The use of inhaled nitric oxide to treat persistent pulmonary hypertension of the newborn in a tertiary public hospital in South Africa from 2010-2014: morbidity, mortality and cost

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Submitted in fulfillment of the requirements of the degree
MMed (Paediatrics)
Faculty of Health Sciences
University of Cape Town

Date of submission: 29 August 2017

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DECLARATION

I, Alastair McAlpine, hereby declare that this dissertation is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university.

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The layout, style and citation method of this manuscript have been standardised according to manuscript submission requirements for the South African Medical Journal (SAMJ).

In terms of authorship of this paper:

I wrote the protocol, did the literature review, collected the data, analysed some of the data and wrote the manuscript.

Prof. Alan Horn supervised all stages of the project, assisted with data analysis and critically reviewed the manuscript.

Dr. Lloyd Tooke maintained the database, was involved in protocol development and also critically reviewed the manuscript.

Signature:

Date: 31 August 2017

ABSTRACT

Background and rationale: Inhaled nitric oxide (iNO) is recommended for the treatment of severe persistent pulmonary hypertension of the newborn (PPHN) because it reduces the need for extracorporeal membrane oxygenation (ECMO). There is insufficient evidence that iNO reduces mortality in the absence of ECMO. Although neonates in some South African public hospitals have access to iNO, ECMO is not available. Oral sildenafil can be effective in settings where iNO is not available, but its effect on outcome and cost of treatment in this setting have not been described.

The literature review in the first part of this thesis describes five studies reporting short-term outcomes of PPHN in the absence of ECMO. No studies from South Africa were identified. Only two studies described outcomes after iNO – the co-administration of Sildenafil with iNO was only reported in one small study. There were insufficient published data to guide management in settings where ECMO is not available.

Aim: To describe a cohort of term and near term neonates with PPHN who were treated with iNO, with or without sildenafil, in a tertiary neonatal unit in South Africa

Objectives: (i) to describe the characteristics at birth, the clinical course, and short-term outcomes; (ii) to determine if any variables were associated with mortality; (iii) to describe the relationship between the use of sildenafil and cost of care, represented by the duration of intubation and iNO use; and (iv) to describe the frequency of sildenafil prescription.

Methods. A retrospective review was carried out on folders of neonates with PPHN who were treated with iNO in Groote Schuur Hospital, Cape Town, South Africa, between January 2010 and December 2014.

Results. Forty neonates were included – most were full term (85%). Meconium aspiration syndrome (MAS) was the commonest cause of PPHN (50%), followed by intrapartum hypoxia (20%), sepsis (17.5%), pulmonary hypoplasia (7.5%) and idiopathic (5%). Fourteen neonates (35%) died. Pulmonary hypoplasia and pneumothorax were associated with mortality (p=0.037 and p=0.004 respectively). An FiO_2 of 1.0 and an iNO dose of $\geq$ 20 ppm at 24 and 48 hours respectively, both predicted death (specificity 89% vs. 100%, sensitivity 67% vs. 43% and p=0.003 vs. p=0.007 respectively). Sildenafil was prescribed more often after 2011 (83% vs. 65%) and was associated with increased survival (p=0.018) – early administration was associated with a shorter time to extubation (p=0.012) and a shorter course of iNO (p=0.044).

Conclusion. The treatment of PPHN with iNO in the absence of ECMO was associated with high mortality, particularly in neonates with congenital lung abnormalities. The FiO_2 and iNO requirements at 24 and 48 hours respectively could be used to identify neonates who are unlikely to benefit from continued treatment. Sildenafil was prescribed with increasing frequency during the study. The

combination of iNO with sildenafil was associated with more cost-effective care and improved short term outcomes. These findings provide a potential basis for cost-saving measures and resource allocation.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to:

Professor Alan Horn: for your wise, and endlessly patient, advice and supervision, even when I lost my way. There are not enough words to express my thanks.

The UCT research committee, for their assistance with funding, which was used to partly remunerate the costs of this paper.

Reshma Kassanjee, and the UCT Statistics Department, for assistance with the data and statistical analysis.

My parents, who always told me to follow my dreams.

My wife, Nicola, without whom, none of this would have been possible.

CONFLICTS OF INTEREST

None of the authors have any conflicts of interest

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ABBREVIATIONS

aEEG – Amplitude-integrated electroencephalogram

AKI – Acute kidney injury

CDH – Congenital diaphragmatic hernia

Echo - Echocardiogram

ECMO – Extracorporeal membrane oxygenation

FDA – Food and Drug Administration

FiO_2 – Inspired concentration of oxygen

HFOV – High frequency oscillatory ventilation

HIE – Hypoxic-ischaemic encephalopathy

HIV – Human immunodeficiency virus

LR – Likelihood ratio

HMD – Hyaline membrane disease

iNO – Inhaled nitric oxide

IQR – Interquartile range

IVH – Intraventricular haemorrhage

MAS – Meconium aspiration syndrome

MBP – Mean blood pressure

MCC – Medicines Control Council

NICU – Neonatal intensive care unit

PaO_2 – Partial pressure of oxygen

PPHN – Persistent pulmonary hypertension of the newborn

ppm – Parts per million

OI – Oxygenation Index

SD – Standard deviation

Chapter 1: Introduction and Literature review

1.1 INTRODUCTION

Persistent pulmonary hypertension of the newborn (PPHN) is an important neonatal condition associated with substantial morbidity and mortality - it is estimated that PPHN accounts for up to 10% of neonatal intensive care unit (NICU) admissions in the Western World, and has a mortality of 4 - 33%, depending on the setting.[1]

PPHN is defined as: 'the failure of the normal circulatory transition that occurs after birth'[2,3]; it is characterized by excessive right-to-left extrapulmonary shunting of blood, often associated with severe hypoxaemia.[3] Pulmonary hypertension complicates the clinical course of more than 10% of all neonates with respiratory failure – poor cardiac output and shock are frequently associated in severe cases.[1]

Various approaches have been suggested for the classification of PPHN - one common approach is to classify it into three categories[3]:

1) Secondary PPHN with abnormally constricted pulmonary vasculature due to lung parenchymal diseases – such as meconium aspiration syndrome (MAS), hyaline membrane disease (HMD), or pneumonia; or transient pulmonary artery vasoconstriction following hypoxaemia, particularly intrapartum hypoxaemia;
2) Idiopathic PPHN with normal lung parenchyma and remodeled pulmonary vasculature;
3) Primary PPHN with hypoplastic pulmonary vasculature as seen in congenital diaphragmatic hernia.

Secondary PPHN is the commonest type (60 - 80%), whilst idiopathic pulmonary hypertension is responsible for only 10 - 20% of all infants with PPHN, but there is significant overlap between the groups.[3]

Inhaled nitric oxide (iNO) is recommended for the treatment of severe PPHN due to reversible causes (secondary PPHN), when the oxygenation index (OI) is > 25 – this recommendation is based on the reduction in the need for extracorporeal membrane oxygenation (ECMO) in treated infants.[4] However, iNO is very expensive,[5] and there are very limited data describing outcomes following the use of iNO for PPHN in settings where ECMO is not available – such as South African state hospitals.

Since the evidence for efficacy of iNO is based on the availability of ECMO, the outcomes of neonates after being treated with iNO in settings where ECMO is *not* available should be studied to monitor the appropriateness and cost effectiveness of this treatment.

1.2 AIM AND OBJECTIVES

The aim of this literature review was to identify and compare the outcomes of studies of cohorts of neonates with acquired and idiopathic PPHN in settings where ECMO was not available.

The objectives of the review were to:

i) describe the diagnostic criteria used to identify PPHN;
ii) describe the mortality associated with PPHN;
iii) describe the demographic and clinical characteristics at birth of neonates with PPHN;
iv) describe the causes of PPHN;
v) describe the treatment modalities for PPHN;
vi) describe the complications and/or non-causal morbidity associated with PPHN:

This review focused specifically on short-term outcomes to provide reference data for comparison with outcomes in similar settings, such as South Africa.

1.3 METHODS

1.3.1 Data source and search strategy

PubMed was searched from the time that the database started, in 1946, until 10 March 2017, using the following search string:
(((((((persistent pulmonary hypertension) AND (neonat* OR newborn)) AND (outcome OR morbidity OR mortality OR demographic))). Foreign-language studies were excluded.

1.3.2 Inclusion and exclusion criteria

Papers describing cohorts of neonates with acquired and idiopathic PPHN that met the following inclusion criteria were included:

i) The population studied consisted of neonates diagnosed with PPHN;
ii) The study described demographic and/or clinical characteristics, causes of PPHN, and outcome at hospital discharge;
iii) The study included term infants (studies describing lower gestation in addition to term infants were not excluded);
iv) The study cohort was managed in a setting where ECMO was not available;
v) The study reported inclusion/diagnostic criteria;

The following types of studies were excluded:

a) Studies which included predominantly non-pulmonary causes of PPHN (e.g. cardiac causes, or congenital diaphragmatic hernia)
b) Studies focused exclusively on single, isolated causes of PPHN (e.g. idiopathic PPHN).
c) Studies without the full text of the study available in English

1.4 RESULTS

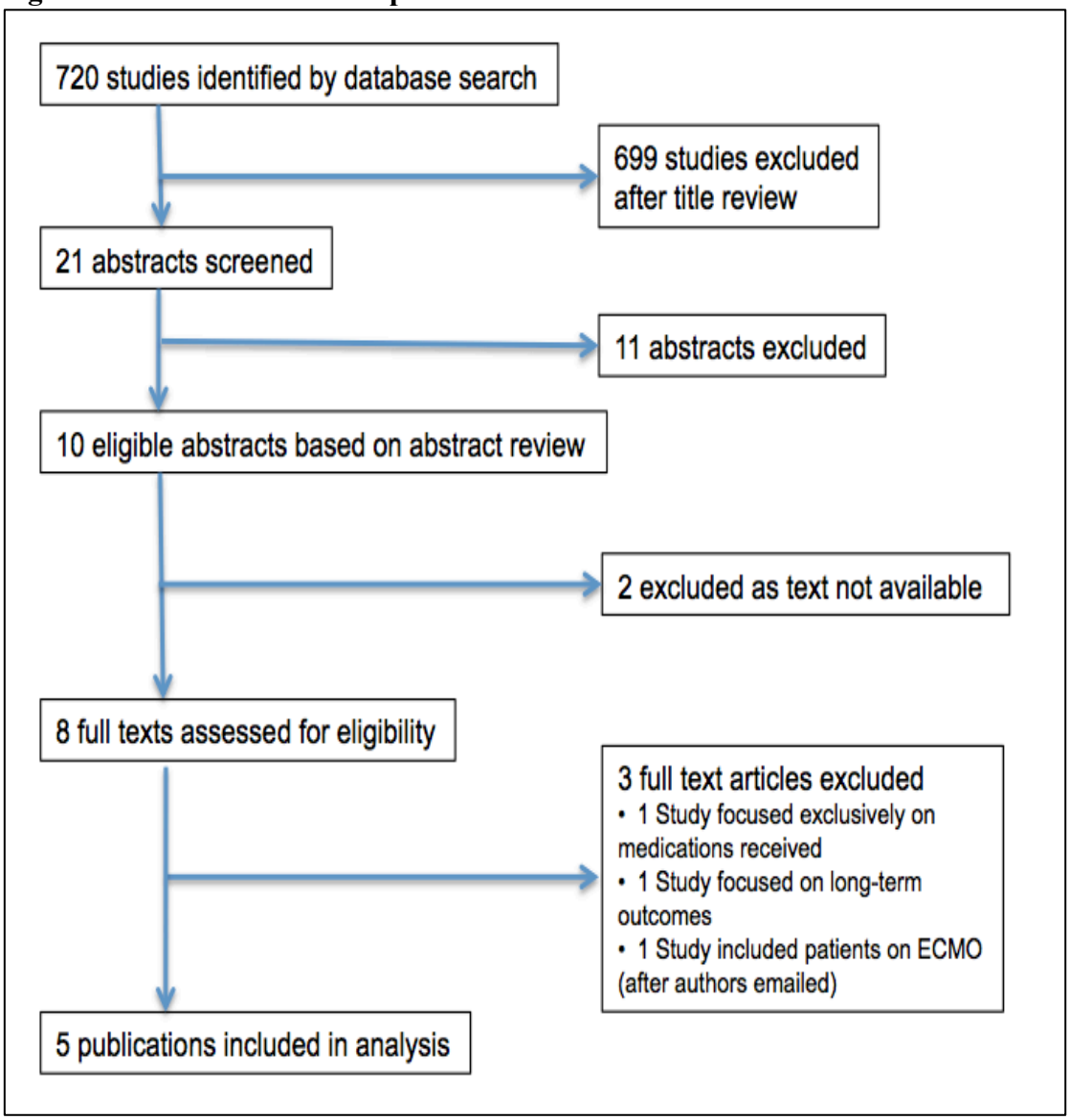
1.4.1 Literature review process

The search string identified 720 articles. Twenty-one potentially relevant studies were identified after title review. Eleven studies were excluded after abstract review. Two further studies were excluded because the full texts were not available either online or through the university library – these two were the oldest of all the studies and were published in the 1980s.[6,7] The full texts of eight studies were reviewed and when it

was not clear from the text or setting whether ECMO was available, the authors were emailed for clarification. Three further studies were excluded: one small study was excluded because it only focused on the medications received by the cohort[8]; the second study focused predominantly on long-term outcomes[9]; and the third study was excluded because the author confirmed that although ECMO was not mentioned in the study, the majority of infants would have had access to it.[10]

The remaining five studies formed the basis of this review: one study was from North America[11] (ECMO may have been available, but the availability of ECMO was not stated and the email address for the corresponding author was no longer valid), three from Asia,[12-14] and one from North Africa.[15] All were peer-reviewed. The process of identification of studies for inclusion is shown in Figure 1.1.

Figure 1.1: Literature review process



The study types, years and dates of publication of the included studies are shown in Table 1.1.

Table 1.1: Type and dates of included studies included in review

Author (publication date)	Type of Study	Setting (study date)
Hsieh (2001) [13]	Retrospective cohort study	Taipei, Taiwan (1990-1998)
Hernandez-Diaz (2007) [11]	Retrospective case-control study	Massachusetts, USA (1998 – 2003)
Razzaq (2013) [14]	Prospective cohort study	Multan, Pakistan (2011-2012)
Abdel-Mohsen (2013) [15]	Prospective cohort study	Al-Minya, Egypt (2009-2012)
Nakwan (2015) [12]	Retrospective cohort study	Hat Yai, Thailand (2010-2014)

USA – United States of America

1.4.2 Diagnosis of PPHN

There was wide variation amongst the studies in the diagnostic criteria for PPHN. The different criteria are summarized in Table 1.2.

One study relied predominantly on clinical criteria,[12] the others used a combination of clinical criteria and echocardiography.[11,13-15] The clinical diagnostic criteria differed between studies: one used a PaO_2 difference in the pre- and post-ductal limbs of 15 mmHg,[13] another used 20 mmHg.[12] Similarly, one study utilised a saturation differential between the pre- and post-ductal limbs of 5%,[11] another used 10%.[12] Echocardiographic criteria also varied: one study only required evidence of pulmonary hypertension (a peak velocity of the tricuspid regurgitation jet of > 40 mmHg),[14] while the others required visual demonstration on echocardiogram of right-to-left shunting at atrial and/or ductal level.

Table 1.2: Variation in diagnostic criteria for PPHN

Study (year)	Hsieh [13] (2001)	Hernandez-Diaz [11] (2007)	Razzaq [14] (2013)	Abdel-Mohsen [15] (2013)	Nakwan [12] (2016)
Method of PPHN diagnosis	Clinical shunting indicated by: PaO_2 difference of ≥ 15 mmHg AND/OR Right to left shunting on Echo	Clinical shunting indicated by: O_2 saturation difference ≥ 5% AND Right to left shunting on Echo	Clinical hypoxaemia (PaO_2 < 50 mmHg) AND Pulmonary hypertension indicated by tricuspid regurgitation	Clinical criteria not stated Right to left shunting on Echo	Clinical hypoxaemia AND ONE OF: Right to left shunting on Echo OR Saturation difference ≥ 10% OR PaO_2 difference of ≥ 20mmHg

Echo- Echocardiogram

1.4.3 Mortality

The demographic and clinical characteristics, causes, treatment and associated morbidity of PPHN are shown in Table 1.3.

The mortality rate ranged from 3 to 39.4% - it was highest in lower socio-economic settings, ranging from 25 to 39.4%. The only study with a mortality of less than 25% took place in USA, and it was not clear whether ECMO was used in this study.[11]

1.4.4 Demographic and clinical characteristics at birth

Only two studies described the proportion of out born patients,[12,13] which ranged from 27.7 to 75.9%. Sex was documented in four studies[12-15] - the majority of babies born with PPHN were male. The difference in sex proportions reached statistical significance in three studies.[11,14,15]

Gestational age was specifically reported on in four studies.[11-14] The other study stated that all babies were term without further detail.[15] Two studies *only* included term children (> 37 weeks),[12,15] but in the other three studies, the majority of the infants were also term (69% - 84%).[11,13,14] Prematurity was significantly associated with developing PPHN in the study that examined it.[11] Most infants had a birth weight above 2500 g. A low weight at birth was not independently associated with PPHN, unless the infant was premature.[11]

The mode of delivery was documented in four studies.[11,12,14,15] Caesarean section rates ranged from 42.9% to 64.6%, and two studies showed a significant association between caesarean section and PPHN.[11,15] None of the studies commented on whether the caesarean sections were elective or emergency in nature.

The need for resuscitation at birth, in the form of intermittent positive pulmonary ventilation, was only recorded in two studies[12,14] and in neither study was a significant association found with PPHN. The incidence of 'Birth asphyxia' was reported in two studies[13,14]; it ranged from 24% to 40%, but the term was not clearly defined.

1.4.5 Causes of PPHN

While the following pathologies are described in the literature as causes of PPHN, and referred to the same in this review, it is difficult to determine if they are causes or associated morbidities.

The most common cause of PPHN in four of the studies was MAS.[12-15] This was diagnosed by finding evidence of meconium-stained liquor (stained fingernails, stained skin, and/or meconium on the vocal cords) and respiratory distress - the proportion of affected infants ranged from 24.4 to 54.6%. The only study that included preterm infants found HMD to be the most common cause.[11]

Although sepsis was an important cause of PPHN in most studies, accounting for 13.8 - 43.8% of cases, only two studies provided diagnostic criteria for it.[11,14] Sepsis was diagnosed in one study on the basis of a suggestive history combined with abnormal septic markers in the serum,[14] in the other study, sepsis was described as suspected or culture-proven.[11]

Only three studies reported on congenital pneumonia (defined as having a septic risk factor, combined with respiratory distress and suggestive findings on Chest X-ray) as a separate entity[11,12,15] (the other studies included it in the 'sepsis' category), but it nevertheless remained an important cause of PPHN, accounting for 15.1 - 31.3% of cases.

Hyaline membrane disease accounted for 0.8 - 50.4% of cases of PPHN, but in the studies that only included term infants, HMD was an infrequent cause of PPHN.

Only two studies reported on idiopathic PPHN[12,13] – it was an uncommon cause, accounting for 0.8 - 7% of cases.

1.4.6 Treatment Modalities

The use of mechanical ventilation (defined as being ventilated via an endotracheal tube using a mechanical ventilator) varied greatly between studies – from as low as 20% in one study,[14] to 100% in two others.[11,13] The percentage of infants requiring high-frequency oscillatory ventilation (HFOV) ranged from 9.4% to 80.7%. One centre did not provide HFOV.[14] Access to iNO was only reported in two studies.[11,12] In one study iNO was used frequently (67%),[11] the other far less so (31%).[12] Neither study reported the criteria that were used to determine which patients were given iNO, nor if the oxygenation index ([inspired concentration of oxygen (%) x mean airway pressure]/post-ductal arterial partial pressure of oxygen in mmHg) was utilised. Only one study reported on outcome[12] – iNO was used in a higher proportion of those who died than in survivors ($p=0.01$), but the association may reflect illness severity rather than lack of effect.

Sildenafil was used in two studies[12,15] – it was given to 50 - 69% of patients, and in one study both iNO and sildenafil were available.[12] In one study, the administration of intragastric sildenafil was used more frequently in those who did not survive than in those who did ($p<0.01$).[12] In the other, half of the patients were randomly assigned the intragastric sildenafil, and 62.5% of patients who received it were reported to 'show improvement', but this was not clearly defined.[15]

Tolazoline (an alpha receptor antagonist which causes peripheral arterial dilatation), was used in one study in 83% of the neonates, and an improvement in their hypoxaemia was reported in 67% of them, but this improvement was not defined, nor was further statistical analysis done.[13]

The use of inotropic agents was variably reported. Only three studies stated that inotropic agents were used in the treatment centres[12,13,15]; of these, only two specified which inotropic agent was used. One centre made use of dopamine exclusively[13]; while the other used dopamine (all patients), adrenaline (98.3%), dobutamine (9.2%) and nor-adrenaline for a minority of their neonates (6.7%).[12] Only one study investigated an association between mortality and inotrope use – treatment with nor-adrenaline was associated with increased mortality ($p=0.03$),[12] but given multiple confounding factors, this finding is difficult to interpret.

1.4.7 Complications/morbidity

Only one study reported on the short-term complications or additional morbidity associated with PPHN[12] – in this study, acute kidney injury (AKI) and pneumothorax were both associated with poor outcomes ($p<0.01$).

Table 1.3: Demographic and clinical characteristics, causes, treatment and associated morbidity of PPHN

Author (year)	Nakwan[12] (2015)	Razzaq [14] (2013)	Abdel-Mohsen [15] (2013)	Hernandez-Diaz [11] (2007)	Hsieh[13] (2001)
N	119	79	32	377	29
Outborn n (%)	33 (27.7)	†	†	†	22 (75.9)
Male n (%)	75 (63)	57 (72.1)	18 (56.2)	†	18 (62.1)
Gestational Age Mean GA or % term	39 wks	77%	††	84%	37.1 wks
Birth-weight (g) Mean or % >2500 g	3044	†	†	92%	2707
Caesarean Section n (%)	51 (42.9)	43 (54.2)	20 (64.6)	231 (61.3)	†
IPPV at birth n (%)	2 (1.7)	35 (44.2)	†	†	†
Birth Asphyxia n (%)	†	32 (40.5)	†	†	7 (24.1)
Sepsis n(%)	22 (18.5)	23 (29.1)	14 (43.8)	122 (32.4)	4 (13.8)
MAS n (%)	65 (54.6)	28 (35.4)	16 (50)	179 (47.5)	8 (27.6)
Pneumonia n (%)	18 (15.1)	†	10 (31.3)	116 (30.8)	†
Idiopathic n (%)	1 (0.8)	†	†	†	2 (6.9)
HMD n (%)	1 (0.8)	11 (13.9)	6 (18.8)	190 (50.4)	5 (17.2)
Mechanical Ventilation n (%)	75 (63)	16 (20)	12 (37.5)	377 (100)	29 (100)
HFOV	96 (80.7)	16 (20)	3 (9.4)	264 (70)	4 (13.8)
Inhaled Nitric Oxide	37 (31.1)	0	0	253 (67.1)	0
Sildenafil	83 (69.7)	0	16 (50)	0	0
Tolazoline	0	0	0	0	24 (82.7)
Inotropes	119 (100)	0	††	††	27 (93)
Pneumothorax	57 (49)	†	†	†	†
Acute Kidney injury	31 (26)	†	†	†	†
Mortality n (%)	47 (39.4)	21 (26.6)	8 (25)	11 (3)	8 (27.6)

† - Variable not reported in the study; †† - Data unclear. Study mentions the presence of variable, but does not quantify or elaborate, HFOV – high frequency oscillatory ventilation; HMD – hyaline membrane disease ; IPPV – intermittent positive pulmonary ventilation; MAS – meconium aspiration syndrome; wks – weeks

1.5 DISCUSSION

There are very few published studies describing the short-term outcomes of neonates with PPHN in centres which do not have access to ECMO. Only one study from Africa (Egypt) was identified.[15] Diagnostic criteria varied widely and the mortality ranged from 25 to 39% in centres where ECMO was definitely not available. Mortality was 3% in one North American study where ECMO may have been used.[11] Clinical characteristics, treatment and morbidities varied widely and were inconsistently reported.

The variable mortality rate, with the lowest mortality occurring in a high-income

country with possible access to ECMO, is similar to the mortality quoted in other global reviews (3 - 33%).[1] The only study with mortality < 25% collected data from three states in North America where ECMO was probably available – ECMO use was not specifically described and the corresponding author could not be contacted.[11] The higher mortality in middle-income countries may have been due to limited equipment, staff and/or specifically due to the lack of ECMO. A meta-analysis of systematic reviews found that the mortality in infants with severe PPHN, in the absence of CDH, decreased from 48% in control infants to 16% in the group treated with ECMO,[16] and mortality in neonates with PPHN was reduced from 28% to 11% when ECMO was introduced in a centre in Chile (an upper middle income country).[17]

Two of the studies reported that many patients with PPHN were not born in a tertiary setting[12,13] – this may have increased their mortality due to: (a) the limited treatment they received in transit and/or (b) the length of time it took for the transfer to take place. Staff shortages were specifically mentioned as being a barrier to providing quality health care.[12,14] In one hospital, a single neonatologist and a single cardiologist were responsible for over 120 infants. There was no consistent relationship between mortality and the extent of iNO use.

Demographic and clinical criteria at birth were not consistently reported across all studies. The majority of infants with PPHN were male and previous studies have shown that being female is protective against developing HMD (a potential cause of PPHN), due to advanced fetal lung maturity.[18,19] The association found in one of the studies between late prematurity and PPHN has been attributed by some authors to a higher rate of HMD after delivery without antenatal steroids in these neonates, and a higher incidence of sepsis, compared to term babies.[20-22] Birth weights were similar across the studies – this is not unexpected, as most of the studies excluded premature babies from their analysis. No associations were found between birth weight and mortality when gestational age was taken into consideration.[11]

Previous studies have identified Caesarean section as an independent risk factor for the development of PPHN.[23] There is echocardiographic evidence of decreased pulmonary vascular resistance following vaginal delivery compared to neonates born by elective Caesarean section,[24] - this may be related to prostaglandin release induced by labour.[25] Other authors have proposed that catecholamine release during normal vaginal deliveries may improve lung compliance, placing those delivered via Caesarean section at risk of developing PPHN.[26] Most of the babies with PPHN in this review were delivered via Caesarean section, and some of the smaller studies described a significant association with PPHN and Caesarean sections.

Although MAS was the most common associated morbidity or cause of PPHN[12,13,15]; sepsis/pneumonia, birth asphyxia, and HMD were also significant causes. Very few of the studies considered the potential for overlap between the causes, which other authors have highlighted,[3] and distinguishing cause from association is difficult in this setting. It is also possible that the diagnosis of MAS was over-called because in many of the studies, if a neonate showed signs of respiratory distress, evidence of exposure to meconium-stained liquor (as evidenced by staining of the skin or fingernails) was accepted as diagnostic of the condition, without further investigation.

Sepsis may also have been over-diagnosed in several studies, for two reasons: first, only one study reported separately those patients in whom sepsis was a risk (e.g. born to a mother with chorioamnionitis), and those in whom it was confirmed on blood culture[11]; second, some of the studies relied on septic markers rather than blood culture, but these have inadequate sensitivity and specificity for neonatal sepsis,[28] especially in the first 12 hours of life, where both MAS and perinatal hypoxia can raise C-Reactive Protein levels.[28] Some studies documented congenital pneumonia separately from sepsis[11,12,15] – but if congenital pneumonia is included in the diagnosis of sepsis then the most common cause (or associated morbidity) of PPHN in this review, is sepsis at birth.

Idiopathic PPHN is estimated to occur in 10 - 20% of cases of PPHN,[3] but only two studies in this review reported on it.[12,13] This suggests that it may be a condition that is being under diagnosed, and some reported causes (e.g. MAS) might be associations rather than causal.

There was substantial variation in the treatments offered across the studies, in keeping with the resources available. Ventilation with HFOV is a longstanding recommendation[29] but not all centres offer it and many hospitals in poorly-resourced environments lack sufficient conventional ventilators to meet their patients' needs. This explains the vast discrepancies in both the number of patients ventilated and those who received HFOV, and may also be a contributing factor to the high mortality in some of the studies.

Only two of the studies included centres that offered iNO,[11,12] probably due to the very high cost of the treatment, and there was insufficient description of treatment protocols and weaning strategies. The Cochrane Collaboration advises using it in term or near-term, mechanically ventilated neonates with an OI > 25.[4] Up to 30% of infants with PPHN will not respond to iNO,[31] but no mention was made in either study about how these patients were identified or managed. Prognostic criteria and potential interactions with other therapies were also not described in these studies.

Sildenafil, a phophodiesterase inhibitor, is recommended as adjunctive therapy for PPHN in resource-limited settings,[30] due to the cost of iNO.[31] It has been shown to both reduce mortality and improve oxygenation,[31] but only two of the studies made use of it,[12,15] while experimental vasodilators such as tolazoline and magnesium sulphate were used in some other studies.[13,15] The mixed association between sildenafil use and mortality in the studies is difficult to assess because the sample sizes were both small, but the results are not in keeping with meta-analysis that demonstrates clear benefit.[31] The timing of the sildenafil administration, and its effects on mean blood pressure (MBP), were also not reported on. Although there is concern that sildenafil may lower MBP, and individual case studies have shown this effect,[32] other small studies indicate that in the doses generally provided (1.5 - 4.5mg/kg/dose), sildenafil has no major effect on it.[33] In addition, neither study in this review looked at any additional benefits the sildenafil provided (such as improvement in oxygenation). Only one study used both sildenafil and iNO,[12] but an analysis of their interaction was not done. In animal models, the combination of iNO at 20 parts per million (ppm) and intravenous sildenafil (2 mg/kg over 2 hours) produced significant pulmonary artery vasodilatation, but with concomitant systemic

vasodilatation and an unacceptable loss of systemic oxygenation.[34] In human infants post cardiac surgery, the combination of iNO at 20 ppm and intravenous sildenafil (0.35 mg/kg over 20 minutes), either before or after the iNO, resulted in systemic hypotension and impaired tissue oxygenation.[35]

Only one study commented on complications or non-causal morbidities[12] – acute kidney injury and pneumothorax were independently associated with poor outcomes. However, due to the exploratory nature, and the large number of variables examined in the study, the 'significant' differences may have been detected by chance.

A number of general limitations make the findings of this review difficult to interpret: first, most studies were small, and the studies which took place in single centres may have been prone to referral bias; second, there was substantial heterogeneity of not only the type of patients studied, but also the treatment modalities and resources available; third, the variation in diagnostic criteria suggests that certain studies may have either over- or under-diagnosed cases of PPHN and there is likely to be variation in the severity of PPHN assessed across the studies.

1.6 CONCLUSION

There are inadequate data describing the mortality of neonates with PPHN in settings where ECMO is not available; but the mortality is substantially higher than settings where ECMO is available, even if iNO is available. In particular, there are insufficient data to guide management of neonates with PPHN in settings where ECMO is not available but iNO and Sildenafil are available; the severity of PPHN needs to be quantified more objectively; and more data are needed to determine which demographic and clinical characteristics at birth or treatment modalities are associated with a good outcome.

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Chapter 2: Publication-ready manuscript for SAMJ submission

'The use of inhaled nitric oxide to treat persistent pulmonary hypertension of the newborn in a tertiary public hospital in South Africa from 2010-2014: morbidity, mortality and cost'

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ABSTRACT

Background. There is insufficient evidence that inhaled nitric oxide (iNO) reduces the mortality of persistent pulmonary hypertension of the newborn (PPHN) when extracorporeal membrane oxygenation (ECMO) is not available. Some hospitals in South Africa have access to iNO for PPHN, but not ECMO. Sildenafil is an alternative to iNO, but there are insufficient data to inform management of neonates treated with iNO, with or without sildenafil, in this setting.

Objectives. (i) To describe the characteristics at birth, clinical course, and short-term outcomes of a cohort of term and near term neonates with PPHN, who were treated with iNO, with or without sildenafil, in a tertiary neonatal unit; (ii) to determine if any variables were associated with mortality; (iii) to describe the relationship between the use of sildenafil and cost of care, represented by the duration of intubation and iNO use; and (iv) to describe the frequency of sildenafil prescription.

Methods. A retrospective review was carried out on folders of neonates with PPHN who were treated with iNO in Groote Schuur Hospital, Cape Town, South Africa, between January 2010 and December 2014.

Results. Forty neonates were included; 85% were full term. Meconium Aspiration Syndrome (MAS) was the commonest cause of PPHN (50%), followed by intrapartum hypoxia (20%), sepsis (17.5%), pulmonary hypoplasia (7.5%) and idiopathic (5%). Fourteen neonates (35%) died. Pulmonary hypoplasia and pneumothorax were associated with mortality (p=0.037 and p=0.004 respectively). An FiO_2 of 1.0 and an iNO dose of $\geq$ 20 ppm at 24 and 48 hours respectively, predicted death (specificity 89% vs. 100%, sensitivity 67% vs. 43% and p=0.003 vs. p=0.007 respectively). Sildenafil was prescribed more often after 2011 (83% vs. 65%) and was associated with increased survival (p=0.018) – early administration was associated with a shorter time to extubation (p=0.012) and a shorter course of iNO (p=0.044).

Conclusion. The treatment of PPHN with iNO in the absence of ECMO was associated with high mortality, particularly neonates with congenital lung abnormalities. The FiO_2 and iNO requirements at 24 and 48 hours respectively could be used to identify neonates who are unlikely to benefit from continued treatment. Sildenafil was prescribed with increasing frequency during the study. The combination of iNO with sildenafil was associated with more cost-effective care and improved short term outcomes. These findings provide a potential basis for cost-saving measures and resource allocation.

2.1 BACKGROUND

Persistent pulmonary hypertension (PPHN) occurs due to a failure of the circulatory transition that should occur after birth; it is characterized by abnormally constricted pulmonary vessels and excessive right-to-left extra-pulmonary shunting of blood, often associated with severe hypoxaemia.[1] It is estimated that PPHN accounts for up to 10% of neonatal intensive care unit (NICU) admissions, and has a mortality rate of 4–33%, depending on the setting.[2]

The commonest causes of PPHN are secondary; due mainly to intrapartum hypoxia, or lung pathologies such as meconium aspiration syndrome (MAS), hyaline membrane disease (HMD) and pneumonia.[3] Other causes are: idiopathic PPHN with normal lung parenchyma and remodeled pulmonary vasculature, and primary PPHN with hypoplastic pulmonary vasculature.[3]

Inhaled nitric oxide (iNO), a pulmonary artery vasodilator, is recommended by the United States Food and Drug Administration (FDA) and the European Medical Equipment Agency for the treatment of term and near-term neonates less than 14 days old, with severe PPHN.[4,5] However, meta-analysis of eight randomised control trials demonstrated that although iNO significantly decreased the need for extracorporeal membrane oxygenation (ECMO) in newborns with secondary PPHN, it did not decrease mortality.[6]

Neonates in some South African public hospitals have access to iNO, but ECMO is not available; there are few published data on mortality of neonates in this setting and data are predominantly derived from retrospective studies.[7-11] Despite the availability of iNO in two of these studies, the mortality was usually high (25–40%).[9,10] Only one study (in a developed setting) reported a mortality of less than 25%, but the availability of ECMO in this study was not clearly stated.[8] The sample sizes and definitions in these studies were too variable to determine outcome predictors and treatment efficacy.[10,11] Permission from the Medicines Control Council (MCC) and parental consent are required to use iNO in South Africa, and it is expensive. A study in North America in 2009, estimated the cost of iNO to range from United States (US) $ 1 200 to US $ 24 000 per patient.[12]

At the time of writing, there are no published reports examining the use and cost of iNO for PPHN in South Africa. A Cochrane systematic review and meta-analysis concluded that sildenafil, a phosphodiesterase-five inhibitor which induces pulmonary vasodilatation, could be used in settings where iNO is not available, but the use of iNO and sildenafil together requires further research.[13]

This study aimed to describe a cohort of neonates with PPHN, who were treated with iNO, with or without sildenafil, between January 2010 and July 2014 in a tertiary neonatal unit at Groote Schuur Hospital (GSH), Cape Town.

2.2 OBJECTIVES

(i) To describe the characteristics at birth, the clinical course, and short-term outcomes;
(ii) To determine if any variables were associated with mortality;
(iii) To describe the relationship between the use of Sildenafil and cost of care, represented by the duration of intubation and iNO use; and
(iv) To describe the relative frequency of sildenafil prescription

2.3 METHODS

2.3.1 Design

The study was a descriptive, retrospective cohort, folder review, from which baseline data were collected. A convenience sample was used, based on a period when data were prospectively entered into a register that was maintained to comply with MCC requirements, and for cost-audit purposes. Neonates were identified using this register and further data were extracted from the folders. The register and retrospective folder review were approved by the Human Research Ethics Committee of the Health Sciences Faculty of the University of Cape Town, and the designated provincial authority.

2.3.2 Setting and participants

The GSH Neonatal Unit is one of two public tertiary neonatal units within the Western Cape Province, South Africa. This 75-bed unit has approximately 200 admissions per month and is the tertiary referral centre for the west metropole area.

A Bedfont NOxBOX (Bedfont® Scientific Ltd, Kent) was used to monitor and administer iNO, according to the unit protocol, in neonates with PPHN meeting all of the following criteria:

i) Gestation $\geq$ 35 weeks;
ii) Oxygen saturation < 90% at FiO_2 > 0.8, despite ventilation with tidal volumes of 4-6ml/kg or treatment with high frequency oscillation ventilation (HFOV);
iii) Suspected PPHN with right to left shunting based on echocardiography and/or clinical signs (hypoxaemia unresponsive to increasing concentrations of FiO_2, but responsive to increased systemic blood pressure or a pre- and post-ductal oxygen saturation differential of $\geq$ 10%);
iv) Absence of cyanotic heart disease and known severe congenital anomalies – neonates with congenital diaphragmatic hernia (CDH) were excluded from 2011 when the departmental policy changed.

Neonates were included in the study as follows:

Inclusion criteria: Neonates meeting the above criteria and treated with iNO for PPHN at GSH between January 2010 and December 2014. Data for oxygenation index were unavailable, and this was not used.

Exclusion criteria: Neonates whose hospital records were not adequate / available.

Treatment with iNO was started at 20 parts per million (ppm); weaning commenced when a sustained fall in FiO_2 occurred and/or when FiO_2 was < 0.6. Sildenafil (started at 0.5 mg/kg/dose 6 hourly via intragastric tube, and increased to a maximum of 2 mg/kg/dose, based on response), was used in some infants, particularly those with stable blood pressures, or who were failing to wean off iNO. However, sildenafil was used more frequently after 2011 when an internal review showed unsustainable use of iNO relative to the budget.

2.3.3 Data collection, outcome measures and analysis

Data were collected on a case record form and analysed using Stata v12 (Stata Corporation, Texas, United States of America).

Characteristics at birth included: birth weight; gestational age (late preterm was defined by gestational age at birth of 35–36 completed weeks based on Ballard or early ultrasound); place of birth; resuscitation at birth and Apgar scores; blood gas parameters in the first hour of life; Human immunodeficiency virus (HIV) exposure; and the presence of sepsis risk factors (prolonged rupture of membranes > 18 hours, chorioamnionitis or maternal sepsis).

The primary cause of the PPHN was recorded using the following definitions: 'mortality' was defined as death due to PPHN or its immediate complications, 'confirmed sepsis' was defined as the growth of a pathological organism on a blood culture taken within the first 72 hours of life; 'congenital pneumonia' was defined as the presence of respiratory distress, sepsis risk at birth and suggestive chest X-ray findings; 'presumed sepsis' was defined as being clinically unwell in the presence of elevated septic markers but no positive blood culture and a normal chest X-ray; 'any sepsis' included infants with confirmed or presumed sepsis and those with congenital pneumonia; 'intrapartum hypoxia' was defined as the need for resuscitation at birth and suggestive maternal history, or a base deficit in the first hour of life of 10 or more; 'MAS' was defined as the presence of respiratory distress, meconium-stained liquor and suggestive X-ray findings; 'pulmonary hypoplasia' was defined as having a small lung capacity relative to ventilator support – this was a clinical judgement.

The clinical course and short-term outcomes were recorded, including: inotrope treatment; surfactant treatment; hydrocortisone treatment; treatment with high frequency oscillatory ventilation (HFOV); FiO_2 at initiation, 12 hours, and 24 hours; iNO dose at initiation, 12 hours, 24 hours and 48 hours; duration of iNO treatment; duration of intubation; sildenafil use and timing of initiation relative to iNO; presence of a pneumothorax; pneumonia, nosocomial sepsis and presumed nosocomial sepsis (defined as stated above for pneumonia, sepsis and presumed sepsis, but presenting at ≥ 72 hours of life); intraventricular haemorrhage (IVH); hypoxic ischaemic encephalopathy (HIE) – defined as the presence of clinical seizures or an abnormal amplitude-integrated electroencephalogram (aEEG) in a neonate with a history of

perinatal hypoxia; and mortality. Sildenafil use was divided into three groups: 'none'; 'early' (before or at the same time as initiation of iNO); and 'late' (after iNO had been commenced).

Association with mortality was analysed using Fisher's exact test for categorical variables, and student's t-test or the Kruskal Wallis test for normally and non-normally distributed continuous variables respectively. Receiver operator curve (ROC) analysis was used to determine threshold levels of iNO dose and FiO_2 that predicted mortality. The diagt statistical module was used to calculate sensitivity, specificity, and likelihood ratio (LR). The relationship between sildenafil use, duration of mechanical ventilation and iNO use was explored using the Kruskal Wallis test. P values of < 0.05 were considered significant.

No formal cost-saving analysis was performed, so proxy-measures, in the form of duration and concentration of iNO use (combined with the known cost of iNO as provided by the supplier), were used.

2.4 RESULTS

A total of 43 patients met inclusion criteria – adequate records were not available for three neonates. Data for the remaining 40 neonates were analysed; 26 survived to hospital discharge and 14 died. The survivors were mechanically ventilated for a median period of 4.5 (IQR 3–7) days.

2.4.1 Characteristics at birth and causes of PPHN

The characteristics of the neonates and causes of PPHN are shown in Table 2.1. Most neonates were term gestation (85%), with a normal birth weight (2970±560 g) and the majority of them were out-born (78%). There were no significant differences in the characteristics at birth between survivors and non-survivors.

Meconium Aspiration Syndrome (MAS) was the commonest cause of PPHN (50%), followed by intrapartum hypoxia (20%), sepsis (18%), pulmonary hypoplasia (8%) and idiopathic (5%). However, only one of the seven neonates with a diagnosis of sepsis had a positive blood culture. Twenty neonates (50%) had a low 5-minute Apgar score and/or received assisted ventilation at birth, but only 13 of these neonates and seven in the rest of the cohort had a blood gas within the first hour of life. Although intrapartum hypoxia was the suspected *primary* cause of PPHN in eight neonates, at least 10 of the 20 neonates with MAS also met criteria for intrapartum hypoxia.

Pulmonary hypoplasia was significantly associated with death ($p=0.037$) – all three affected neonates died before 24 hours of age. The causes of pulmonary hypoplasia were CDH, severe oligohydramnios associated with renal failure, and idiopathic. Both the neonates with idiopathic PPHN were term infants – one was underweight for gestational age (1860 g), the other had a normal birth weight of 2800 g.

Table 2.1: Characteristics at birth and causes of PPHN

Variable	All (N=40)	Alive (N=26)	Dead (N=14)	P-Value
	*Data are n(%) / mean (±SD)**			
Characteristics at birth				
Birth-weight in grams	2970 (±560.4)	2966 (±524.9)	2977.5 (±642)	0.955
Late preterm	6 (15)	6 (23)	0	0.074
Inborn	13 (32.5)	10 (38.5)	3 (21.4)	0.316
5 minute Apgar <7	16 (40)	11 (42.3)	5 (35.7)	0.746
Assisted ventilation at birth	17 (42.5)	9 (34.6)	8 (57.1)	0.198
Low Apgar or ventilation at birth	20 (50)	12 (46)	8 (57)	0.741
Blood Gas in 1st hour	20 (50)	11 (42.3)	9 (64.3)	0.320
Lowest pH in 1st hour	7.1 (±0.1) (n=20)	7.1 (±0.2) (n=11)	7.2 (±0.1) (n=9)	0.357
Lowest base deficit in 1st hour	11.7 (±5.5) (n=20)	13 (±6.3) (n=11)	10 (±4) (n=9)	0.209
HIV exposed	10 (25)	8 (30.8)	2 (14.3)	0.446
Sepsis risk at birth	6 (15)	5 (19.2)	1 (7.1)	0.399
Primary Cause of PPHN				
Any sepsis	7 (17.5)	6 (23)	1 (7)	0.387
Confirmed sepsis	1 (2.5)	1 (4)	0	1
Congenital pneumonia	2 (5)	2 (8)	0	0.553
Presumed sepsis	4 (10)	3 (12)	1 (7)	1
Intrapartum hypoxia	8 (20)	5 (19.2)	3 (21.4)	1
All MAS	20 (50)	15 (58)	5 (36)	0.185
MAS plus intrapartum hypoxia	10 (25)	6 (23)	4 (20)	0.718
Idiopathic	2 (5)	0	2 (14.3)	0.117
Pulmonary hypoplasia	3 (7.5)	0	3 (21)	0.037

*For normally distributed data, PPHN – Persistent pulmonary hypertension of the newborn, HIV – Human Immunodeficiency virus, MAS – Meconium aspiration syndrome,

2.4.2 Clinical course and short-term outcomes

At the time iNO was commenced, all neonates were receiving an FiO_2 of > 0.9, two inotropic agents and HFOV. Other aspects of the clinical course and short-term outcomes are shown in Table 2.2.

Most neonates were treated with surfactant (maximum of two doses), hydrocortisone for hypotension, and sildenafil. Administration of surfactant and sildenafil was associated with survival (p=0.003 and p=0.018 respectively). Those who were not given surfactant had either pulmonary hypoplasia or idiopathic PPHN. Most of the neonates who were treated with sildenafil received it before 48 hours (range 4–96 hours).

The median age at initiation of iNO was 12 (range 1–60) hours. The median duration of iNO was 36 (range 5–192) hours. There was no difference between survivors and non-survivors in the proportion of neonates receiving iNO at 48 hours. Survivors tended to have a longer duration of iNO treatment (p=0.059). However, the dose of iNO at 48 hours was significantly higher in those who died (median doses of 9.2 and 0 ppm respectively and p=0.044). The median FiO_2 at 12 and 24 hours was significantly lower in survivors (p=0.007 and p=0.017 respectively).

All three neonates with a 48-hour dose of iNO of ≥ 20 ppm died – ROC and diagt analysis showed that this threshold correctly predicted death in 88% of neonates, with a sensitivity and a specificity of 43% and 96%; and positive and negative likelihood ratios of 22.8 and 0.6 respectively. Similar analysis of FiO_2 at 24 hours showed that a threshold of 1.0 correctly predicted death in 83% of neonates, with a sensitivity and a specificity of 67% and 89%; and positive and negative LRs of 5.8 and 0.4 respectively. An FiO2 of 1.0 was documented at 24 hours in all three neonates with a 48 hour iNO dose of ≥ 20 ppm.

The co-morbidities of HIE, IVH, and nosocomial sepsis occurred infrequently. Pneumothorax complicated the course of seven neonates (18%) and was strongly associated with death (p=0.004) – two of the six affected neonates who died also had pulmonary hypoplasia, however the association remained significant when neonates with pulmonary hypoplasia were excluded (p=0.021).

Of the neonates with HIE, none met the criteria for initiating therapeutic hypothermia.

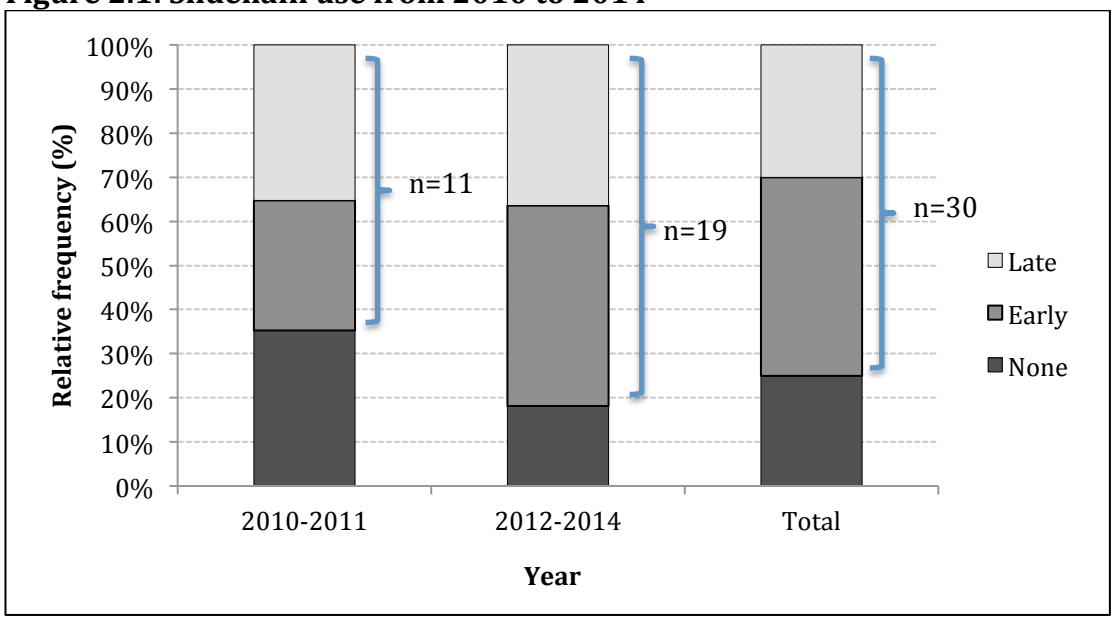
Table 2.2: Clinical course and short-term outcomes

Variable	All (N=40)	Alive (N=26)	Dead (N=14)	P-Value
	Data are n/N (%) or median (IQR as 1st – 3rd quartile)			
Surfactant	35/40 (87.5)	26/26 (100)	9/14 (64.2)	0.003
Hydrocortisone	33/40 (82.5)	21/26 (80.8)	12/14 (85.7)	1.000
Sildenafil	30/40 (75)	23/26 (88.5)	7/14 (50)	0.018
Early sildenafil	18/30 (60)	14/23 (60.9)	4/7 (57.1)	1.000
Age in hours at sildenafil initiation	21.5 (9-48) (n=30)	21 (9-48) (n=23)	22 (9-36) (n=7)	0.902
Age in hours at iNO initiation	12 (8-24) (n=40)	13 (9-24) (n=26)	11(6-22) (n=14)	0.153
iNO dose, ppm at 12 hours	17.5 (11.25-20) (n=28)	16 (11-20) (n=19)	20 (19.2-20) (n=9)	0.086
iNO dose, ppm at 24 hours	10 (0-15.1) (n=34)	9.5 (0-15) (n=26)	14.35 (4.1-20) (n=8)	0.268
iNO dose, ppm at 48 hours	0 (0-9.1) (n=32)	0 (0-6) (n=25)	9.2 (0-20) (n=7)	0.044
iNO for >48 hours	15/40 (37.5)	10/26 (38.5)	5/14 (35.7)	1.000
Duration in hours of iNO	36 (22-60) (n=40)	36 (30-72) (n=26)	24 (10-60) (n=13)	0.079
$Fi0_2$ at 12 hours	70 (50-100) (n=37)	60 (40-95) (n=25)	100 (85-100) (n=12)	0.007
$Fi0_2$ at 24 hours	60 (40-100) (n=35)	45 (40-80) (n=26)	100 (50-100) (n=9)	0.017
HIE/Seizures	4/40 (10)	4/26 (15.4)	0/14 (0)	0.278
Intraventricular haemorrhage	3/40 (7.5)	3/26 (11.5)	0/14 (0)	0.539
Pneumothorax	7/40 (17.5)	1/26 (3.8)	6/14 (42.9)	0.004
Nosocomial sepsis	5/40 (12.5)	4/26 (15.4)	1/14 (7.1)	0.640

iNO – Inhaled nitric oxide, FiO_2 – Inspired concentration of oxygen, HIE – Hypoxic ischaemic encephalopathy

Sildenafil was prescribed in 65% and 83% of infants before and after the end of 2011 respectively – these data (together with the absolute numbers of neonates who received sildenafil) are shown Figure 2.1.

Figure 2.1: Sildenafil use from 2010 to 2014



2.4.3 Sildenafil use and cost of care

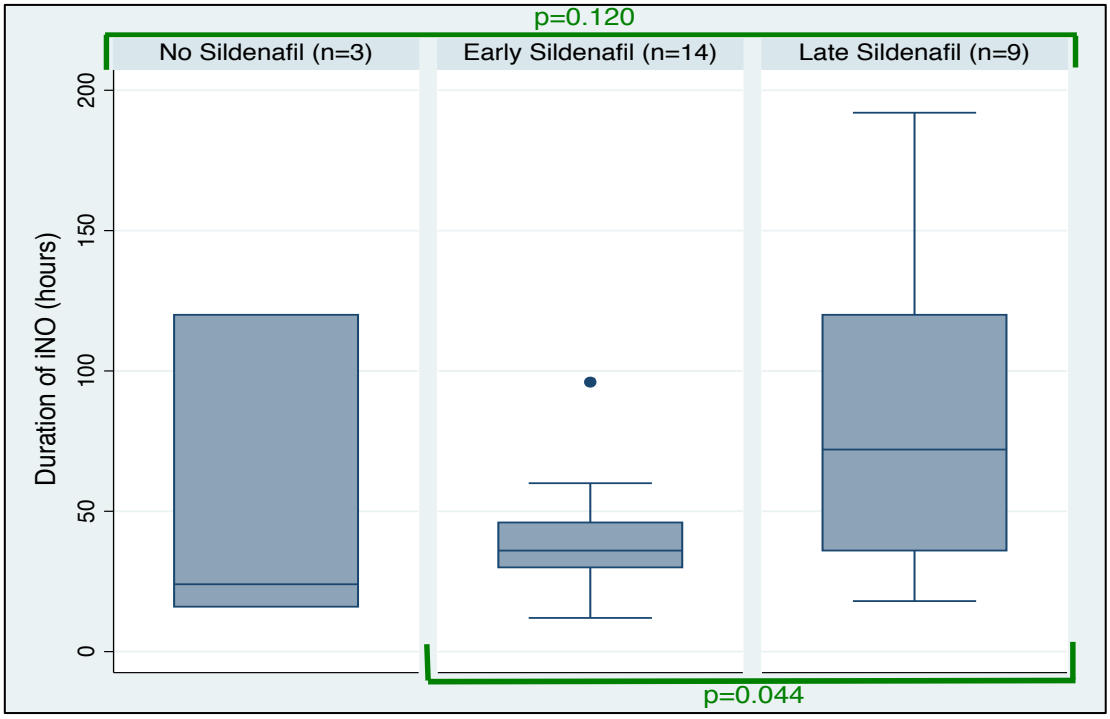
The relationships between sildenafil use and the duration of iNO treatment and intubation in survivors are shown in Figures 2.2 and 2.3 respectively.

The duration of iNO treatment in the three survivors who did not receive sildenafil ranged from 16 to 120 hours. Neonates who were treated with sildenafil early required iNO for a median of 36 (IQR 30–46) hours vs. 72 (IQR 36–120) hours in those who were treated late (p=0.044).

The duration of intubation (and ventilation) of the three survivors who did not receive sildenafil ranged from 3–9 days. Neonates who were treated with sildenafil early remained intubated for a median of 3.5 (IQR 3–5) hours vs. 7 (IQR 7–8) hours in those who were treated late (p=0.012). There was a significant difference across all three categories (p=0.043).

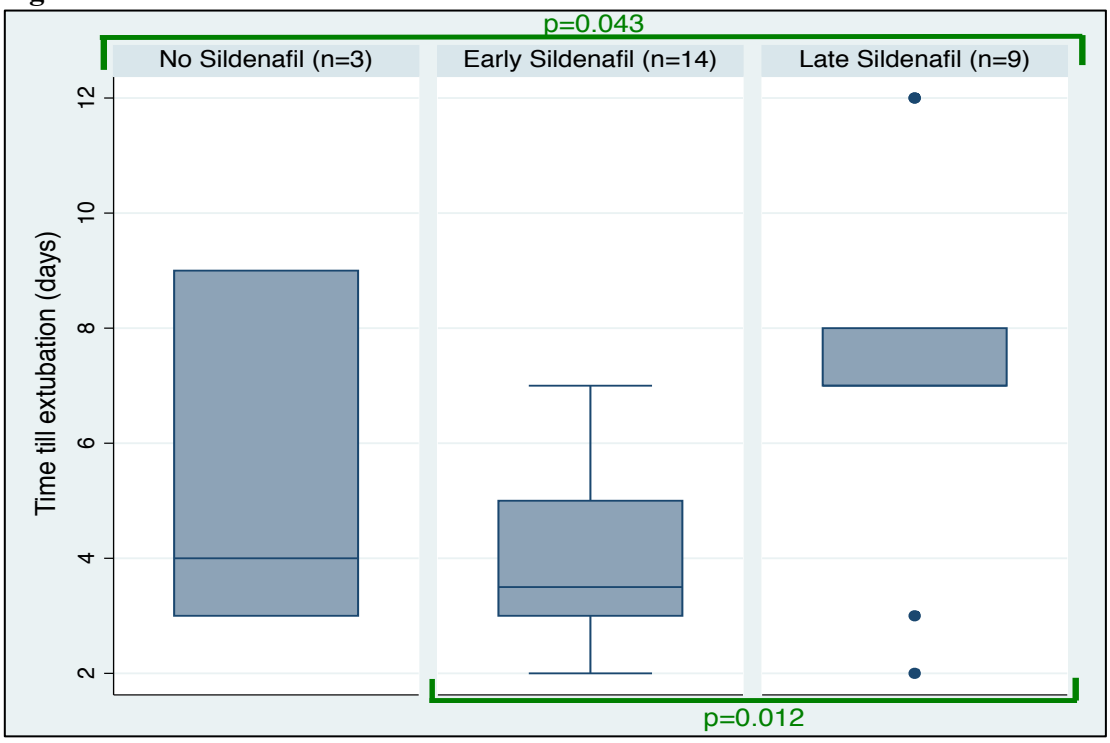
According to the single supplier in South Africa, each cylinder of NO is filled with 1500 L of gas, and the cost to public hospitals at the time of writing (May 2017), is R 11.83/L (before tax). The delivery of approximately 25 ml of NO gas/L ventilator gas flow will achieve a dose of 20 ppm. Therefore, the NO consumption at a ventilator flow rate of 20 L/minute, is approximately 20 x 0.025 x 60=30 L/hour; this equates to a cost of R 355/hour or approximately R 18 per each ppm per hour, but may vary based on different types of ventilators, leaks, and mode of ventilation.

Figure 2.2: Sildenafil and duration of iNO in survivors



iNO – Inhaled nitric oxide

Figure 2.3: Sildenafil and duration of Intubation in survivors



2.5 DISCUSSION

This retrospective cohort study demonstrates that neonates with PPHN who were treated with iNO, in a setting where ECMO was not available, had a high mortality (35%). Data of the age at the time of death were not collected. The commonest causes of PPHN were MAS and intrapartum hypoxia; and most neonates were out-born. The variables significantly associated with death were: pulmonary hypoplasia, pneumothorax, absence of sildenafil administration, absence of surfactant administration, and maximum doses of FiO_2 and iNO at 24 and 48 hours respectively.

The high proportion of out-born neonates (67%) is expected, because neonates with PPHN require tertiary care. Studies in developing countries report out-born proportions ranging from 28 to 76%.[9,10] There was no association between birth weight and mortality in our study – such an association has been reported in preterm neonates with PPHN,[8] but we did not include neonates under 35 weeks' gestation.

The high mortality and the frequency of MAS and intrapartum hypoxia are in keeping with findings from other studies in developing countries[7,9-11] – in contrast to developed countries, where sepsis is causal in the majority of cases of PPHN.[8] The role of intrapartum hypoxia as a primary cause may have been under-estimated in our study due to the frequent co-existence of MAS and intrapartum hypoxia. Moreover, only half of the cohort had early blood gas data, possibly due to the lack blood gas machines at the birth sites of the out-born neonates.

Idiopathic PPHN is associated with normal lung parenchyma and abnormal remodeling of the fetal pulmonary vasculature.[3] The low birth weight of one of the two infants with idiopathic PPHN in our study may represent an associated chronic underlying cause – inborn errors of metabolism and abnormal genetic mutations causing 'idiopathic' PPHN and low birth weight have been described.[14] Data from animal studies suggest an association between idiopathic PPHN and maternal non-steroidal anti-inflammatories (NSAIDs) or selective serotonin reuptake inhibitor (SSRI) antidepressants, but human data are limited and conflicting.[15]

Pulmonary hypoplasia was an uncommon cause of PPHN, but it was the only cause associated with mortality – this is in keeping with the published literature, which shows a high mortality worldwide.[16] The diagnosis was a clinical one, based on small lung fields relative to ventilation, and may have been subject to observer bias. One of the neonates had pulmonary hypoplasia secondary to CDH. This neonate was born in 2010 when iNO was still used for CDH in GSH; that approach was possibly based on a report at the time showing potential benefit[17] and a guideline that supported iNO use in some situations.[18] The unit practice changed from 2011 after reviewing previous, more robust evidence suggesting lack of efficacy[19]; thereafter, neonates with CDH were no longer treated with iNO. A recent update of this evidence supports the continued stance of not treating CDH with iNO.[20]

Pneumothorax was the only morbidity associated with death. In two of these neonates it complicated pulmonary hypoplasia, which is well described,[21,22] but may be a confounder. However, even when these patients were excluded, pneumothorax was

still significantly associated with death (p=0.021). A study of PPHN in Thailand found a similar association.[10]

Treatment with surfactant in our cohort was strongly associated with survival; this was probably due to the fact that three of the five neonates who did not receive surfactant had pulmonary hypoplasia, which is independently associated with a high mortality rate.[16] When the analysis was repeated with these three infants excluded, the association was no longer significant (p=0.083). Surfactant administration to term infants with PPHN has been shown to reduce the need for ECMO when parenchymal disease is present (the majority of our cohort) but, similar to iNO, it has not been shown to decrease mortality.[23] A recent meta-analysis of surfactant for MAS found that although hospital stay and the requirement for ECMO were significantly reduced, there was no effect on mortality, morbidity or duration of ventilation.[24] A Cochrane review in 2012 was unable to identify any suitable studies to determine if surfactant was beneficial to term and near-term neonates with pneumonia.[25]

Most infants in the cohort were treated with hydrocortisone for refractory hypotension – this approach is supported by limited neonatal data,[26] but is in keeping with global trends.[27] Although glucocorticoids are not recommended for improving respiratory outcomes of MAS,[28-30] hydrocortisone has been shown to improve oxygenation and reduce pulmonary vasoconstrictor levels in lambs with PPHN[31]; and two small randomised controlled trials, enrolling non-intubated neonates with MAS, found that both intravenous methylprednisone and inhaled budesonide resulted in improved oxygenation and shorter hospital stays with no effect on mortality.[32,33]

The neonates in our cohort who died tended to have a shorter duration of iNO, despite it being initiated at a similar time to survivors – this is probably due to early death (seven of the 14 neonates who died, did so before 48 hours) and/or early cessation of iNO due to lack of effect. A poor response to iNO in those who were still alive at 48 hours, but died later, is suggested by the significantly higher dose of iNO in non-survivors vs. survivors (median 9.2 and 0 ppm respectively); an iNO dose of $\geq$ 20 ppm at 48 hours correctly predicted death in 88% of neonates. Most of the non-responders (i.e. those who did not survive) in this cohort could probably have been identified earlier by the persistently high FiO_2 at 24 hours. The infants who died had a significantly higher FiO_2 at 24 hours compared to survivors (median 0.45 and 1.0 respectively); an FiO_2 of 1.0 at 24 hours correctly predicted death in 83% of infants.

There are few published data on the use of sildenafil together with iNO, specifically for PPHN, and none on the effect that combining these has on mortality for this condition.[10] The authors of a Cochrane review concluded that sildenafil may be used in resource-limited environments, but they make no recommendation for the use of sildenafil *with* iNO.[34] Data from animal models and small studies in infants post cardiac surgery suggest that when iNO and sildenafil are used together, a significant drop in systemic oxygenation and blood pressure can occur.[35,36]

The data from this study indicate a significant reduction in mortality in those who were treated with sildenafil, but these data are difficult to interpret because some neonates with persistent refractory hypotension may not have received sildenafil due to concerns about potential exacerbation of hypotension. However, this confounding effect is unlikely to be substantial because the majority of neonates were treated with

sildenafil, particularly in the last 3 years (83%); and most neonates who received hydrocortisone also received sildenafil. Data showing the earlier weaning of iNO and the shorter periods of ventilation experienced by the neonates who were treated earlier compared to those who were treated later, may have been confounded by similar selection bias. In 2015, Limjoco and co-authors published data showing that the practice of withholding enteral sildenafil from hypotensive neonates with PPHN may not be appropriate; they described a cohort of 17 neonates treated with iNO, inotropes and enteral sildenafil – there was no significant decrease in blood pressure, nor increase in inotrope requirement after treatment with sildenafil, but there was a wide variation in the extent of inotrope treatment.[37]

Currently, iNO costs R18 per ppm per hour. The cost of iNO at 20 ppm is therefore R8 640 per day. Since all neonates in our cohort with this requirement at 48 hours died and also had FiO2 of 1.0 at 24 hours, these neonates should be considered as non-responders – substantial cost savings could be made by stopping iNO at 48 hours or earlier. The shorter duration of iNO in those who received early sildenafil (median 36 vs. 72 hours) may represent a potential cost-saving of between R3 196 (at 5 ppm for 36 hours) and R12 780 (at 20 ppm for 36 hours) per patient. These numbers are subject to inaccuracies due to circuit leaks, different types of ventilators, incorrect monitoring, or faulty equipment. Sildenafil was also associated with a reduced time to extubation, which suggests further potential cost savings.

In addition to the potential selection bias associated with Sildenafil treatment, this study has further limitations: the small cohort size and wide confidence intervals; and the lack of data regarding the reason for not using sildenafil, make the associated outcomes difficult to interpret. The lack of data regarding the oxygenation index makes it difficult to ascertain which infants would have benefited from ECMO. In addition, the multiple drugs and treatment modalities may have introduced the potential for confounding the effects of iNO. To eliminate them all to assess pure survival in iNO at different doses, however, would have been impossible.

Despite the limitations, this is the first study (to our knowledge) in sub-Saharan Africa to document outcomes of neonates with PPHN who were treated with iNO and sildenafil; and it describes data from a sufficient period to detect changes in clinician prescribing behavior. Another strength of this study is that it identifies robust clinical markers that strongly predict a poor outcome at 24 and 48 hours and it provides some data to measure the cost of continuing iNO in these neonates.

2.6 CONCLUSION

The treatment of PPHN with iNO in the absence of ECMO was associated with a high mortality, particularly in neonates with congenital lung abnormalities. An FiO_2 of 1.0 at 24 hours and an iNO dose of $\geq$ 20ppm at 48 hours could be used to identify neonates who are unlikely to benefit from treatment, particularly when both are present. The combination of iNO with Sildenafil may be associated with cost-effective care and improved short term outcomes, but a formal cost-saving analysis is needed to confirm this due to the presence of many other factors.

These findings provide a potential basis for cost-saving measures and resource allocation. The combination of iNO and sildenafil as treatment for neonates with PPHN should be studied further in prospective randomized controlled trials.

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2.8 APPENDICES

2.8.1 HREC Ethics approval and extension

UNIVERSITY OF CAPE TOWN
Faculty of Health Sciences
Human Research Ethics Committee

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03 December 2014

HREC REF: 897/2014

A/Prof A Horn
Paediatrics
Neonatology
Room 65, H46
OMB

Dear A/Prof Horn

PROJECT TITLE: THE USE OF INHALED NITRIC OXIDE TO TREAT PERSISTENT PULMONARY HYPERTENSION OF THE NEWBORN IN A TERTIARY PUBLIC HOSPITAL IN SOUTH AFRICA FROM 2010-2014: MORBIDITY, MORTALITY AND COST (MMED-candidate-Dr A Mc Alpine)

Thank you for submitting your study to the Faculty of Health Sciences Human Research Ethics Committee for review.

It is a pleasure to inform you that the HREC has **formally approved** the above-mentioned study.

Approval is granted for one year until the 30th December 2015.

Please submit a progress form, using the standardised Annual Report Form if the study continues beyond the approval period. Please submit a Standard Closure form if the study is completed within the approval period.
(Forms can be found on our website: www.health.uct.ac.za/fhs/research/humanethics/forms)

Please quote the HREC REF in all your correspondence.

Please note that the ongoing ethical conduct of the study remains the responsibility of the principal investigator.

Please note that for all studies approved by the HREC, the principal investigator **must** obtain appropriate institutional approval, where necessary, before the research may occur.

We acknowledge that the student, Dr Alistair McAlpine will also be involved in this study.

Yours sincerely

PROFESSOR M BLOCKMAN
CHAIRPERSON, FHS HUMAN RESEARCH ETHICS COMMITTEE
Federal Wide Assurance Number: FWA00001637.
Institutional Review Board (IRB) number: IRB00001938

HREC 897/2014

This serves to confirm that the University of Cape Town Human Research Ethics Committee complies to the Ethics Standards for Clinical Research with a new drug in patients, based on the Medical Research Council (MRC-SA), Food and Drug Administration (FDA-USA), International Convention on Harmonisation Good Clinical Practice (ICH GCP), South African Good Clinical Practice Guidelines (DoH 2006), based on the Association of the British Pharmaceutical Industry Guidelines (ABPI),and Declaration of Helsinki (2013) guidelines.
The Human Research Ethics Committee granting this approval is in compliance with the ICH Harmonised Tripartite Guidelines E6: Note for Guidance on Good Clinical Practice (CPMP/ICH/135/95) and FDA Code Federal Regulation Part 50, 56 and 312.

Western Cape Government
Health

GROOTE SCHUUR HOSPITAL

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Professor Alan Horn
Neonatology
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E-mail: alan.horn@uct.ac.za

Dear Professor Horn

RESEARCH PROJECT EXTENSION: The Use Of Inhaled Nitric Oxide To Treat Persistent Pulmonary Hypertension Of The Newborn In A Tertiary Public Hospital In South Africa From 2010 To 2014: Morbidity, Mortality And Cost

Your recent communication to the hospital refers.

The extension of your research has been approved in accordance with UCT Ethics clearance, until **30 December 2017**.

As previously mentioned:

a) Your research may not interfere with normal patient care.
b) Hospital staff may not be asked to assist with the research.
c) No hospital consumables and stationary may be used.
d) **No patient folders may be removed from the premises or be inaccessible.**
e) Please provide the research assistant/field worker with a copy of this letter as verification of approval.
f) Confidentiality must be maintained at all times.
g) Once the research is complete, please submit a copy of the publication or report.

I would like to wish you every success with the project.

Yours sincerely

DR BERNADETTE EICK
CHIEF OPERATIONAL OFFICER
Date: 31 March 2017
BE/vms

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2.8.2 Author agreement form

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Manuscript Number:

Re: Manuscript (final typeset PDF) entitled: **(the "Work")**
The use of inhaled nitric oxide to treat persistent pulmonary hypertension of the newborn in a tertiary public hospital in South Africa from 2010-2014: morbidity, mortality, and cost

For publication in: The South African Medical Journal (SAMJ) **(the "Journal")**

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Author's signature:

Date:

2.8.3 SAMJ Instructions to authors

(From: http://www.samj.org.za/index.php/samj/about/submissions, accessed on 26th May 2017)

Manuscript preparation

Preparing an article for anonymous review

To ensure a fair and unbiased review process, all submissions are to include an anonymised version of the manuscript. The exceptions to this are Correspondence, Book reviews and Obituary submissions.

Submitting a manuscript that needs additional blinding can slow down your review process, so please be sure to follow these simple guidelines as much as possible:

• An anonymous version should not contain any author, affiliation or particular institutional details that will enable identification.

• Please remove title page, acknowledgements, contact details, funding grants to a named person, and any running headers of author names.

• Mask self-citations by referring to your own work in third person.

General article format/layout

Accepted manuscripts that are not in the correct format specified in these guidelines will be returned to the author(s) for correction, which will delay publication.

General:

• Manuscripts must be written in UK English.

• The manuscript must be in Microsoft Word or RTF document format. Text must be single-spaced, in 12-point Times New Roman font, and contain no unnecessary formatting (such as text in boxes).

• Please make your article concise, even if it is below the word limit.

• Qualifications, full affiliation (department, school/faculty, institution, city, country) and contact details of ALL authors must be provided in the manuscript and in the online submission process.

• Abbreviations should be spelt out when first used and thereafter used consistently, e.g. 'intravenous (IV)' or 'Department of Health (DoH)'.

• Include sections on Acknowledgements, Conflict of Interest, Author Contributions and Funding sources. If none is applicable, please state ‘none’.

• Scientific measurements must be expressed in SI units except: blood pressure (mmHg) and haemoglobin (g/dL).

• Litres is denoted with an uppercase L e.g. 'mL' for millilitres).

• Units should be preceded by a space (except for % and ºC), e.g. '40 kg' and '20 cm' but '50%' and '19ºC'.

• Please be sure to insert proper symbols e.g. μ not u for micro, a not a for alpha, b not B for beta, etc.

• Numbers should be written as grouped per thousand-units, i.e. 4 000, 22 160.

• Quotes should be placed in single quotation marks: i.e. The respondent stated: '...'

• Round brackets (parentheses) should be used, as opposed to square brackets, which are reserved for denoting concentrations or insertions in direct quotes.

• If you wish material to be in a box, simply indicate this in the text. You may use the table format – this is the only exception. Please DO NOT use fill, format lines and so on.

SAMJ is a generalist medical journal, therefore for articles covering genetics, it is the responsibility of authors to apply the following:

- Please ensure that all genes are in italics, and proteins/enzymes/hormones are not.

- Ensure that all genes are presented in the correct case e.g. TP53 not Tp53.

**NB: Copyeditors cannot be expected to pick up and correct errors wrt the above, although they will raise queries where concerned.

- Define all genes, proteins and related shorthand terms at first mention, e.g. '188del11' can be glossed as 'an 11 bp deletion at nucleotide 188.'

- Use the latest approved gene or protein symbol as appropriate:

• Human Gene Mapping Workshop (HGMW): genetic notations and symbols

• HUGO Gene Nomenclature Committee: approved gene symbols and nomenclature

• OMIM: Online Mendelian Inheritance in Man (MIM) nomenclature and instructions

• Bennet et al. Standardized human pedigree nomenclature: Update and assessment of the recommendations of the National Society of Genetic Counselors. J Genet Counsel 2008;17:424-433: standard human pedigree nomenclature.

Preparation notes by article type

Research

Guideline word limit: 4 000 words

Research articles describe the background, methods, results and conclusions of an original research study. The article should contain the following sections: introduction, methods, results, discussion and conclusion, and should include a structured abstract (see below). The introduction should be concise – no more than three paragraphs – on the background to the research question, and must include references to other relevant published studies that clearly lay out the rationale for conducting the study. Some common reasons for conducting a study are: to fill a gap in the literature, a logical extension of previous work, or to answer an important clinical question. If other papers related to the same study have been published previously, please make sure to refer to them specifically. Describe the study methods in as much detail as possible so that others would be able to replicate the study should they need to. Results should describe the study sample as well as the findings from the study itself, but all interpretation of findings must be kept in the discussion section, which should consider primary outcomes first before any secondary or tertiary findings or post-hoc analyses. The conclusion should briefly summarise the main message of the paper and provide recommendations for further study.

Select figures and tables for your paper carefully and sparingly. Use only those figures that provided added value to the paper, over and above what is written in the text. Do not replicate data in tables and in text .

Structured abstract

• This should be 250-400 words, with the following recommended headings:

◦ Background: why the study is being done and how it relates to other published work.

◦ Objectives: what the study intends to find out

◦ Methods: must include study design, number of participants, description of the intervention, primary and secondary outcomes, any specific analyses that were done on the data.

◦ Results: first sentence must be brief population and sample description; outline the results according to the methods described. Primary outcomes must be described first, even if they are not the most significant findings of the study.

◦ Conclusion: must be supported by the data, include recommendations for further study/actions.

• Please ensure that the structured abstract is complete, accurate and clear and has been approved by all authors.

• Do not include any references in the abstracts.

Here is an example of a good abstract:

Background. Despite enormous strides in preventing hepatitis B virus (HBV) infection, perinatal transmission still contributes significantly to HBV epidemiology worldwide; this could account for approximately 50% of chronically infected individuals.

Objective. To assess the need for HBV screening in antenatal clinics in the HIV/AIDS era.

Methods. This was a retrospective study conducted at the antenatal clinic of 1 Military Hospital, Tshwane, South Africa. Laboratory data for HBV, HIV and CD4 count were obtained and analysed for the period January 2008 December 2013.

Results. A total of 2 513 patients' results were retrieved and 2 368 patients were enrolled as both their HBV and HIV serology results were available. The mean age of participants was 29 years (range 14 46). HIV prevalence in this study was 20.5% (95% confidence interval (CI) 0.189 0.222). The median CD4 count in HIVinfected patients was 522 cells/μL (interquartile range 370 711). There was an overall HBV prevalence of 0.8% (95% CI 0.005 0.011). The hepatitis B surface antigen (HBsAg) prevalence was significantly higher (2.1%) among HIV coinfected compared with HIVuninfected patients (0.4%) (p=0.0001). Hepatitis e antigen (HBeAg) positivity was 30% in the HIV coinfected compared with 37.6% in the HIVuninfected individuals (p=0.7400).

Conclusion. This study showed a significantly higher HBV prevalence in HIVinfected compared with HIVuninfected patients. The comparable HBeAg prevalence between the two groups indicates that both were at an increased risk of vertical transmission, therefore demonstrating a need for antenatal screening for HBV. Since antenatal screening is often not affordable in lowincome countries, administration of HBV vaccine at birth is needed for prevention of vertical transmission.

Main article

All articles are to include the following main sections:

Introduction/Background, Methods, Results, Discussion, Conclusions.

The following are additional heading or section options that may appear within these:

• Objectives (within Introduction/Background): a clear statement of the main aim of the study and the major hypothesis tested or research question posed

• Design (within Methods): including factors such as prospective, randomisation, blinding, placebo control, case control, crossover, criterion standards for diagnostic tests, etc.

• Setting (within Methods): level of care, e.g. primary, secondary, number of participating centres.

• Participants (instead of patients or subjects; within Methods): numbers entering and completing the study, sex, age and any other biological, behavioural, social or cultural factors (e.g. smoking status,

socioeconomic group, educational attainment, co-existing disease indicators, etc)that may have an impact on the study results. Clearly define how participants were enrolled, and describe selection and exclusion criteria.

• Interventions (within Methods): what, how, when and for how long. Typically for randomised controlled trials, crossover trials, and before and after studies.

• Main outcome measures (within Methods): those as planned in the protocol, and those ultimately measured. Explain differences, if any.

Results

• Start with description of the population and sample. Include key characteristics of comparison groups.

• Main results with (for quantitative studies) 95% confidence intervals and, where appropriate, the exact level of statistical significance and the number need to treat/harm. Whenever possible, state absolute rather than relative risks.

• Do not replicate data in tables and in text.

• If presenting mean and standard deviations, specify this clearly. Our house style is to present this as follows:

• E.g.: The mean (SD) birth weight was 2 500 (1 210) g. Do not use the ± symbol for mean (SD).

• Leave interpretation to the Discussion section. The Results section should just report the findings as per the Methods section.

Discussion

Please ensure that the discussion is concise and follows this overall structure – sub-headings are not needed:

• Statement of principal findings

• Strengths and weaknesses of the study

• Contribution to the body of knowledge

• Strengths and weaknesses in relation to other studies

• The meaning of the study – e.g. what this study means to clinicians and policymakers

• Unanswered questions and recommendations for future research

Conclusions

This may be the only section readers look at, therefore write it carefully. Include primary conclusions and their implications, suggesting areas for further research if appropriate. Do not go beyond the data in the article.

Illustrations/photos/scans

• If illustrations submitted have been published elsewhere, the author(s) should provide consent to republication obtained from the copyright holder.

• Figures must be numbered in Arabic numerals and referred to in the text e.g. '(Fig. 1)'.

• Each figure must have a caption/legend: Fig. 1. Description (any abbreviations in full).

• All images must be of high enough resolution/quality for print.

• All illustrations (graphs, diagrams, charts, etc.) must be in PDF or jpeg form.

• Ensure all graph axes are labelled appropriately, with a heading/description and units (as necessary) indicated. Do not include decimal places if not necessary e.g. 0; 1.0; 2.0; 3.0; 4.0 etc.

• Scans/photos showing a specific feature e.g. Intermediate magnification micrograph of a low malignant potential (LMP) mucinous ovarian tumour. (H&E stain). –include an arrow to show the tumour.

• Each image must be attached individually as a 'supplementary file' upon submission (not solely embedded in the accompanying manuscript) and named Fig. 1, Fig. 2, etc.

Tables

• Tables should be constructed carefully and simply for intelligible data representation. Unnecessarily complicated tables are strongly discouraged.

• Large tables will generally not be accepted for publication in their entirety. Please consider shortening and using the text to highlight specific important sections, or offer a large table as an addendum to the publication, but available in full on request from the author

• Embed/include each table in the manuscript Word file - do not provide separately as supplementary files.

• Number each table in Arabic numerals (Table 1, Table 2, etc.) and refer to consecutively in the text.

• Tables must be cell-based (i.e. not constructed with text boxes or tabs) and editable.

• Ensure each table has a concise title and column headings, and include units where necessary.

• Footnotes must be indicated with consecutive use of the following symbols: * † ‡ § ¶ ‖

then ** †† ‡‡ etc.

Do not: Use [Enter] within a row to make ‘new rows’:

Rather:

Each row of data must have its own proper row:

Do not: use separate columns for n and %:

Rather:

Combine into one column, n (%):

Do not: have overlapping categories, e.g.:

Rather:

Use <> symbols or numbers that don’t overlap:

References

NB: Only complete, correctly formatted reference lists in Vancouver style will be accepted. Reference lists must be generated manually and not with the use of reference manager software. Endnotes must not be used.

• Authors must verify references from original sources.

• Citations should be inserted in the text as superscript numbers between square brackets, e.g. These regulations are endorsed by the World Health Organization,[2] and others.[3,4-6]

• All references should be listed at the end of the article in numerical order of appearance in the Vancouver style (not alphabetical order). • Approved abbreviations of journal titles must be used; see the List of Journals in Index Medicus.

• Names and initials of all authors should be given; if there are more than six authors, the first three names should be given followed by et al.

• Volume and issue numbers should be given.

• First and last page, in full, should be given e.g.: 1215-1217 not 1215-17.

• Wherever possible, references must be accompanied by a digital object identifier (DOI) link). Authors are encouraged to use the DOI lookup service offered by CrossRef:

On the Crossref homepage, paste the article title into the 'Metadata search' box.

Look for the correct, matching article in the list of results.

Click Actions > Cite

Alongside 'url =' copy the URL between { }. Provide as follows, e.g.: https://doi.org/10.7196/07294.937.98x

Some examples:

• Journal references: Price NC, Jacobs NN, Roberts DA, et al. Importance of asking about glaucoma. Stat Med 1998;289(1):350-355. http://dx.doi.org/10.1000/hgjr.182

• Book references: Jeffcoate N. Principles of Gynaecology. 4th ed. London: Butterworth, 1975:96-101.

• Chapter/section in a book: Weinstein L, Swartz MN. Pathogenic Properties of Invading Microorganisms. In: Sodeman WA, Sodeman WA, eds. Pathologic Physiology: Mechanisms of Disease. Philadelphia: WB Saunders, 1974:457-472.

• Internet references: World Health Organization. The World Health Report 2002 - Reducing Risks, Promoting Healthy Life. Geneva: WHO, 2002. http://www.who.int/whr/2002

(accessed 16 January 2010).

• Legal references

• Government Gazettes:

National Department of Health, South Africa. National Policy for Health Act, 1990 (Act No. 116 of 1990). Free primary health care services. Government Gazette No. 17507:1514. 1996.

In this example, 17507 is the Gazette Number. This is followed by :1514 - this is the notice number in this Gazette.

• Provincial Gazettes:

Gauteng Province, South Africa; Department of Agriculture, Conservation, Environment and Land Affairs. Publication of the Gauteng health care waste management draft regulations. Gauteng Provincial Gazette No. 373:3003, 2003.

• Acts:

South Africa. National Health Act No. 61 of 2003.

• Regulations to an Act:

South Africa. National Health Act of 2003. Regulations: Rendering of clinical forensic medicine services. Government Gazette No. 35099, 2012. (Published under Government Notice R176).

• Bills:

South Africa. Traditional Health Practitioners Bill, No. B66B-2003, 2006.

• Green/white papers:

South Africa. Department of Health Green Paper: National Health Insurance in South Africa. 2011.

• Case law:

Rex v Jopp and Another 1949 (4) SA 11 (N)

Rex v Jopp and Another: Name of the parties concerned

1949: Date of decision (or when the case was heard)

(4): Volume number

SA: SA Law Reports

11: Page or section number

(N): In this case Natal - where the case was heard. Similarly, (C) woud indicate Cape, (G) Gauteng, and so on.

NOTE: no . after the v

• Other references (e.g. reports) should follow the same format: Author(s). Title. Publisher place: Publisher name, year; pages.

• Cited manuscripts that have been accepted but not yet published can be included as references followed by '(in press)'.

• Unpublished observations and personal communications in the text must not appear in the reference list. The full name of the source person must be provided for personal communications e.g. '...(Prof. Michael Jones, personal communication)'.