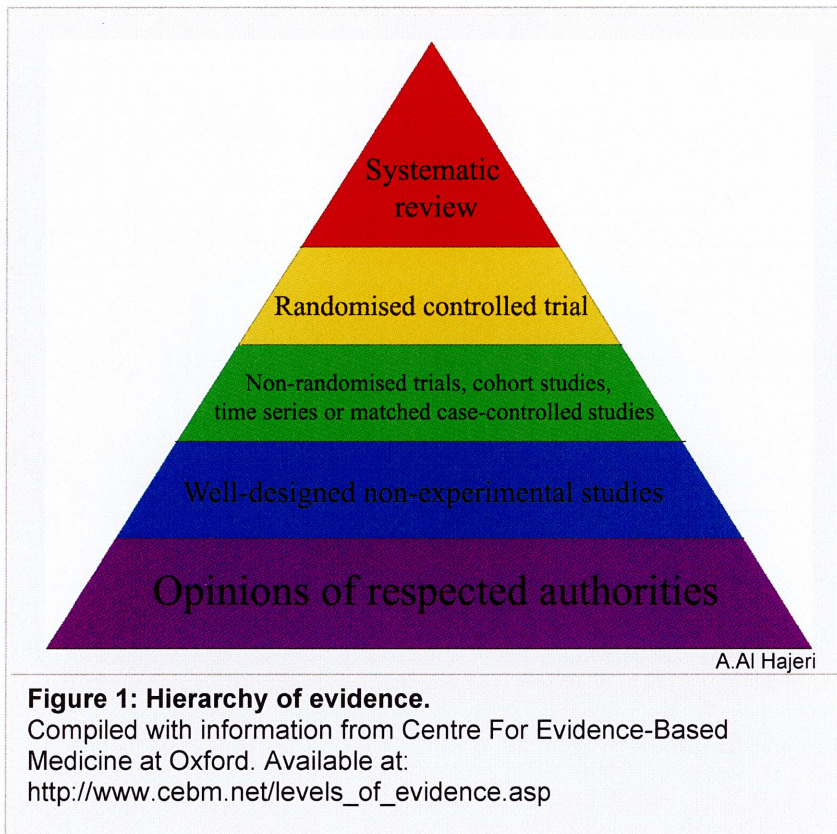
I chose this topic because of several factors. Sickle cell disease is a common hereditary blood disorder in my country and many other countries (Al-Arrayed and Al-Hajeri 2006). It has a considerable social and economic burden on both the individual and at national level. When I was working as a resident in the Internal Medicine department in the ministry of health (Bahrain) I came across numerous cases of young patients with sickle cell disease presenting with acute chest syndrome. I found this to be one of the most fearful complications of SCD. Unfortunately many of those patients did not survive, and it was this combination of factors that drew my attention to this topic.

Rationale for a systematic review

In medical research, systematic reviews of randomized controlled trials occupy the highest level in the hierarchy of evidence (Ebell *et al.* 2004) [Figure 1]. Systematic reviews involve the critical assessment of existing evidence by answering specific clinical questions which are formulated explicitly according to four variables; population, intervention, comparison and the potential outcome (Cook *et al.* 1997). They include a comprehensive literature search, critical appraisal of the quality of the included studies and use specific statistical techniques to combine the valid studies (Ebell *et al.* 2004).

Systematic reviews can also be an excellent research tool in medical education because each step involves many learned lessons (Lang 2004).

With my background as a scientist and a clinician I chose to cover both the clinical and the molecular aspects of medical genetics in the form of applied health research which has direct practical implications.



Consultation with clinicians:

I recently had the opportunity to meet Prof. Graham Serjeant and I asked him about the most recent trends in managing ACS and was told that the use of inhaled nitric oxide to treat ACS is a hot topic at the moment. Dr. Mark Gladwin who has a special interest in the pathophysiology of ACS and Nitric Oxide was also contacted during the course of the review.

What makes this dissertation a unique piece of work

As far as I am aware, this is the first systematic review of the use of inhaled nitric oxide to treat patients with ACS, and the first systematic review submitted as a dissertation for this MSc course.

In addition to the clinical aspects of this research which may ultimately benefit a lot of patients, clinicians and health decision makers, I discuss the scientific-genetic aspects of this review in which I propose a unique approach which has never been sought as a possible therapeutic strategy in the form of gene therapy.

3.1 Evidence Based Medicine: a new era

In 1972 Professor Archie Cochrane a Scottish epidemiologist suggested that randomised controlled trials should be used to test health interventions in his book "*Effectiveness and Efficiency: Random Reflections on Health Services*". His work predated the concept of evidence based medicine (EBM) which has shown increasing acceptance worldwide.

A group of enthusiastic epidemiologists at McMaster University in Canada developed this concept. This group, the Evidence Based Medicine Working Group (EBMWG), lead by David Sackett had recognized the limitations of relying solely on medical expertise in clinical knowledge (EBMWG 1992). They found that many review articles and journal editorials had no experimental basis and were biased and fallacious (Oxman and Guyatt 1988). EBM has been defined by the EBMWG as, "*the conscientious, explicit and judicious use of current best evidence in making decisions about the care of individual patients*" (Sackett 1996).

Sackett (2000) cited four main reasons for the sudden interest in EBM:

- the need for readily accessible and valid information about diagnosis, prognosis, therapy and prevention.
- a better understanding of the limitations of traditional sources for this information.
- a recognition of the discrepancy between diagnostic skills and clinical judgment.

- an awareness of the time constraints faced by clinicians to access this information.

Advances in technology have helped in the rapid spread and ease of use of search engines, not only to find the relevant studies, but also to help with the appraisal of evidence of effectiveness of healthcare interventions. High quality systematic reviews of the effects of healthcare interventions are considered as new strategies which have given clinicians improved opportunities to ensure they are up to date with the best evidence on healthcare interventions.

3.2 Evidence based Genetics

As with other medical specialties the clinical geneticist is faced with pressures for change to his clinical practice, and is increasingly required to apply the concept of EBM. The growing number of randomized controlled trials and systematic reviews in medical genetics has significantly increased the awareness among clinicians and policy makers worldwide. Such studies have directly contributed to the development of clinical guidelines, screening programs for genetic diseases and helped in establishing integrated care pathways and subsequently lead to changes in clinical practice (Campbell 2000).

3.3 The Hierarchy of Evidence (HOE)

The 'hierarchy of evidence' (Guyatt *et al.* 1995) provides ranking of varying levels of evidence in clearly defined terms related to the reliability of relevant studies. It initially focused on therapeutic and preventative

interventions but is increasingly being used in diagnosis, prognosis and harms. Modifications to the HOE are being undertaken at the Centre for Evidence-Based Medicine at Oxford (CEBM) [Figure 1].

This hierarchy emphasizes that not all studies provide equal levels of evidence. There is a wide agreement that the highest levels of evidence in this hierarchy are represented by systematic reviews and randomized controlled trials (Chalmers 1989).

Despite its wide acceptance this hierarchy depends on the methodological quality of the research which ultimately needs to be assessed through the technique of critical appraisal.

3.4 Randomised controlled trial (RCT)

An RCT is defined as “*a study in which people are allocated at random to receive one of several interventions*” (Jadad, 2000, p1).

Randomised controlled trials are considered the ‘gold standard’ for comparing and evaluating different treatments. In randomised controlled trials all participants receive an equal chance of being allocated to any one of the treatment modalities being investigated. This is done by a clearly defined process of randomisation such as using computer generated or table of random numbers. Therefore RCTs have gained wide acceptance because of the precise way of quantifying the level of effect of specific healthcare interventions. Nevertheless, it is also crucial to evaluate RCT’s for the quality and completeness in reporting of their results which may significantly influence any subsequent data synthesis (Altman 1996).

3.5 *Systematic reviews the 'platinum standard'*

"A systematic review is a summary of the medical literature that uses explicit methods to systematically search, critically appraise, and synthesize the data on a specific topic" (Mulrow 1987). Its aim is to minimize systematic bias and to provide a valid summary of the best available evidence for a specific clinical problem.

Systematic reviews are considered the platinum standard (Tugwell 2004). Clarke (2004) suggests that no new piece of research should be done without first conducting a systematic review to find out what is already known. Other commonly used terms are 'new prospective study' and 'research synthesis' (Chalmers, Hedges and Cooper, 2002).

3.6 *Benefits of systematic reviews,*

Benefits of systematic reviews are summarized in table 1.

Table 1: Benefits of systematic reviews
Systematic reviews can help overcome bias
Systematic reviews may increase precision
Systematic reviews are transparent and reproducible

3.7 *Systematic Reviews in Genetics*

The increasing demand for high level evidence of effectiveness of genetic healthcare interventions whether in the form of screening programs or certain therapeutic intervention has lead to a noticeable increase in the

published systematic reviews. These important publications are important to the NHS and other bodies responsible for healthcare decision making.

The Cochrane Cystic Fibrosis and Genetic Disorders Group (CFGD) is one of the most active Cochrane groups. In the Cochrane library there are 63 completed and updated systematic reviews covering a wide range of genetic disorders such as cystic fibrosis, sickle cell disease, phenylketonuria, X-linked hypophosphatemia, haemophilia, thalassemia and familial hypercholesterolemia. The scope of these reviews covers screening, prevention and treatment. (Issue 2 of *The Cochrane Library*, July 2007).

4.1 Aim of the literature review

The aim of this literature review was to provide background information to the research question. My background questions include the following:

What are the aetiology, epidemiology, clinical features and treatment of SCD?

What is ACS?

What are the available therapies for ACS?

What is the role of NO in the pathogenesis of ACS?

What is the role of inhaled NO in the treatment of ACS?

4.2 Sources of background information: clinical reference books:

The primary sources that were used to generate background information in this systematic review were, one standard clinical reference book on SCD and a collection of recent relevant articles.

Clinical Reference book:

Serjeant G, Serjeant B (2001) Sickle Cell Disease. 3rd edition. New York: Oxford.

4.3 Aetiology, epidemiology, clinical features and treatment of SCD

Sickle cell disease is a spectrum of autosomal recessive disorders characterized by the presence of HbS variant of the β -globin gene. Affected individuals have two copies of this variant (*Hb SS*). Individuals affected with other types of SCD are compound heterozygotes where they have a copy of HbS and another copy of a different variant such as Hb β -thalassemia and

HbC. HbS results from a point mutation in the sixth codon of the β -globin gene which leads to the substitution of valine in place of glutamate. This single aminoacid change causes abnormal haemoglobin which distorts red blood cells leading to premature destruction and blockage of small blood vessels.

Sickle cell disease affects millions of people throughout the world. It is common among people from Sub-Saharan Africa, South America, Cuba, Central America, the Arabian-Peninsula, India, and Mediterranean countries such as Turkey, Greece, and Italy (WHO 2005).

It is characterized by recurrent painful attacks, recurrent infections and anaemia. The severity of symptoms range from severe life threatening complications such as stroke and ACS to mild painful attacks.

The disease is treated conservatively with adequate hydration, analgesics, folic acid supplements, occasional blood transfusion and prophylactic antibiotics. Some patients might benefit from hydroxyurea which elevates fetal haemoglobin (Charache *et al.* 1995). Hematopoietic cell transplantation can cure SCD, but it has many serious complications (Vermylen *et al.* 1998).

4.4 Acute chest syndrome: definition and current treatment.

Acute chest syndrome (ACS) is the second most common cause of admission and contributes to 25% of deaths among patients with SCD (Vichinsky *et al.* 2000). ACS is defined as an acute pulmonary condition initiated by infectious and noninfectious events which are exacerbated by the underlying sickling. It is diagnosed when a new infiltrate is seen on chest

radiography and is accompanied by acute respiratory symptoms (Walters *et al.* 2002).

The incidence of ACS is high, about 30% of SCD patients have had at least one attack of ACS (Castro *et al.* 1994) and is often underestimated because relying solely on clinical judgment can be misleading (Walters *et al.* 2002).

In 20% of the cases ACS is caused by pulmonary infarction. Blockage of the blood vessels in the pulmonary microcirculation results in tissue hypoxia leading to infarction. Tissue death activates the inflammatory response which in turn exacerbates lung injury (Stuart 1999) and this is further complicated by vascular constriction.

Nitric oxide is a soluble gas which is produced by NO synthase in the endothelium which then diffuses into the adjacent smooth muscle where it leads ultimately to the activation of cGMP-dependent protein kinases, which sequester calcium and produce vasodilation (Gladwin and Kato 2005). It also plays a key role in vascular homeostasis [Table 2]. In addition to the effects listed in table 2, NO decreases intrapulmonary shunting and dilates the bronchi making it of additional benefit to patients with ACS (Walters *et al.* 2002).

Table 2: Vascular effects of nitric oxide.
(From Gladwin and Kato 2005 with modification).

Vasodilatory	Relaxation of vascular smooth muscle
	Decreased expression of endothelin-1 and endothelin receptor
Anti-adhesive	Decreased endothelial expression of adhesion molecules
Anti-thrombotic	Decreased platelet activation
	Decreased tissue factor activity
	Decreased thrombin generation
Anti-oxidant	Inactivation of reactive oxygen species

Sickle cells and hypoxia inhibit NO production by decreasing NO synthase in the endothelium (Hammerman 1999). Atz and Wessel (1997) reported that the use of inhNO was beneficial in patients with SCD.

Trials on neonates and adults with pulmonary hypertension showed that inhaled NO (inhNO) caused vasodilatation and improved oxygenation (Walters *et al.* 2002) and in this way has shown a promise as a therapeutic intervention in ACS.

5.1 Criteria for considering studies for this review*Types of studies*

Effectiveness of healthcare interventions is best assessed by randomised controlled trials. Therefore, only randomized controlled trials (RCTs) were considered in this review. Random allocation of participants with similar characteristics helps minimize the effect of bias. The methods used to ensure random allocation are described in the section 5.4 *Assessment of methodological quality*.

Types of participants

Only studies which had recruited patients with sickle cell disease SS, SC, S β O, S β + (confirmed by electrophoresis and sickle solubility test, with family studies or DNA tests as appropriate) of all ages and both sexes, in any setting.

The definition of ACS used was according to the clinical signs, symptoms and criteria described by Vichinsky as, "a new infiltrate visible on chest radiograph associated with one or more symptoms, such as fever, cough, sputum production, tachypnea (abnormally fast breathing), dyspnea (shortness of breath), or new-onset hypoxia" (Vichinsky 2000).

Types of intervention and control

The intervention and control of interest for this systematic review were inhaled nitric oxide compared to placebo or standard care for any single or multiple treatment periods and over any time period.

Types of outcome measures

The types of outcome measures are summarized in table 3.

Table 3: types of outcome measures.	
Primary	(1) Chest pain (a) Intensity (expressed as scores obtained through any validated patient reported outcomes instrument either generic or sickle cell disease specific) (b) Duration
	(2) Fever
	(3) Laboratory Investigations (a) Arterial blood gases (b) Pulse oximetry
Secondary	(1) Duration of any assisted ventilation
	(2) Duration of hospitalisation in the intensive care unit (ICU): the number of inpatient days
	(3) Quality of life (e.g. absence from school, lost time at work, mobility) as assessed by any validated questionnaire either generic or SCD specific
	(4) Participant satisfaction with the intervention assessed by any appropriate and validated questionnaire (either generic or SCD specific)

Adverse effects

Reports on any specific adverse effects, systemic or local, toxicity, any clinically diagnosed hypersensitivity or other unacceptable or adverse events associated with this treatment were to be recorded.

5.2 Search Strategy for identification of studies

Generating a search strategy

It is very important that the search strategy is sensitive (able to identify relevant articles) and specific (able to exclude irrelevant articles).

The key components in developing a search strategy are illustrated in figure2:

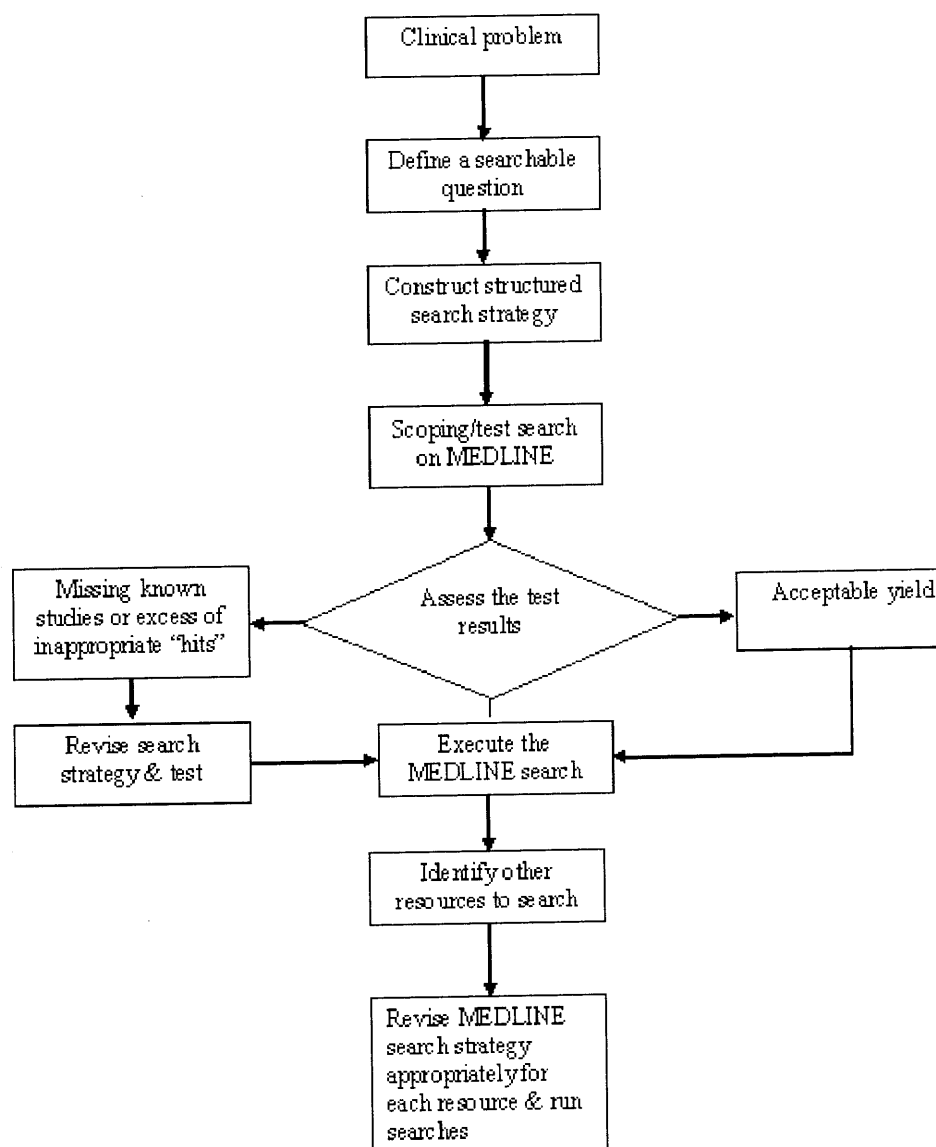


Figure 2: Search strategy flow chart.

(Personal communication with Sylvia Bickley, Cochrane trial search coordinator.
<http://www.ohg.cochrane.org/>)

Databases searched

I sought to identify relevant trials from the Cystic Fibrosis and Genetic Disorders Group's Haemoglobinopathies Trials Register using the terms: sickle cell AND acute chest syndrome.

The Haemoglobinopathies Trials Register is compiled from electronic searches of the Cochrane Central Register of Controlled Trials (Clinical Trials)

(updated each new issue of The Cochrane Library) and quarterly searches of MEDLINE. Unpublished work is identified by searching the abstract books of five major conferences: the European Haematology Association conference; the American Society of Haematology conference; the British Society for Haematology Annual Scientific Meeting; the Caribbean Health Research Council Meetings; and the National Sickle Cell Disease Program Annual Meeting.

The search strategy for systematic review must be documented so that it can be reproduced by any researcher. Therefore, it is important to include the main sources that were used, the search strategy for each database, the date of the search and any language or other constraints.

Medical databases such as MEDLINE are considered to be useful in developing the search strategy because they use thesaurus-derived indexing terms plus a structured vocabulary known as MeSH (Medical Subject Headings) which facilitate the identification of appropriate keywords. See **Appendix A** for search strategy.

The search strategy was developed in conjunction with trial search coordinator at the CFGD. It was conducted on the Cochrane Library Issue 4 October 2006 and updated in May 2007, the results of the records retrieved are presented in **Appendix A**.

5.3 *Finding the trials*

The search strategy identified 9 references. All titles and abstracts were assessed against the inclusion criteria of this systematic review. Only study designs that randomised the participants and used inhaled nitric oxide as an intervention were considered. As none of these studies matched the inclusion criteria of this research topic they were eliminated from further review.

5.4 *Assessment of methodological quality*

Although none of the studies found were relevant each study that reported a randomised trial would have been assessed according to the criterion grading system described in the Cochrane Handbook of Systematic Reviews of Interventions (Higgins and Green, 2005). Using the following parameters [Table 4].

Table 4: Criteria to assess methodological quality.

(Compiled with information from Higgins and Green, 2005).

Randomisation	Adequate (A) if the study uses any one of the following methods of randomisation; computers or table of random numbers, drawing of lots.
	Unclear (B)
	Inadequate method of randomisation (C) if it uses any of the following: record number, date of birth or alternate numbers.
Concealment of allocation	Adequate (A), if it uses either central randomisation or numbered sealed envelopes.
	unclear (B)
	Inadequate (C), if there is an open allocation where participants and physicians are able to foresee the upcoming assignment.
Blinding of outcomes	Yes, if persons assessing the outcome of care are aware of which treatment the participant received. It is graded as yes, no and unclear (detection bias).
Handling of withdrawals and losses	Yes (A), if there was a clear description given of the difference between the two groups of losses to follow up (attrition bias) or unclear (B) or No (C).

5.5 Data collection

Study details were to be entered into the 'Characteristics of included studies' table in The Cochrane Collaboration's RevMan 4.2.2 (<http://www.cc-ims.net/RevMan>)

As there were no trials matching the inclusion criteria no data were extracted.

5.6 Data synthesis

No synthesis of data was performed as none of the studies matched the inclusion criteria.

6.1 *Assessment of search results and description of studies*

The search strategy identified a total of 9 references, through MEDLINE and CENTRAL databases. All were RCT's, but none of them investigated the effectiveness of inhNO in alleviating the symptoms of ACS. Therefore all were excluded from this systematic review. (See table 5).

6.2 *Summary of excluded studies*

The trialists in the excluded trials investigated the effectiveness of several therapeutic modalities which include incentive spirometry, blood transfusions, intravenous dexamethasone and expiratory pressure devices in the management or the prevention of ACS. All the outcomes were encouraging but not definitive. (See **appendix B** for full references).

Table 5: The trials identified by the search strategy.

Author	Type of Study	Interventions/treatment	Outcomes
Bellet 1995	Randomized controlled trial	Incentive spirometry	Incentive spirometry can prevent pulmonary complications associated with ACS.
Bernini 1995	Randomized controlled trial	Intravenous dexamethasone	Intravenous dexamethasone had a beneficial but in some cases short lived effect in children.
Bernini 1998	Randomized controlled trial	Intravenous dexamethasone	Intravenous dexamethasone has a beneficial effect in children with mild to moderately severe ACS.
Hsu 2004	Randomized controlled trial	Expiratory pressure device versus incentive spirometry.	Expiratory pressure device is equivalent to incentive spirometry in preventing ACS.
Hsu 2005	Randomized controlled trial	Expiratory pressure device versus incentive spirometry.	Expiratory pressure device is equivalent to incentive spirometry in preventing ACS.
Rucknagel 1995	Randomized controlled trial	Incentive spirometry	Incentive spirometry can prevent pulmonary complications associated with ACS.
Styles 2004	Randomized controlled trial	Blood transfusion	Blood transfusion has been shown to reduce phospholipase A2 which may prevent ACS.
Styles 2007	Randomized controlled trial	Blood transfusion	Blood transfusion may prevent ACS
Wook 2004	Randomized controlled trial	Intravenous dexamethasone	Intravenous dexamethasone decreased the duration of oxygen therapy and fever.

The aim of this review was to answer a clearly defined research question, by following explicitly developed inclusion criteria, a clear search strategy and a standardised methodology and in this way hoping to provide reliable evidence regarding the effectiveness of inhNO for ACS by examining clinical outcomes from relevant RCT's. The null hypothesis I tested was, that for ACS there was no difference between patients receiving inhNO as compared to those who have received placebo or other treatments.

The conventional therapeutic modalities for ACS include hospitalisation, supplemental oxygen, antibiotics intravenously and/or orally, analgesia and simple or exchange transfusion. However, when tested using study designs based on randomised controlled trials none of these modalities were shown to be effective (Bernini *et al.* 1998). Whilst I found a number of RCTs for other interventions which showed that they were not definitive I was unsuccessful in finding any RCTs for inhNO interventions in my systematic review.

Dr. Mark Gladwin (Chief of the Vascular Medicine Branch in the Division of Intramural Research at NIH's National Heart, Lung, and Blood Institute, USA) was contacted by electronic mail and asked to provide information about any published or unpublished trials in this field and if he was aware of any ongoing research being carried out. Only one randomized,

double blind placebo controlled phase II clinical trial is in progress in which the effectiveness and safety of inhaled nitric oxide for the treatment of sickle cell painful crisis in paediatric patients with SCD is being evaluated. The trial is expected to be completed by September 2007 (Weiner *et al.* 2007) and it is hoped will add to the evidence base of inhaled nitric oxide use in SCD.

The urgent need to report RCTs in a transparent manner has led to two independent international groups of statisticians, epidemiologists and biomedical editors to develop the CONSORT statement. The CONSORT statement is “an important research tool that takes an evidence-based approach to improve the quality of reports of randomized trials” (CONSORT Statement 2007). Many prominent medical journals and editorial groups such as The Lancet, JAMA and the Council of Science Editors (CSE) support the CONSORT statement. It provides a checklist and flow diagram for reporting the conduct of an RCT. They are used as guidance in writing, reviewing, or evaluating reports of RCTs (Moher *et al.* 2001) and therefore provide some assurance in reliable high quality trials.

8.1 *Implications for practice and research*

No RCTs on the use of inhNO in ACS were identified for inclusion in this systematic review. The rather limited evidence we have so far is based on a number of case reports and laboratory based studies. Therefore, well-designed, randomised controlled trials are needed to assess the effectiveness of inhNO in patients with SCD.

The absence of evidence in this systematic review highlights the importance of further high quality research which is required to provide reliable evidence for the effectiveness of this intervention.

9.1 Introduction

Although this systematic review indicates that the amount of data from clinical trials is very limited, the amount of scientific knowledge available on this topic is sufficient enough to highlight the importance of inhNO as a potential therapeutic option in ASC.

Nitric oxide is an important signaling molecule in the body, it is involved in many biological processes such as, vessel dilatation, neurotransmission, hair growth and penile erection. During the course of preparing my dissertation I came across several studies which investigated the transfer of endothelial cell nitric oxide synthase (eNOS) gene to restore the normal expression in grafted or injured vessels for coronary artery disease. This form of future genetic manipulation has exciting potential for patients suffering from ACS.

In the next section I discuss studies which have been conducted to investigate the potential of globin gene therapy to treat or cure SCD.

9.2 Gene therapy for SCD

The only effective approach to cure SCD is stem cell bone marrow transplantation. However, it is only offered for a limited number of patients with SCD, has a high mortality rate and is subject to the availability of suitable donors (Walters *et al.* 1996).

In the absence of an effective and safe cure for SCD a substantial amount of research has been carried out to find an alternative approach

based on gene therapy. Gene therapy is the transfer of a desired gene into target cells. To limit the production of the abnormal haemoglobin that causes sickling, a viral vector is introduced in cell cultures of patients with SCD which carries a therapeutic globin gene so the newly formed red blood cells make normal haemoglobin [Figure 3].

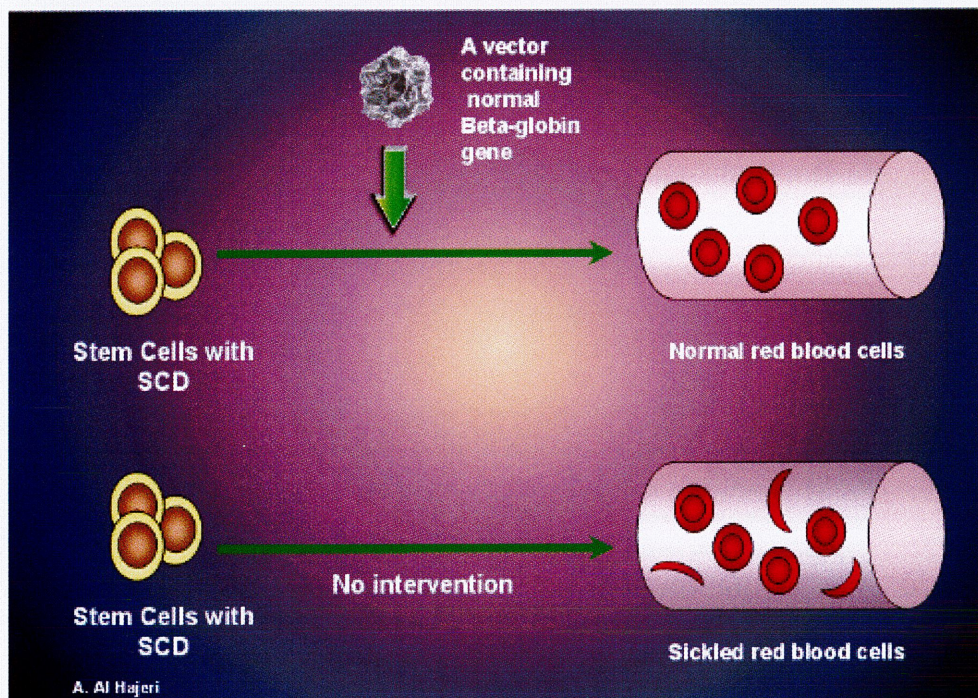
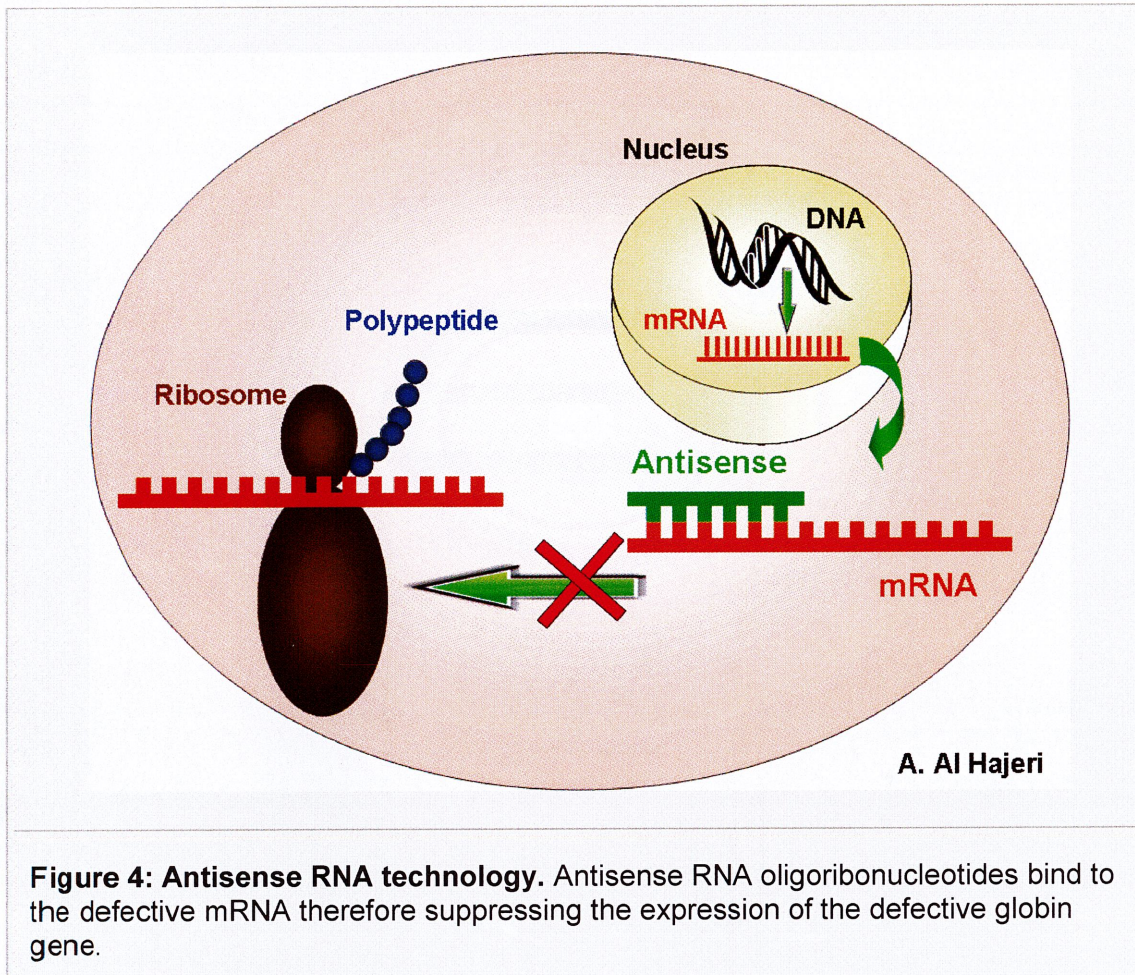


Figure3: The concept of sickle cell gene therapy. Stem cells are infected with viral vectors which harbour the normal globin gene. Therefore, normal red blood cells are produced.

Lentivirus vectors containing human globin genes and regulatory regions from the β -globin locus control region have been found to be effective in expressing normal haematopoietic stem cells and red cells when trans-infected to mouse models of SCD (May *et al.* 2000) and (Levasseur *et al.* 2003). Antisense RNA transcripts have also been investigated as an

alternative strategy to inhibit the abnormal S-globin expression (Xu *et al.* 2000) [Figure 4].



In spite of the advances in gene therapy, many essential challenges face the progression of research. The way we ensure viral transduction into haematopoietic stem cells and the generation of safe, stable, haemoglobin specific gene expression at a sufficient level to have a clinical effect are the most difficult obstacles researchers need to overcome.

As the progression of SCD gene therapy has been very slow over the past 20 years, I decided to look for a potential alternative gene therapy to treat one of the most important complications of SCD which is ACS. I was inspired by this systematic review to propose a new potential modality of treatment in which NO synthase gene can be introduced into lung tissue. In the following section I review several successful studies in which investigators have managed to increase the production of NO in different types of tissues and were able to restore the bioactivity of this vital compound.

9.3 Injured vessels and NO synthase gene therapy

There are three isoforms of the enzyme responsible for the production of NO, neuronal NOS (nNOS), endothelial NO synthase (eNOS) and inducible NO synthase (iNOS) (Ricciardolo *et al.* 2004). The latter two are related to blood vessels which consequently cause relaxation, inhibition of vascular smooth muscle cell proliferation, and inhibition of platelet activation and adhesion. The aim of NOS gene transfer is to increase nitric oxide bioactivity and enhance the anti-atherogenic properties of the vessel wall.

It has been shown that adenovirus harbouring iNOS gene is able to prevent aortic allograft atherosclerosis in rats (Shears *et al.* 1997) and reduce inflammatory cell infiltration and lipid deposition in carotid arteries of cholesterol-fed rabbits (Qian *et al.* 1999). Cooney *et al.* (2007) also illustrated the efficacy of adenoviral-mediated gene delivery of the two nitric oxide synthase isoforms, eNOS and iNOS on endothelial regeneration and intimal hyperplasia following endothelial injury in the rabbit carotid artery. Inducible NO synthase has the advantage of not being dependant on calcium and its levels are sustained (Brooks 2002). West *et al.* (2001) showed that

intraoperative NOS gene transfer has favourable effects on venous bypass graft remodelling. These findings suggest the potential utility of NOS gene transfer as a therapeutic strategy in coronary artery bypass grafting.

9.4 *Erectile dysfunction and NO synthase gene therapy*

Nitric oxide is an essential mediator of penile erection through vascular dilatation and relaxation of penile smooth muscle. This leads to increased blood flow into the cavernous tissue. Studies in rats showed that over expression of NO synthase in corpus cavernosum of the penis can correct erectile dysfunction (Chancellor *et al.* 2001).

The impaired endothelial function in the conditions mentioned above and the relative success of NO synthase gene therapy in the restoration of nitric oxide bioactivity appear to suggest that the same strategy may be applicable for ACS in SCD.

9.5 *Acute chest syndrome and NO synthase gene therapy*

In order to propose a strategy to deliver the NO synthase gene to the pulmonary tissue it is necessary to review the available strategies and their advantages and limitations.

9.5.1 *NO synthase genes*

As mentioned previously eNOS and iNOS produce NO in blood vessels, the gene responsible for eNOS is located on chromosome 7q36 and was first isolated by Janssens *et al.* in 1992. It spans 21 kilobases and encodes an mRNA of 4,052 nucleotides. The translated human protein is

1,294 amino acids long (McKusick *et al.* 2006). Inducible NOS gene is located on chromosome 17q11.2-q12 and is approximately 37 kilobases in length and consists of 26 exons (Chartrain *et al.* 1994)) [Figure 5].

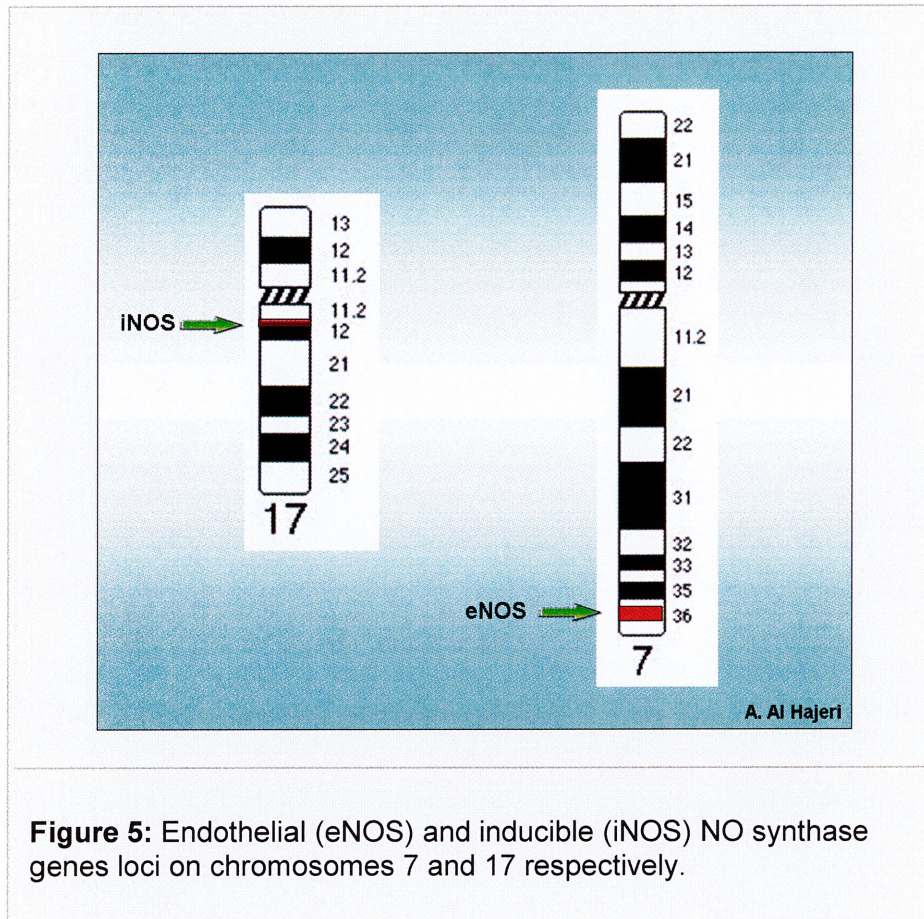


Figure 5: Endothelial (eNOS) and inducible (iNOS) NO synthase genes loci on chromosomes 7 and 17 respectively.

9.5.2 Vectors used for pulmonary gene therapy

The key element in gene therapy is to design an effective and safe vector to transfer the gene. There are many obstacles facing pulmonary gene therapy such as the inflammatory response caused by the vector, targeting the specific tissue, promoter down-regulation, and overcoming the physical barriers in the airway (West and Rodman 2001). Nevertheless, many trials on human gene therapy were completed using adeno-associated virus, and liposomes. Viral and non-viral vectors have been proposed for pulmonary gene therapy. Their advantages and limitations are summarised in table 6.

Table 6: Advantages and limitations of viral and non-viral vectors for pulmonary gene therapy.

(Compiled with information from West and Rodman 2001).

Vector	Advantages	Limitations
Viral:		
Adenovirus	New generations of the virus are devoid of viral antigens thus have little potential for an inflammatory response. Prolonged expression Potential of re-treating patients without the fear of immune response.	Several modifications to the virus need to be made before transfer. Lack of integration
Adeno-associated virus	Can be expressed for several months or longer High titre can be produced.	Lack of integration Small insert size (can't accommodate big genes). Can elicit immunity response, but it's not significant.
Herpes simplex virus	Good for neural tissue (can be used to transduce nerves in the lung).	Needs to be tested for lung tissue transduction to demonstrate its efficiency.
Lentivirus	Can integrate into the DNA of nonreplicating cells.	Needs to be tested for lung tissue transduction to demonstrate its efficiency.
Non-viral: Cationic lipids	Can target the lung circulation after IV injection.	Inefficient transfer of the plasmid to the nucleus. This can be partially overcome by adding protamine and proteins
Others, such as polymers, small synthetic beads and naked DNA	Less inflammatory response.	Low expression level

9.5.3 NOS gene transfer for pulmonary conditions

The closest pulmonary condition to ACS that has been investigated for NOS gene transfer is pulmonary hypertension (Janssens *et al.* 1996). The role of NO is well established in the pathogenesis of pulmonary hypertension (Hampl and Herget 2000). In the lung of patients with pulmonary hypertension the eNOS mRNA level is reduced indicating low expression (Giaid and Saleh 1995).

Janssens *et al.* (1996) studied the expression of recombinant eNOS after adenovirus gene transfer. They found the delivery of recombinant eNOS adenovirus by a single aerosolization enables increased NO production in bronchial and alveolar epithelial cells and vascular adventitial and endothelial cells. It also significantly attenuates hypoxic pulmonary vasoconstriction.

9.5.4 Developing a strategy to transfer NOS gene to treat ACS

As stated above, recombinant eNOS adenovirus successfully induced adequate levels of NO in pulmonary endothelial cells. Therefore, the same principle can be applied to ACS. Trials can be started on sickle cell transgenic mice with hypoxia-induced vaso-occlusion mimicking ACS in humans. Adenovirus is the most commonly used vector for transferring eNOS or iNOS in the conditions mentioned earlier, it is the preferred vector for the advantages mentioned in table 6.

Recombinant eNOS adenovirus can be aerosolized into the lungs of transgenic mice and a control group of mice should be run concomitantly by administering normal saline instead. Immunohistochemistry staining with monoclonal antibodies against eNOS can be used to assess its level of

expression and distribution in the lung tissues at several points of times after gene transfer. This should be performed for both the treatment and the control groups. Endothelial NOS immunostaining should be observed in the endothelial cells of medium-sized and small pulmonary vessels, in epithelial cells lining the large airways and in alveolar cells of the lung parenchyma. Immunoblot techniques can also be used concomitantly to measure the level of eNOS expression. The strategy I propose is illustrated in figure 6.

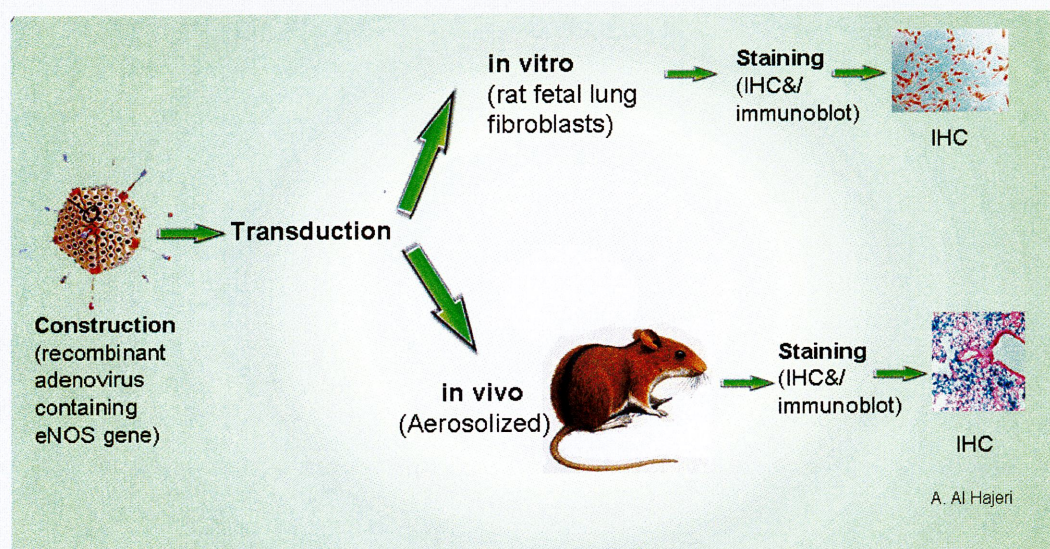


Figure 6: The strategy proposed for eNOS gene therapy in SCD transgenic mice. First, recombinant adenovirus is constructed and purified, then the vector is transferred in vitro (into rat fetal lung fibroblasts) and in vivo (the recombinant eNOS adenovirus is aerosolized into the lungs of transgenic mice). The level of eNOS expression is finally assessed via immunoblot techniques or immunohistochemistry (IHC) staining.

Although developing a strategy to deliver a gene to pulmonary endothelium may sound easy, many issues need to be addressed such as the efficacy and safety of NOS gene therapy, the selection of iNOS versus eNOS,

systemic versus local approach, the duration of gene expression and whether the treatment can be repeated or not.

9.6 Other genetic implications of NO genes

As stated earlier nitric oxide plays a major role in the regulation of vascular tone, blood pressure and many other vital functions. There is a recent trend to study the association between the genetic variation in NOS genes and the susceptibility to certain conditions such as hypertension and systemic lupus erythematosus (SLE) (Serrano *et al.* 2004). Mitchell *et al.* (2007) investigated 18 single nucleotide polymorphisms (SNPs) which account for the majority of underlying variation in NOS3 gene and found modest relation between the variants and arterial stiffness.

Although, no strong association has been discovered until now, the available results might provide insight into the significance of polymorphism at the NOS genes loci. More research needs to be done in order to improve our understanding of the molecular aspects of NOS gene and ultimately to design a superior strategy for gene therapy.

9.7 Conclusion

Gene therapy to restore the normal NO level may hold promise as a future therapeutic strategy for ACS. However, patient safety must come first. Despite the advances in gene therapy, we still have a long way ahead to design an effective strategy, test it on transgenic animals and to conduct human clinical trials.

Glossary*

Amino acid: Any of a class of 20 molecules that are combined to form proteins in living things. The sequence of amino acids in a protein and hence protein function are determined by the genetic code.

Base pair (bp): Two nitrogenous bases (adenine and thymine or guanine and cytosine) held together by weak bonds. Two strands of DNA are held together in the shape of a double helix by the bonds between base pairs.

Bias: Deviation of results or inferences from the truth, or processes leading to such deviation.

Blinding: An important study procedure to keep secret certain study procedures such as which is an active drug and which is a placebo. Bias can result when study subjects and those involved in study procedures are aware of the treatment assignment of individual subjects. Blinding is a method to help avoid the introduction of this kind of bias. Double-blinding refers to when neither patient nor persons performing the intervention or exposure know if the patient is in the study group or the comparison group.

Chromosomes: The self replicating genetic structures of cells containing the cellular DNA that bears in its nucleotide sequence the linear array of genes. In prokaryotes, chromosomal DNA is circular, and the entire genome is carried on one chromosome. Eukaryotic genomes consist of a number of chromosomes whose DNA is associated with different kinds of proteins.

Concealment of allocation: Occurs when the person who is enrolling a participant into a clinical trial is unaware whether the next participant to be enrolled will be allocated to the intervention or control group.

DNA (deoxyribonucleic acid): The molecule that encodes genetic information. DNA is a double-stranded molecule held together by weak bonds between base pairs of nucleotides. The four nucleotides in DNA contain the bases: adenine (A), guanine (G), cytosine (C), and thymine (T). In nature, base pairs form only between A and T and between G and C; thus the base sequence of each single strand can be deduced from that of its partner.

Enzyme: A protein that acts as a catalyst, speeding the rate at which a biochemical reaction proceeds but not altering the direction or nature of the reaction.

Exons: The protein-coding DNA sequences of a gene.

Gene: The fundamental physical and functional unit of heredity. A gene is an ordered sequence of nucleotides located in a particular position on a particular chromosome that encodes a specific functional product (i.e., a protein or RNA molecule).

Genetics: The study of the patterns of inheritance of specific traits.

Heterogeneity: This occurs when there is more variation between the study results (in a systematic review) than would be expected to occur by chance alone.

Heterozygosity: The presence of different alleles at one or more loci on homologous chromosomes.

Human gene therapy: Insertion of normal DNA directly into cells to correct a genetic defect.

Introns: The DNA base sequences interrupting the protein- coding sequences of a gene; these sequences are transcribed into RNA but are cut out of the message before it is translated into protein.

Messenger RNA (mRNA): RNA that serves as a template for protein synthesis.

Nucleotide: A subunit of DNA or RNA consisting of a nitrogenous base (adenine, guanine, thymine, or cytosine in DNA; adenine, guanine, uracil, or cytosine in RNA), a phosphate molecule, and a sugar molecule (deoxyribose in DNA and ribose in RNA). Thousands of nucleotides are linked to form a DNA or RNA molecule. See DNA, base pair, RNA.

Nucleus: The cellular organelle in eukaryotes that contains the genetic material.

Plasmid: Autonomously replicating, extrachromosomal circular DNA molecules, distinct from the normal bacterial genome and nonessential for cell survival under nonselective conditions. Some plasmids are capable of integrating into the host genome. A number of artificially constructed plasmids are used as cloning vectors.

Polymorphism: Difference in DNA sequence among individuals. Genetic variations occurring in more than 1% of a population would be considered useful polymorphisms for genetic linkage analysis.

Protein: A large molecule composed of one or more chains of amino acids in a specific order; the order is determined by the base sequence of nucleotides in the gene coding for the protein. Proteins are required for the structure, function, and regulation of the body's cells, tissues, and organs, and each protein has unique functions. Examples are hormones, enzymes, and antibodies.

Randomization (or random allocation): Method analogous to tossing a coin to assign patients to treatment groups (the experimental treatment is assigned if the coin lands "heads" and a conventional, "control" or "placebo" treatment is given if the coin lands "tails").

Randomized control clinical trial (RCT): Participants are randomly allocated into an experimental group or a control group and followed over time for the variables/outcomes of interest.

Recombinant DNA technologies: Procedures used to join together DNA segments in a cell- free system (an environment outside a cell or organism). Under appropriate conditions, a recombinant DNA molecule can enter a cell and replicate there, either autonomously or after it has become integrated into a cellular chromosome.

Transgenic: An experimentally produced organism in which DNA has been artificially introduced and incorporated into the organism's germ line, usually by injecting the foreign DNA into the nucleus of a fertilized embryo.

Virus: A noncellular biological entity that can reproduce only within a host cell. Viruses consist of nucleic acid covered by protein; some animal viruses are also surrounded by membrane. Inside the infected cell, the virus uses the synthetic capability of the host to produce progeny virus.

*Taken from:

Genome Glossary (http://www.ornl.gov/sci/techresources/Human_Genome/glossary/)

Centre for Evidence-Based Medicine

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(last accessed 07/07/2007)

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Appendix A: Electronic Database Search Strategies

- 1) **MEDLINE:** Searched on Ovid, from 1966 to 1994, on Silver Platter CD-Rom, from 1994 to 2002 and Ovid from 2003 to 1st May 2007.

The following search strategy for Ovid MEDLINE was used:

- #1 randomized controlled trial.pt.
- #2 Randomized Controlled Trials/
- #3 controlled clinical trial.pt.
- #4 Random Allocation/
- #5 Double-Blind Method/
- #6 Single-Blind Method/
- #7 or/1-6
- #8 clinical trial.pt.
- #9 exp Clinical Trials/
- #10 (clin\$ adj25 trial\$).tw.
- #11 ((singl\$ or doubl\$ or trebl\$ or tripl\$) adj25 (blind\$ or mask\$)).tw.
- #12 PLACEBOS/
- #13 placebo\$.tw.
- #14 random\$.tw.
- #15 Research Design/
- #16 or/8-15
- #17 Comparative Study/
- #18 exp Evaluation Studies/
- #19 Follow-Up Studies/
- #20 Prospective Studies/
- #21 (control\$ or prospectiv\$ or volunteer\$).tw.
- #22 or/17-21
- #23 animal/ not (human/ and animal/)
- #24 7 or 16 or 22
- #25 24 not 23

- #26 exp HEMOGLOBINOPATHIES/
- #27 exp Hemoglobin, Sickle/
- #28 (hemoglobinopath\$ or haemoglobinopath\$).tw.
- #29 (thalassemia or thalassaemia).tw.
- #30 (hemoglobin h disease or haemoglobin h disease).tw.
- #31 lepore.tw.
- #32 lepore \$.au.
- #33 31 not 32
- #34 (barts and (blood or plasma)).tw.
- #35 hydrops fetalis.tw.
- #36 sickle cell.tw.
- #37 sickle-cell.tw.
- #38 meniscocytic.tw.
- #39 drepanocytosis.tw.
- #40 (hemoglobin s or haemoglobin s).tw.
- #41 (hemoglobin sc disease or haemoglobin sc disease).tw.
- #42 (sickling and (blood or plasma)).tw.
- #43 (hemoglobin c disease or haemoglobin c disease).tw.
- #44 (hemoglobin d disease or haemoglobin d disease).tw.
- #45 (hemoglobin e disease or haemoglobin e disease).tw.

#46 (or/26-30) or (or/33-45)

#47 25 and 46

Lines #26 to #46 identify records relating to haemoglobinopathies; lines #1 to #25 reflect the Cochrane highly sensitive search strategy for RCTs and line #47 combines these components together to identify RCTs/CCTs on haemoglobinopathies.

2. **CENTRAL**: Searched on issue 2 2007 of The Cochrane Library.

#1HEMOGLOBINOPATHIES

#2HEMOGLOBIN SICKLE

#3THALASSEMIA

#4HYDROPS FETALIS

#5ANEMIA SICKLE CELL

#6hemoglobinopath*

#7haemoglobinopath*

#8(thalassemi* or thalassaemi*)

#9(hemoglobin next h next disease)

#10(haemoglobin next h next disease)

#11lepore

#12lepore:au

#13 (#11 and (not #12))

#14(barts and (blood or plasma))

#15(hydrops next fetalis)

#16(sickle next cell)

#17sickle-cell

#18(sickled or sickling)

#19meniscocytic

#20drepanocytosis

#21(hemoglobin next s)

#22(haemoglobin next s)

#23(hemoglobin next sc next disease)

#24(haemoglobin next sc next disease)

#25(sickling and (blood or plasma))

#26(hemoglobin next c next disease)

#27(haemoglobin next c next disease)

#28(hemoglobin next d next disease)

#29(haemoglobin next d next disease)

#30(hemoglobin next e next disease)

#31(haemoglobin next e next disease)

#32(#1 or #2 or #3 or #4 or #5 or #6 or #7 or #8 or #9 or #10 or #13 or #14 or #15 or #16 or #17 or #18 or #19 or #20 or #21 or #22 or #23 or #24 or #25 or #26 or #27 or #28 or #29 or #30 or #31)

#33(#32 and (not sr-cf))

Lines #1 to #32 identify records relating to haemoglobinopathies. Line #33 identifies records which are not already on the CFGD haemoglobinopathies register. Search terms shown in capitals are MeSH terms.

HANDSEARCHING ACTIVITIES

(A) Conference Proceedings

HAEMOGLOBINOPATHIES AND COAGULOPATHIES RELATED CONFERENCES

European Hematology Association

(Searched abstracts on CD-Rom) (NB. Searched only for RCT/CCTs relevant to the scope of the Group) from 1996 to 2006.

American Society of Hematology

(Searched abstracts on CD-Rom) (NB. Searched only for RCT/CCTs relevant to the scope of the Group) from 1995 to 2006.

British Society for Haematology

From 1995 to 2007

Standing Advisory Committee For Medical Research In The British Caribbean Meeting

From 1968 to 1972.

Commonwealth Caribbean Medical Research Council Meeting

From 1973 to 1998

Caribbean Health Research Council Meeting

From 1999 to 2006

National Sickle Cell Disease Program Annual Meeting

From 1989 to 2006.

(B) JOURNALS

British Journal of Haematology (1995 to 1996; Vol 105 Suppl 1 (1999); 2000)

Appendix B: References to excluded studies

Study	Reason for exclusion
Bellet 1995	The intervention was incentive spirometry rather than inhaled nitric oxide
Bernini 1995	The intervention was intravenous Dexamethasone rather than inhaled nitric oxide
Bernini 1998	The intervention was intravenous Dexamethasone rather than inhaled nitric oxide
Hsu 2004	The study compares the effectiveness of expiratory pressure device compared to incentive spirometry.
Hsu 2005	The study compares the effectiveness of expiratory pressure device compared to incentive spirometry.
Rucknagel 1995	The intervention was incentive spirometry rather than inhaled nitric oxide
Styles 2004	The intervention was blood transfusion rather than inhaled nitric oxide
Styles 2007	The intervention was blood transfusion rather than inhaled nitric oxide
Wook 2004	The intervention was intravenous Dexamethasone rather than inhaled nitric oxide

References of the excluded studies:

Bellet PS, Kalinyak KA, Shukla R, Gelfand MJ, Rucknagel DL (1995) Incentive spirometry to prevent acute pulmonary complications in sickle cell diseases [see comments]. *New England Journal of Medicine*. **333**: 699-703.

Bernini JC, Rogers ZR, Sandler ES, Reisch JS, Quinn CT, Buchanan GR. (1998) Beneficial effect of intravenous dexamethasone in children with mild to moderately severe acute chest syndrome complicating sickle cell disease. *Blood*. **92**(9): 3082-9.

Bernini JC, Tkaczewski I, Rogers ZR, Sandler E, Reisch JS, Buchanan GR. (1995) Dexamethasone therapy for children with acute chest syndrome (ACS) complicating sickle cell disease (SCD): a randomized, double-blind, placebo-controlled pilot study [abstract]. *Blood*. **86**(10 Suppl 1): 142a.

Hsu LL, Batts B. (2004) Positive expiratory pressure device acceptance by hospitalized children with sickle cell disease is comparable to incentive spirometry [abstract]. *American Journal of Respiratory and Critical Care Medicine*. **169**(7): A379.

Hsu LL, Batts BK, Rau JL (2005) Positive expiratory pressure device acceptance by hospitalized children with sickle cell disease is comparable to incentive spirometry. *Respiratory Care*. **50**(5): 624-7.

Rucknagel DL, Bellet PS, Kalinyak KA, Gelfand MJ, Shukla R (1995) Prevention of acute pulmonary complications by incentive spirometry in children with sickle cell diseases [abstract]. *The National Sickle Cell Disease Program Annual Meeting Conference Proceedings*; March. 1995. 236.

Styles LA, Abboud M, Larkin S, Lo M, Kuypers FA (2004). Transfusion prevents acute chest syndrome predicted by elevated secretory phospholipase A2 [abstract]. *27th Annual Meeting of the National Sickle Cell Disease Program*; 2004 April 18-21; Los Angeles, California. 72.

Styles LA, Abboud M, Larkin S, Lo M, Kuypers FA (2007) Transfusion prevents acute chest syndrome predicted by elevated secretory phospholipase A2. *British Journal of Haematology*. **136**(2): 343-4.

Wook Huh W, Quinn CT, Rogers ZR, Dale JC, Williams BF, Buchanan GR. Follow-up study of intravenous dexamethasone therapy for children with acute chest syndrome complicating sickle cell disease [abstract]. *27th Annual Meeting of the National Sickle Cell Disease program*; 2004 April 18-21; Los Angeles, California. 2004. 73.

Appendix C: Declaration of Own Work



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Dissertation: MSc (Med Sci) in Medical Genetics

This sheet must be filled in (each box ticked to show that the condition has been met), signed, dated and bound into your Dissertation.

Name: Amani Ali Al Hajeri

Matriculation number: 0605695

I confirm that this Dissertation is entirely my own work, except where I have indicated otherwise in the Dissertation, and that I have:

Read, understood and complied with both the Guidelines on Plagiarism supplied with this *Declaration of Own Work* form, and the information on Plagiarism in the Course Information Document:

☐

Clearly referenced all sources:

☐

Read all sources that I have cited:

☐

Referenced and put in inverted commas all quoted text (from books, web, journals, and other sources):

☐

Used my own words (except where indicated by inverted commas) and not simply paraphrased the source material:

☐

Given the sources of all illustrations, tables, data, *etc* that are not my own:

☐

Not made use of the essays or dissertations of any other student, either past or present:

☐

Acknowledged in appropriate places any and all help that I have received from others (fellow students, University staff, NHS staff, external sources):

☐

Not sought or used the help of any external professional agencies for the work:

☐

Understood that any false claim for any of the above will lead to penalties in accordance with University regulations:

☐

Signature:

Date: