GENE EXPRESSION PROFILES ASSOCIATED WITH CONGENITAL HEART DISEASE AMONG CHILDREN AGED 0-59 MONTHS AT MUHIMBILI NATIONAL HOSPITAL AND JAKAYA KIKWETE CARDIAC INSTITUTE, DAR ES SALAAM, TANZANIA.

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Muhimbili University of Health and Allied Sciences
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By

Haika Kanike Mariki

A Dissertation Submitted in (Partial) Fulfillment of the Requirements for the Degree of Master of Medicine (Paediatrics and Child Health) of

Muhimbili University of Health and Allied Sciences
October, 2017
CERTIFICATION

The undersigned certify that they have read and hereby recommend for acceptance by Muhimbili University of Health and Allied Sciences a dissertation entitled: “Gene expression profiles associated with Congenital Heart Disease among children aged 0-59 months at Muhimbili National Hospital and Jakaya Kikwete Cardiac Institute, Dar es salaam, Tanzania”, in (partial) fulfillment of the requirements for the degree of Master of Medicine (Paediatrics and Child Health) of the Muhimbili University of Health and Allied Sciences.

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Dr. C. Pallangyo ..............

Date............................................................

(Supervisor 3)
DECLARATION AND COPYRIGHT

I, Haika Kanike Mariki, declare that this dissertation is my own original work and that it has not been presented and will not be presented to any other University for a similar or any other degree award.

Signature ........................................................................................................ Date ........................................

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Above all, I thank God for seeing me through the process, and for providing me with amazing family and friends who have been supportive in one-way or another.
DEDICATION

To Science
ABSTRACT

Background: Congenital heart diseases (CHD) represent the most common birth defect in paediatric population. The majority of cases are caused by a combination of complex genetic alterations and environmental influences. There is still a large proportion of cardiac malformations with unknown precise origin. There is insufficient data in Africa, particularly in Tanzania on the profile of gene expression implicated to cause CHD.

Objective: To determine the gene expression profiles associated with CHD among children aged 0 – 59 months at Muhimbili National Hospital and Jakaya Kikwete Cardiac Institute in Dar es Salaam, Tanzania.

Methodology: A cross-sectional hospital based study was carried out at the Paediatric units at Muhimbili National Hospital (MNH) and Jakaya Kikwete Cardiac Institute (JKCI), which are part of the tertiary hospital in Dar es Salaam, Tanzania. Ethical clearance and permission to conduct the study was sought from MUHAS ethical committee, JKCI and MNH administration respectively. Written informed consents were administered prior to any study procedures. A structured questionnaire was used and blood sample were collected for Real-time reverse transcription Polymerase Chain Reaction (RT-PCR) and baseline routine investigations. Echocardiogram was done to confirm the presence and type of CHD. RT-PCR was performed on a Light Cycler 480 Real Time PCR system (Roche) using standard RT-PCR conditions. Results were analyzed using the Light Cycler 480 Software v1.5. Primer sequences used for RT PCR

Results: A total of 220 children were enrolled into the study, highest percentage (58%) 1 month – 2 year. RT-PCR was performed on 163 samples. NKX2-5 and GATA4 were found to be expressed low in most of the subtypes of CHD. GATA4 and HAND1 were lowly expressed most in septal defects (GATA4 in 34% of Ventricular Septal Defects (VSD) and 27% of Atrial Septal Defects (ASD), while HAND1 in 31% of VSD and 22% of ASD). In conotruncal defects, NKX2-5, TBX5, GATA4 and GATA6 were lowly expressed, with the highest being in TBX5 (29% in Tetralogy of Fallot and 27% in Truncus Arteriosus).
Conclusion and Recommendation: Expression of NKX2-5, TBX5, GATA4, HAND1 and GATA6 may signify a role in development of CHD. Further studies are recommended on protein sequencing of the genes that were lowly expressed in this study so as to know the exact mutations.
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<table>
<thead>
<tr>
<th>Acronym</th>
<th>Abbreviation</th>
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<tbody>
<tr>
<td>AAP</td>
<td>American Association of Paediatrics</td>
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<tr>
<td>ACE</td>
<td>Angiotensin Converting Enzyme</td>
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<td>ACHD</td>
<td>Acyanotic Congenital Heart Disease</td>
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<td>AHA</td>
<td>American Heart Association</td>
</tr>
<tr>
<td>ASD</td>
<td>Atrial Septal Defect</td>
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<tr>
<td>AV</td>
<td>Atrio-ventricular</td>
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<td>AVS</td>
<td>Aortic Valve Stenosis</td>
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<tr>
<td>BUN</td>
<td>Blood Urea Nitrogen</td>
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<td>CAVC</td>
<td>Complete Atrioventricular Canal Defect</td>
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<tr>
<td>CCHD</td>
<td>Cyanotic Congenital Heart Disease</td>
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<td>cDNA</td>
<td>Complementary Deoxy-ribonucleic Acid</td>
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<tr>
<td>CHD</td>
<td>Congenital Heart Disease</td>
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<td>CPL</td>
<td>Central Pathology Laboratory</td>
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<td>CRF</td>
<td>Case Record Form</td>
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<td>DNA</td>
<td>Deoxy-ribonucleic Acid</td>
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<td>ECHO</td>
<td>Echocardiogram</td>
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<td>HIV</td>
<td>Human Immunodeficiency Virus</td>
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<td>ICU</td>
<td>Intensive Cardiac Unit</td>
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<tr>
<td>JKCI</td>
<td>Jakaya Kikwete Cardiac Institute</td>
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<tr>
<td>MGL</td>
<td>Muhimbili Genetics Laboratory</td>
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<tr>
<td>MNH</td>
<td>Muhimbili National Hospital</td>
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<tr>
<td>MRI</td>
<td>Magnetic Resonance Imaging</td>
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<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>mRNA</td>
<td>Messenger Ribonucleic Acid</td>
</tr>
<tr>
<td>MUHAS</td>
<td>Muhimbili University of Health Allied Sciences</td>
</tr>
<tr>
<td>NPC</td>
<td>New Paediatric Complex</td>
</tr>
<tr>
<td>PDA</td>
<td>Patent Ductus Arteriosus</td>
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<tr>
<td>PVS</td>
<td>Pulmonary Valve Stenosis</td>
</tr>
<tr>
<td>RBC</td>
<td>Red Blood Cells</td>
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<tr>
<td>RT-PCR</td>
<td>Real-time reverse transcription Polymerase Chain Reaction</td>
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<tr>
<td>SDG</td>
<td>Sustainable Developmental Goal</td>
</tr>
<tr>
<td>TA</td>
<td>Truncus Arteriosus</td>
</tr>
<tr>
<td>TOF</td>
<td>Tetralogy of Fallot</td>
</tr>
<tr>
<td>VSD</td>
<td>Ventricular Septal Defect</td>
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<tr>
<td>WBC</td>
<td>White blood Cells</td>
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CHAPTER ONE

1.0 INTRODUCTION

1.1 Overview of CHD

Congenital Heart Disease (CHD) comprises of a heterogeneous group of cardiac malformations affecting the structure of the heart. It is one of the most common birth defects and one of the major causes of children mortality.

CHD are usually divided into two main groups depending on the presence or absence of cyanosis. Acyanotic congenital heart diseases (ACHD) refers to CHD that present without cyanosis and cyanotic congenital heart diseases (CCHD) are those presenting with cyanosis. The different types of CHD according to the American Heart Association (AHA) are summarized in Table 1 (1).

| Table 1: Types of cyanotic and acyanotic congenital heart diseases |
|------------------|------------------|
| **ACHD** | **CCHD** |
| 1. Atrial Septal Defect (ASD) | 1. Tetralogy of Fallot (TOF) |
| 2. Ventricular Septal Defect (VSD) | 2. Truncus Arteriosus |
| 3. Patent Ductus Arteriosus (PDA) | 3. d-Transposition of the great arteries |
| 4. Pulmonary Valve Stenosis (PVS) | 4. l-Transposition of the great arteries |
| 5. Coarctation of the Aorta | 5. Ebstein’s Anomaly |
| 6. Aortic Valve Stenosis (AVS) | 6. Total Anomalous Pulmonary Venous Return |
| 7. Complete Atrioventricular Canal defect (CAVC) | |

Source: American Heart Association (AHA)
1.2 Causes of CHD

Many cases of CHD are multifactorial, and result from a combination of both genetic predisposition and environmental stimulus (2). There are some chromosomal abnormalities that are related to a small percentage of congenital cardiac lesions, such as trisomy 21, 13 and 18. The recognized non-genetic causes include environmental teratogens, maternal exposures (e.g. alcohol, antiseizure medications) and infectious agents such as rubella. Other maternal factors include Diabetes Mellitus and Phenylketonuria. Familial CHD mutations occur as autosomal dominant, autosomal recessive or X-linked traits (3).

There are genes that have been implicated to cause CHD. These include NKX2.5, MeF2, TBX5, GATA4, HAND1, HAND2, GATA6, NOTCH1 and TFAP28 (4). Deletion of a large area of chromosome 22q11.2 (known as DiGeorge critical region) is a well characterized genetic cause of CHD. At least 30 genes have been mapped on the deleted region (2).

Most CHD are still relegated to a multifactorial inheritance pattern, resulting to a low risk of recurrence. The incidence of CHD increases to 2-6% with a subsequent pregnancy after birth of a child with CHD or if one of the parents is affected (2).

1.3 Clinical presentation for CHD

Clinical presentation of CHD depends on the degree of extent of structural defects. Range of symptoms may be mild, moderate or severe. It is unfortunate that most of the children we see in our setting present at very late stages. However, most infants would present with one of the following four presentation (5):

1. Asymptomatic murmur.

This may be caused by regurgitant valves (mitral or tricuspid) or by lesions producing turbulence in a great artery (pulmonic stenosis, aortic stenosis, non- physiological peripheral pulmonary stenosis and supravalvular aortic stenosis). An infant will usually be asymptomatic unless a critical stenosis of the pulmonary or aortic valve exists.
2. Cyanosis
Severe cyanosis after birth can be observed in transposition of great arteries, pulmonary valve atresia (with or without a VSD) once the ductus arteriosus starts to close and Ebstein’s malformation.

3. Gradually progressing symptoms of heart failure
This manifests by a collection of signs that result from excessive pulmonary blood flow or inadequate systemic blood flow. It normally develops after the natural fall of the pulmonary vascular resistance. Findings include tachypnea, sweating (especially of the forehead), difficulty feeding, failure to thrive, gallop rhythm and hepatomegaly.

4. Catastrophic heart failure and shock
These may be brought by compromised cardiac output. The symptoms can be seen in critical coarctation of the aorta, interrupted aortic arch, critical aortic valve stenosis and hypoplastic left heart syndrome. The only clues in the newborn nursery may be a single and loud S2, a marked increase in right ventricular activity on precordial palpation or a minimally abnormal post-ductal pulse oximetry reading.

1.4 Diagnostic modalities of CHD
After thorough history taking and physical examination, there are several modalities that can be used to diagnose CHD in suspected patients.

Pulse Oximeter
A pulse oximeter is an important tool that can be used to assess for cyanosis and differential cyanosis. There is a strategy of newborn pulse oximetry screening endorsed by the America Association of Pediatrics (AAP) and AHA (6). Oxygen saturation (SpO₂) should be measured in the right hand (pre-ductal) and either foot (post-ductal). A positive screen using the American Association of Pediatrics include any of the following:

- SpO2 measurement <90% in either extremity
- SpO2 measurement <95% in both upper and lower extremities on three measurements, each separated by one hour
• SpO2 difference >3% between the upper and lower extremities on three measurements each separated by one hour

**Chest radiography**

A plain *Chest X-ray* is a radiograph that is useful in CHD. It gives a simple, quick and cheap shape of the heart, size, and lung pathology (7). A cardio-thoracic ratio (CT ratio) can be calculated from it aiding in detection of enlargement of cardiac silhouette. CT ratio is the ratio of maximum horizontal cardiac diameter to maximum horizontal thoracic diameter. A normal ratio should be less than 0.5, although in the very young, it can be less than 0.6 if taken in Antero-Posterior view. The cardiac silhouette may point towards CHD, for example ‘snowman’ or ‘cottage loaf’ in total anomalous pulmonary venous drainage (7).

**Electrocardiogram**

An *Electrocardiogram* measures the heart’s conductivity. It demonstrates anatomic and hemodynamic features principally by changes in QRS and T-wave morphological features (2). A normal axis of the heart can be obtained for an electrocardiogram, and axis deviation is observed in chamber enlargement, which can be seen in CHD. Rate and Rhythm of the heart can also be assessed in this test.

**Echocardiogram**

An *Echocardiogram* a common modality used to diagnose congenital heart disease. It is a test that uses high frequency sound waves to make pictures of the heart. One will be able to visualize the pericardium, the large vessels that enter and leave the heart, presence of blood clots in the chambers and also abnormal openings between the chambers. The size and shape of the heart can be obtained, the heart’s pumping strength, size, thickness and movement of heart walls. Heart valves can also be visualized, and regurgitation, stenosis and vegetations can be seen if present. This has replaced invasive studies such as cardiac catheterization for the initial diagnosis of CHD (2).
**Other Diagnostic Modalities**

Other diagnostic modalities include cardiac catheterization and angiogram, transesophageal echocardiogram, Magnetic Resonance Imaging (MRI) and Computerized Tomography (CT) of the heart. Cardiac catheterization is an invasive procedure whereby a catheter is inserted into a chamber of vessel of the heart. A number of procedures can be performed once the catheter is in place, and these include coronary angioplasty, balloon septostomy, electrophysiology studies or catheter ablation.

**1.5 Treatment and outcome of CHD**

**Treatment**

Most of the patients who have mild CHD require no treatment, and a normal life is expected with no restrictions of the child’s activities necessary (2).

For those that are symptomatic and have progressed to heart failure, anti-failure medications are initiated before definitive treatment (surgery) is employed. The recommended anti-failure regimes include a combination of:

- Pre-load reduction agents for example Furosemide.
- Potassium sparing diuretic - Spironolactone.
- After-load reduction ACE inhibitors (e.g. Captopril).
- Increase heart contractility - Digoxin.

A child may need open heart surgery if the defect cannot be fixed using a catheter procedure. Number of surgeries depends on the complexity of the defect. Others one surgery can fix the defect completely, while others need a series of surgeries over months or years to fix the problem. Surgery may be done to:-

- Close the holes in the heart with stitches or with a patch
- Repair or replace heart valves
- Widen arteries or openings to heart valves
- Repair complex defects, such as problems with where the blood vessels near the heart are located or how they developed
Surgery of very serious CHD, such as TGA, must be done in infancy in order to minimize complications. However, some of the defects do not become a problem until many years later.

**Outcome**

With proper and timely intervention, survival of children with CHD has been improving, and more of them reach adulthood and reproduce. A retrospective population-based cohort study in infants born with structural CHD looking at temporal survival concluded that the one year survival for infants has been improving over time (8).

Children with CHD in Africa have a dramatically different prognosis to those living in developed nations, in terms of morbidity and mortality (9). A recent review of the profile of CHD in newborns in Bizerte, Tunisia, a middle-income country, revealed that medical treatment was indicated in 46.3% and surgery in 22.5% of patients with a very high case-fatality rate of 23.8% over a 9-year period (10).

Based on clinical observation and experience at Muhimbili National Hospital, prognosis of most Tanzanian children is poor due to late presentation and late diagnosis/referral. Most of the children we see present with complications that contribute to delayed surgical treatment.

Chances for definitive surgical treatment, is usually low due to cost, and the dependence of visiting surgical teams to perform some of the procedures, which are not done by the local surgical team at our institute. At Muhimbili National Hospital (MNH), performance of paediatric cardiac surgeries at the Jakaya Kikwete Cardiac Institute (JKCI) started in July 2015, where 12 patients were operated that month. Proportion of diseases requiring surgery at the institute were 36%, 24%, 11% and 6% for PDA, ASD/VSD, TOF and PVS respectively.
1.6 The Burden of CHD

The birth prevalence of CHD is thought to be relatively similar worldwide with variations between regions and countries due to genetic, environmental and epigenetic differences (11).

Several recent publications have profiled the epidemiology of CHD in children and adults in Africa, emphasizing the burden of CHD among patients referred with suspected heart disease (12–14). Among children and adults with suspected heart disease the prevalence appears to be dramatically much higher according to recent data from Sudan (15). Out of the five hundred and twenty two patients, 435 had abnormal hearts, with 87% being CHD (15). In Cameroon, 13.1% of patients with suspected cardiac pathologies, aged between 2 months and 41 years (mean age 10±9 years), during a 4-year study period, were diagnosed with CHD (16). A survey conducted in Mozambique provided an opportunity to assess the prevalence of CHD in the general population of public school children in Maputo (17). Five children (out of 2170) had CHD giving a prevalence of 2.3 in 1000 of which 80% were newly discovered.

Data from northern Nigeria reported that among 1312 patients (aged 9 days to 35 years) with abnormal echocardiograms, 122 (9.3%) had CHD (18). The predominant lesions noted were ventricular septal defect and among cyanotic lesions, Tetralogy of Fallot.

There is very few data on children with CHD in Tanzania. In a study done by Sanga (2007), looking at pattern of cardiac disease at MNH from patients 7 years old and above, 4.4% of the study participants had CHD (19). JKCI is the only cardiac institute in the country, and it caters for both adults and children. In the first three months of doing paediatric surgeries in June 2015, they conducted 32 surgeries, with a current estimate of performing 120 paediatric surgeries a year. In December 2015, there were a total of 440 admissions in the two general paediatric wards at MNH as reported in the ward’s report books. Out of all the admissions, 72 (%) were due to CHD. There were 32 deaths in that month, with 9 deaths (28%) due to CHD as underlined cause of death.
1.7 Genetics of CHD and the role of genetic screening.

Cardiac development is a fine-tuned process governed by complex transcriptional networks, in which transcription factors interact with other regulatory layers. The core cardiac transcription factors include Gata, Hand, NKx2, Mef2, Tbx and Srf. Their disruption leads to various cardiac phenotypes in mice and mutations in humans have been associated with Congenital Heart Defects (4).

Genetic counseling of couples with either a child with CHD or a family member with CHD is very important. This is because the risk of having a child with CHD increases with such a family history. Genetic screening may follow the counseling. With this risk in mind, foetal echocardiography can be done early during pregnancy, and if positive, plans for intervention of the unborn child can be done early, thus saving the child and avoiding complications.

RT-PCR has revolutionized the quantification process of DNA and RNA fragments, allowing precise quantification of these nucleic acids with greater reproducibility (20). The technique provides a sensitive method for the accurate quantification of individual species, which could be very relevant to the diagnosis of pathogens and genetic diseases. Two small primers are usually used in the process. A primer is a small oligonucleotide that is complementary to a portion of the message being looked for. Oligonucleotides are relatively short stretches of DNA, about 20 bases. Advantages of RT-PCR include the ease of quantification, greater sensitivity, reproducibility and precision, rapid analysis, better control of quality in the process and a lower risk of contamination(20).
Despite advances in clinical and surgical management, understanding the etiology of CHD remains incomplete. Advances in understanding the molecular aspects of normal heart development, including the identification of transcriptional regulators, signaling molecules, and structural genes have mainly come from animal experiments in mice, zebra, fish, and frogs. Despite the differences in the structure of the hearts in these animals and many others, many of the genes involved in regulating cardiogenesis are highly conserved amongst all higher vertebrates.
CHAPTER TWO

2.0 LITERATURE REVIEW

The epidemiological patterns of heart disease differ greatly between developed nations and sub-Saharan countries. The birth prevalence of CHD is thought to be relatively similar the world over, with variations between regions and countries due to genetic, environmental and epigenetic differences (11). The estimate of ~8 per 1000 live births is generally accepted as the most reliable, however a recent systematic review emphasized potential significant differences, with the lowest prevalence rate observed in Africa, particularly among lowest-income populations (21). The low prevalence may be explained by the lack of data.

In the systematic review by Van der Linde et al, total CHD birth prevalence worldwide was increasing substantially over time, from 0.6 per 1,000 live birth in 1930 to 1934 to 9.1 per 1,000 live births in 1995 (21). Over the last 15 years, stabilization occurred, corresponding to 1.35 million newborns with CHD every year. Significant geographical differences were found. Asia reported the highest CHD birth prevalence, with 9.3 per 1,000 live births, with relatively more pulmonary outflow obstructions and fewer left ventricular outflow tract obstructions. Reported total CHD birth prevalence in Europe was significantly higher than in North America (8.2 per 1,000 live births vs. 6.9 per 1,000 live births).

CHD and cardiac dysmorphogenesis can be caused by cardiac teratogens, which are environmental or physical factors. Generally, teratogens are thought to be environmental toxins, to which the mother and fetus are exposed, but human conditions, such as different demographic categories and maternal diseases, can be considered as risk factors. In a review of the current state of knowledge regarding non-inherited risk factors for structural cardiac anomalies, maternal environmental exposure such as organic solvent, herbicides, pesticides, air quality, water chlorination byproducts were found to be possible risk factors (22).
Advanced maternal age (35-40 years) was found to be associated with increased risk of all heart defects (OR 1.12; 95% CI 1.03 to 1.22). Maternal stress measured by maternal reports of job loss, divorce, separation or death of a close relative or friend was found to be associated with increased risk of conotruncal heart defects (22).

While there have been many studies that correlate maternal influences with congenital disorders, a review by Jonathan Day et al showed that paternal influences can cause birth defects via epigenetic mechanisms such as DNA methylation, histone modification and miRNA expression (23).

A small percentage of CHD are related to known chromosomal abnormalities in particular trisomy 21 (40 - 50% of patients), trisomy 13 & 18 (>90% of patients) and Turner syndrome (20 - 50% of patients) (24–26). A well-characterized genetic cause of congenital heart disease is the deletion of the large region of chromosome 22q 11.2, known as Di George critical region, with TBX1 as the implicated gene.

In 1998, Schott et al described heterozygote mutations in NKX2.5 that resulted in atrioventricular (AV) conduction block; many genotype-positive individuals also had a secundum atrial septal defect (ASD) (27). Based on findings in fly and mouse, these results were unexpected. Subsequent family studies identified extensive variable expressivity; in addition to AV block and ASD, genotype positive individuals also had ventricular septal defect, Tetralogy of Fallot (TOF), double outlet right ventricle, and tricuspid valve abnormalities, including Ebstein anomaly (28). Together these results suggest an essential role for NKX2.5 in atrial, ventricular, and conotruncal septation, AV valve formation, and maintenance of AV conduction.

NKX2.5 is an NK-homeobox transcription factor (29). Expression of a murine Nkx2.5 homeobox gene (also described as cardiac-specific homeobox gene) is observed in early cardiac mesoderm and in heart muscle lineage throughout life (30–32).
Khositseth A et al performed a study to determine the frequency of 22q11 deletions and associated phenotypic features and abnormalities in conotruncal heart defects (33). Sixty-one patients with the defects were enrolled, and screened for 22q11 deletions by the fluorescence in situ hybridization technique. Nine of the 61 patients had 22q11 deletions. The deletions were seen in 100% of Interrupted Aortic Arch patients, 50% of Truncus Arteriosus patients, 33.3% of Subpulmonary VSD patients, 33.3% of Pulmonary Atresia/VSD patients and 3.1% of TOF patients.
2.1 Problem Statement

Congenital heart disease (CHD) defines a large set of structural and functional deficits that arise during cardiac embryogenesis. Causes are often portioned into genetic and non-genetic categories.

Data on CHD in Tanzania is still scarce, despite the burden of the disease. While early postoperative mortality in children deemed fit for surgery may be as low as 4%. The long-term outcome of children sent abroad for surgery is associated with a high mortality rate (18.8%) in part related to the delay prior to definitive treatment (34). Yet, the majority of congenital cardiac lesions are simple and potentially correctable with suitable medical and surgical facilities. Instead, children living with these lesions in many parts of Africa will die in early teens or adulthood from the long-term effects of cyanosis, pulmonary hypertension and cardiac failure (35). Advances in clinical care, such as surgical intervention, have enabled most patients to reach adulthood, however, it has been achieved at a cost as many patients suffer late complications (with heart failure and arrhythmias being prominent(36)).

There have been speculations on the risk factors for CHD, but are these the same in our settings? There is no data on causative risks in Tanzania and Africa as a whole. No genetic studies have also been done in our settings and therefore we do not know if the speculated implicated genes that cause CHD will be the same.

With the burden on infectious diseases in Tanzania, there is less concentration on the burden of non – communicable diseases. The 4th target of 3rd sustainable development goal states that “By 2030, reduce by one third premature mortality from non-communicable diseases through prevention and treatment and promote mental health and well being”(37). As progress is made towards achieving this goal, further reductions of premature mortality from non-communicable diseases will be achieved by early interventions, such as gene therapy. Cardiovascular gene therapy is the third most popular application for gene therapy, at 8.4% of trials (38). Implicated genes to cause CHD such as NKX2.5 are termed “modifiers” because their effect on the disease trait, i.e., normal or abnormal, are only manifest in the presence of the deleterious mutation. If the genetic modifiers that permit an
embryonic heart to develop normally despite a serious mutation were understood, a drug might be developed that mimics a protective gene. The drug could be prescribed to expectant mothers to prevent a heart defect from forming in their child.

2.2 Conceptual Frame Work

Several factors have been linked to increase the risk of developing CHD. Genetic mutation is one of the factors. These mutations lead to abnormal cardiomyocyte differentiations, leading to CHD. Without interventions, the CHD lead to long-term morbidity, and in some cases, mortality.
2.3 Rationale

In Africa, the vast majority of congenital cardiac lesions are not diagnosed prior to birth, due to severely limited antenatal screening for CHD. Early diagnosis of simple lesions can result in timely referral before onset of permanent sequelae yet remains hampered by limited resources.

Advances in understanding the molecular aspects of normal heart development, including the identification of transcriptional regulators, signaling molecules, and structural genes have mainly come from animal experiments in mice, zebra, fish, and frogs. Despite the differences in the structure of the hearts in these animals and many others, many of the genes involved in regulating cardiogenesis are highly conserved amongst all higher vertebrates. More research needs to be directed in investigating humans to better understand the pathogenesis of human CHD.

This study investigated the prevalence of genetic mutations among children with CHD, which provide an overview of the association. Furthermore, it shows the relative expressions of the genes implicated in causing CHD.

The findings of this study support the knowledge in screening for CHD, thus early diagnosis and early intervention in the management of CHD. Early genetic screening can be easy than doing an ECHO as blood samples can be easily collected and transported to Muhimbili Genetic Laboratory (MGL) for analysis. Genetic associations support the understanding of the etiology, finding novel treatments and precautions. Positive screens can further be investigated for follow up ECHO even in absence of symptoms. This can minimize the problem currently faced by health care providers of receiving children with CHD at a very late step with complications. With future pregnancies, these women may be prioritized for prenatal fetal CHD echocardiography screening, which currently cannot be performed to all pregnant women at JKCI.
The findings of this study contribute to academic literature, considering the scarcity of data in Tanzania on CHD, especially as we move towards attaining the fourth target of the third SDG. Despite remarkable progress in understanding cardiac development, the mechanisms underlying the cardiac anomalies are largely unknown, even when related to mutation of a single gene (39). It is an eye opener to further studies in this particular field.

Due to complications of advanced interventions such as surgery, alternative treatment towards correcting abnormal developing hearts, such as prenatal cellular and genetic therapy, will offer tremendous potential in the future (40,41). Understanding the genetic alteration and molecular pathways underlying CHD is essential to develop novel therapeutic strategies.
2.4 Research Question and Objectives

2.4.1 Research Question
Are there any gene expressions associated with development of Congenital Heart Disease in our setting?

2.4.2 Broad Objective
To determine the genetic mutations associated with CHD amongst children aged 0 – 59 months at Muhimbili National Hospital and Jakaya Kikwete Cardiac Institute in Dar es Salaam, Tanzania.

2.5 Specific Objectives
1. To describe the pattern of CHD in children aged 0 – 59 months presenting at MNH and JKCI.
2. To determine the types of genes lowly expressed in patients with CHD.
CHAPTER THREE

3.0 RESEARCH METHODOLOGY

3.1 Study Area

The study was conducted at MNH and JKCI, both located in Ilala district in Dar es Salaam, Tanzania. MNH is a 1,400-bed facility and is national referral and teaching hospital for the country. It serves as a second level referral hospital for the whole country. JKCI is the only cardiac institute in the country within the National hospital. The JKCI receives international collaboration from SACH Israel, Open Heart International, Australia & Mending Kids International, California (permanent) and Saudi & Turkish (Sporadic). Future collaborations are anticipated from Germany, Ireland and USA.

The paediatric cardiac unit is at the New Paediatric Complex (NPC), in one of the two general wards (Ward A). The unit receives all confirmed cardiac cases from other wards. Children are also seen at JKCI everyday, where ECHO is performed. After cardiac surgery, these children are usually admitted at JKCI until stable. The Muhimbili Neonatal Unit is located in the Maternity block. It is divided in three main areas, the premature ward, birth asphyxia ward and the infectious ward. Neonates confirmed to have CHD are admitted in any of the wards depending on the category that fits best.

3.2 Study Design

This was a hospital based cross sectional study.

3.3 Study Population

The study population included all children under five years of age admitted at Paediatric cardiac unit at MNH and those attending Paediatric cardiac clinic at JKCI.

3.4 Study Duration

The study was done in a period of three months, from December 2016 to March 2017.
3.5 Sample Size
Sample size was estimated using the Leslie Kish formula (42):

\[ n = \frac{z^2 p(100 - p)}{\varepsilon^2} \]

Where:

\( Z = \) Level of confidence (1.96 for 95% confidence level)
\( P = \) Expected proportion = 14.6% (Khositseth A et al, prevalence of chromosome 22q11 deletion among patients with conotruncal heart defects (33). This study was used because there were no other studies in our setting that talked about genetic part of CHD.
\( \varepsilon = \) Margin of error = 5%

Therefore,

\[ n = \frac{1.96^2 \times 14.6 (100 - 14.6)}{5^2} \]
\[ = 192. \]

3.6 Inclusion Criteria
All children seen as in-patients or outpatients at MNH and JKCI, and whose mothers consented were included.

3.7 Exclusion Criteria
- Children with CHD and with obvious dysmorphic features such as low set ears, large protruding tongue, flat nasal bridge, and webbed neck.
- Children diagnosed with infections that predispose to CHD such as Congenital Rubella Syndrome.

3.8 Sampling Procedure
All patients who met the inclusion criteria were consequently sampled until the desired sample size was reached.
3.9 Data Collection
All children included in the study were assigned a code number and given a card of participation. This was to avoid repetition of patients for example during re-admission. All information was recorded in a structured Case Record Form (CRF) considering the objectives of the study.

ECHO was done at JKCI by a Paediatric Cardiologists using a GE Vivid S5 machine or with Siemens SC2000 with 3D, 2D & TEE probes. For neonates, Philips SONOS 7500 was used because it has a neonatal probe.

3.10 Blood Specimen Collection
Blood collection was done from the anterior cubital fossa after swabbing the area with alcohol and allowing it to fully dry before carrying out the procedure. Blood (one – two millimeters) was drawn and immediately sequestered in a purple-capped vacu-container test tube to prevent clotting, then transferred into special tubes for storage awaiting analysis. The syringes, swabs and gloves were disposed in an appropriate and safe manner. The storage tubes were clearly labelled with the patient’s identifying code number. The blood sample was then transported to the MUHAS microbiology laboratory for storage at -80°C within 2 hours from time of collection. Analysis of the samples was done in the Muhimbili Genetics Laboratory (MGL), where RT-PCR was done.

For the baseline investigations (HIV serology test, Hepatitis Panel, Sickling test, Full Blood Picture and Serum Urea & Creatinine), the same procedures for drawing blood was done, but the samples were taken to Muhimbili Central Pathology Laboratory (CPL) for analysis.

3.11 RNA extraction and RT-PCR analysis
RT-PCR was done looking at the most implicated genes to cause CHD, which were NKX2-5, GATA4, GATA6, HAND1, HAND2, MEF-2, NOTCH1, TBX-5 and TAFP2B. Total RNA was prepared from the tissue samples using QIAamp RNA Blood Mini Kit (Qiagen) following manufacturer’s recommendations, 1µg of the total RNA was reverse transcribed into cDNA using SuperScript II Reverse Transcriptase First-Strand cDNA Synthesis kit also according to the manufacturer’s protocol (Invitrogen).
Real Time PCR was performed on a LightCycler 480 Real Time PCR system (Roche) according to the manufacturer’s protocol using primers indicated in the table below and 2x SYBR Green MasterMix (Roche). GAPDH was used as the house-keeping gene and the results were analyzed using the $\Delta \Delta^Ct$ method (43) and presented as relative gene expression.

The PCR reaction was performed using standard RT-PCR conditions: 50°C for 2min, 10min 95°C, followed by 40 cycles of 95°C for 30s and 60°C for 1min. The results were analyzed using the LightCycler 480 Software v1.5.

Primer sequences used for RT PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward 5'→3'</th>
<th>Reverse 5'→3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>GTCAGTGGTGACATCCTGACCT</td>
<td>TGCTGTAGCCAAATTGCTTG</td>
</tr>
<tr>
<td>GATA4</td>
<td>TCAAACCGAGAAGACGGAAG</td>
<td>CTGTGAGGCTAGTGAGATGA</td>
</tr>
<tr>
<td>GATA6</td>
<td>CTCTTCCCTCGTCCTCCCTCCT</td>
<td>GTCAGGTCAGTGAAACAGCA</td>
</tr>
<tr>
<td>HAND1</td>
<td>AAAGGCCCTACTTCAGAGGC</td>
<td>TGCCTTGAAATGCTCTCAG</td>
</tr>
<tr>
<td>HAND2</td>
<td>ACGCTGCTACCTGCTGAC</td>
<td>TGGTTTTCTTGTGCTGCTG</td>
</tr>
<tr>
<td>MEF-2</td>
<td>AGCTCCTAGACGACCAACCAA</td>
<td>GGAGGAGGAGACTTTTGAGGG</td>
</tr>
<tr>
<td>NKX2-5</td>
<td>CCAGATGCTCAAAGAC</td>
<td>AAAGGACACGACACACTTG</td>
</tr>
<tr>
<td>NOTCH1</td>
<td>ACTGTGAGGACCTGGGAC</td>
<td>TTGTAGGTGTGGGGAGGTC</td>
</tr>
<tr>
<td>TBX-5</td>
<td>AGCTCTCTCCACCTCATCCA</td>
<td>TCCAAGCTGTGGATGTCTCCA</td>
</tr>
<tr>
<td>TFAP2B</td>
<td>GCCTCAATGCATCTCTCCTC</td>
<td>GTGCTGAGGGTTCAATACT</td>
</tr>
</tbody>
</table>

The statistical analysis was performed using GraphPad Prism 5 (GraphPad Software, La Jolla, CA). Genes that had a mean relative expression less than 0.5 (meaning it was expressed less than 50%) was found to be significant, meaning expressed low and vice versa.
Analysis was done on the subtypes of CHD that had more than 10 samples each, making a total of 163. These included ASD, VSD, TOF, TGA, PDA and Truncus Arteriosus. Mean of relative expression of each was stated.

3.12 Data Collection Tools
Case record forms, syringes, sterile vacutainers, 70% alcohol swabs, dry swabs. Case record forms were edited for consistency and quality check.

3.13 Ethical Consideration
Ethical clearance was obtained from the Senate Research and Publications Committee of MUHAS, the MNH administration and JKCI.

Every parent/guardian of each participant was requested to sign an informed consent form prior to recruitment. Informed consent explained to the parent/guardian the importance of the study and its benefits, of the procedures that will be carried out and the risks involved if they consent to their child participating in the study.

All children received proper management regardless of whether they participated or abstained from the study. For the newly diagnosed children with CHD, proper channeling was done for them to see a paediatric cardiologist, and for proper continuum of care.

For the children who were suspected to have CHD but had normal ECHO were counseled about the results, and given the detailed results to take back to their respective doctors for further management.
CHAPTER FOUR

4.0 RESULTS

Out of the 200 children that were studied, 115 (57.5%) were male. Most of them (58%) were between 1 month and 2 years of age, with a mean age of 23.72 months. Majorities were HIV negative.

Table 1: Baseline Characteristics of Study Population

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Frequency (n = 200)</th>
<th>Percentage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>115</td>
<td>57.5</td>
</tr>
<tr>
<td>Female</td>
<td>85</td>
<td>42.5</td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 1 month</td>
<td>7</td>
<td>3.5</td>
</tr>
<tr>
<td>1 month - 2 yrs</td>
<td>116</td>
<td>58</td>
</tr>
<tr>
<td>&gt;2 - 3 yrs</td>
<td>33</td>
<td>16.5</td>
</tr>
<tr>
<td>&gt;3 - 4 yrs</td>
<td>20</td>
<td>10</td>
</tr>
<tr>
<td>&gt;4 - &lt;5 yrs</td>
<td>24</td>
<td>12</td>
</tr>
<tr>
<td>Mean age (months)</td>
<td>23.72 ± 18.092</td>
<td></td>
</tr>
<tr>
<td>HIV Status</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>197</td>
<td>98.5</td>
</tr>
<tr>
<td>Positive</td>
<td>1</td>
<td>0.5</td>
</tr>
<tr>
<td>Exposed</td>
<td>2</td>
<td>1</td>
</tr>
</tbody>
</table>

According to the National Birth Defects Prevention Study (44), CHD was classified into the following types:- Septal Defects, atrioventricular septal defects, conotruncal defects, left ventricular outflow tract obstruction, right ventricular outflow tract obstruction, single ventricle and PDA. Most of the CHD were septal defects that constituted 37%. Among the septal defects, VSD had the highest percentage (25.5%).
Table 2 shows the pattern observed, constituting 87% of the types of CHD. Others included Tricuspid Atresia 8(4%), Complex Cardiac malformation 5(2.5%), Ectopia Carditis 1 (0.5%) and the rest were mixed septal defects and PDA 12(6%).

Table 2: Pattern of CHD of Study Population

<table>
<thead>
<tr>
<th>CHD Classification</th>
<th>Frequency</th>
<th>Percentage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Septal Defect</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ASD</td>
<td>17</td>
<td>8.5</td>
</tr>
<tr>
<td>VSD</td>
<td>51</td>
<td>25.5</td>
</tr>
<tr>
<td>VSD + ASD</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td>AVSD</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>Conotruncal Defects</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TOF</td>
<td>31</td>
<td>15.5</td>
</tr>
<tr>
<td>D-TGA</td>
<td>11</td>
<td>5.5</td>
</tr>
<tr>
<td>TA</td>
<td>17</td>
<td>8.5</td>
</tr>
<tr>
<td>DORV</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td><strong>LVOTO</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CoA</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>AS</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>IAA, A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CoA + AS</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>RVOTO</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PS/PA</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td><strong>LVOTO + RVOTO</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AS + PS</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>Single Ventricle</strong></td>
<td>1</td>
<td>0.5</td>
</tr>
<tr>
<td><strong>PDA</strong></td>
<td>30</td>
<td>15</td>
</tr>
</tbody>
</table>

ASD atrial septal defect, VSD ventricular septal defect, AVSD atrioventricular septal defect, TOF tetralogy of Fallot, D-TGA d-transposition of the great arteries, TA Truncus Arteriosus, DORV Double Outlet Right Venticle, COA coarctation of the aorta, AS aortic stenosis, IAA interruption arterial arch, PS pulmonary stenosis, PA pulmonary atresia, PDA patent ductus arteriosus, LVOTO Left ventricular outflow tract obstruction, RVOTO Right ventricular outflow tract obstruction.
Figure 1 shows the means of relative expressions of the implicated genes to cause CHD. RT-PCR was done looking on nine genes. GATA4 and NKX2-5 was observed to be expressed the lowest in most of the CHD types.

Most genes (NKX2-5, TBX5, GATA4, HAND2 and GATA6) were observed to have the lowest relative expression in TOF. This was followed by PDA, where NKX2-5, TBX5, GATA4, MEF2 and TFAP2B were lowly expressed, with the later two genes being expressed just below 50%.

All the analyzed genes were seen to have a relative expression that is above 50% in TGA.

NOTCH1 was seen to not be one of the possible implicated genes to cause CHD in this study. Other genes that had only one type if CHD expressed relatively low were MEF2 (PDA), HAND2 (TOF) and TFAP2B (PDA).
Figure 1: Means of Relative Gene Expression of implicated genes among the study population.
Table 3 shows percentage of study population that had lower gene expressions. Highest percentage was seen in GATA4 for VSD (34%), ASD (31%) and TOF (29%), as compared to TBX5, which was highest also in TOF (29%) and Truncus Arteriosus (TA) (27%)

**Table 3: Percentage of study population with significant low gene-expression levels**

<table>
<thead>
<tr>
<th></th>
<th>VSD</th>
<th>TOF</th>
<th>PDA</th>
<th>ASD</th>
<th>TA</th>
<th>TGA</th>
</tr>
</thead>
<tbody>
<tr>
<td>NKX2-5</td>
<td>13%</td>
<td>27%</td>
<td>12%</td>
<td>25%</td>
<td>19%</td>
<td>0%</td>
</tr>
<tr>
<td>MEF-2</td>
<td>0%</td>
<td>0%</td>
<td>7%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>TBX5</td>
<td>0%</td>
<td>29%</td>
<td>11%</td>
<td>0%</td>
<td>27%</td>
<td>0%</td>
</tr>
<tr>
<td>GATA4</td>
<td>34%</td>
<td>29%</td>
<td>14%</td>
<td>31%</td>
<td>25%</td>
<td>0%</td>
</tr>
<tr>
<td>HAND1</td>
<td>27%</td>
<td>0%</td>
<td>0%</td>
<td>22%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>HAND2</td>
<td>0%</td>
<td>8%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>GATA6</td>
<td>0%</td>
<td>13%</td>
<td>0%</td>
<td>13%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>NOTCH1</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>TFAP2B</td>
<td>0%</td>
<td>0%</td>
<td>4%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
</tr>
</tbody>
</table>
CHAPTER FIVE

5.0 DISCUSSION

This was a cross sectional study looking at relative gene expression of the most implicated genes to cause CHD. Although more sophisticated methods such as sequencing would provide more answers on the mutation variants, findings of this study still provides an overview on this subject, thus laying a foundation.

Out of the 200 patients enrolled in the study, RT-PCR was done on 163 patients with CHD subsets VSD, ASD, TOF, Truncus Arteriosus, TGA and PDA. Frequency of septal defects was highest, with VSD (25.5%) being top of the list. In cyanotic CHD, TOF, which accounted for 15.5% of all the patients, was the most common. This is in keeping with a prospective hospital based study that was done in Nigeria where VSD was the most prominent CHD accounting for 34.1% and TOF being the most common cyanotic CHD (12).

The number of neonates in the study was only seven, constituting only 3.5% of the study population. This could be explained by the fact that no screening is usually done during pregnancy, and no early neonatal screening, therefore contribute to late presentation of these children. Another explanation could be early mortality due to no timely intervention.

Out of the 9 genes tested, 5 genes were observed to be generally expressed low across the subtypes. These were NKX2-5, TBX5, GATA4, HAND1 and GATA6. This may mean that these transcriptional factors may be a risk in developing CHD if expressed differently due to either environmental factors or inheritable mutations. In a study by Tong 2016, both NKX2-5 and GATA4 gene mutations and gene expression were detected via DNA sequencing and RT-PCR respectively in 185 cases of CHD and 210 cases of healthy individuals (45). Gene expression of both genes was found to be lower in the cases with CHD as compared to controls, and also gene mutation was found in the CHD group. This is in keeping with the gene expression of the two genes in this study population, which may mean they participate in the development of CHD.

GATA4 and HAND1 were lowly expressed most in septal defects. GATA4 was lowly expressed in 34% of VSD and 27% of ASD, while HAND1 in 31% of VSD and 22% of
ASD. In conotruncal defects, NKX2-5, TBX5, GATA4 and GATA6 were lowly expressed, with the highest being in TBX5 (29% in TOF and 27% in TA). In a study by Yoshida et al 2016, looking at genetic mutation analysis in non-syndromic patients with CHD, they found an almost similar picture with conotruncal defects (46). TBX5 variation p.Pro108Thr, located in the T-box domain, was identified in a patient with tricuspid atresia and an exon-intron boundary variation of GATA4 (IVS4+5G>A) was detected in a Tetralogy of Fallot.

All the tested genes were expressed more than 50% in TGA, one of the common subtype in this population. This could mean that there are other risk factors that may lead to development of TGA, and are not genetically related.

The strength of this study is that it is the first of its kind thus gives an overview on whether the proposed genetic mutations associated with CHD are found in our setting. This creates more opportunity for further studies such as sequencing which will show the exact mutation variants. It also raises awareness of academia, clinicians, and geneticist in the country.

There were limitations to this study. It was taken at one centre, thus results cannot be generalized but can provide baseline information as the centre receives referred from the whole country. Due to the nature of the study, it is not possible to ascertain the causal relationship between genetic mutation and CHD, and also because the relative gene expression gives an estimate as it may be environmentally influenced, or a true mutation. The nine genes studied were mostly implicated to cause CHD in the developed world, which could not be the same in our African setting.
CHAPTER SIX

6.0 CONCLUSION

Septal defects were the most common observed CHD in the study population, with VSD having the highest percentage. Myogenic transcription factors NKX2.5, TBX5, GATA4, HAND1 and GATA6 may play a role in development of CHD if lowly expressed during cardiac development.

6.1 Recommendations

1. Further studies on protein sequencing of the genes that were lowly expressed in this study such as NKX2.5, GATA4 and TBX5 so as to know the exact mutations.

2. Genetic counseling for couples with a relative with a child with CHD, or who already have a child with CHD. This can best be done prenatally. Genetic testing may be done after the counseling and early intervention plan.
REFERENCES

1. Association AH. Symptoms & Diagnosis of Congenital Heart Defects [Internet]


Appendix 1: Consent forms (English Version)

ID NO……………………..

PERMISSION FOR A CHILD TO TAKE PART IN RESEARCH

Study title: GENE EXPRESSION PROFILES ASSOCIATED WITH CONGENITAL HEART DISEASE AMONG CHILDREN AGED 0-59 MONTHS AT MUHIMBILI NATIONAL HOSPITAL AND JAKAYA KIWETE CARDIAC INSTITUTE, DAR ES SALAAM, TANZANIA.

Introduction:
My name is Dr. Haika. I am a university student working on my thesis. Your child is being asked to take part in this study because I am trying to determine genetic mutations associated with congenital heart diseases.

Your decision whether or not to allow your child to take part will have no effect on the quality of your child’s medical care. Please ask questions if there is anything about this study you do not understand.

Will you benefit from taking part in this study?
Your child will be a volunteer and might not personally benefit from being in this research study. However, the study may help other children in the future. If you choose not to take part in the study, your child will continue to receive regular care at the hospital regardless.

What does this study involve?
Your child’s evaluation as part of this study will be done in the ward and the cardiac institute. An ECHO will be done to confirm that your child has a heart disease. I will also draw blood to test for genetic mutations that might be associated with the heart disease.

What are the risks involved with taking part in this study? I will be drawing blood from your child in order to be able to test the presence of possible genetic mutations. This is among the normal procedures for the child.
Leaving the study: You may choose to stop taking part in this study at any time for any reason. If you decide to stop taking part, it will have no effect on your child’s medical care.

New Information: New information related to this study and specifically new information about your child will be made known to you when it becomes available.

How will your privacy be protected?
The information you and your child provide as well as the test results will be kept strictly confidential. The study information will be stored in protected computer files and in paper records stored in locked filing cabinets. Only study staff will have access to the information.
The information will be maintained indefinitely.

Who may use or see your health information?
By signing this form, you allow the research team to use your child’s health information and give it to others involved in the research. The research team includes the study director plus others working on this study at MUHAS and the lab.
Your permission to use your child’s health information for this study will not end until the study is completed. You may ask for your child’s study data at any time.

It is possible for a court or government official to order the release of study data including information about your child.

What if you decide not to give permission to use and share your personal health information?
If you do not allow use of your child’s health information for this study, you may not take part in this study.
If you choose to stop taking part in this study, you may cancel permission for the use of your child’s health information. You should let the researcher know if you want to cancel your permission. The study team will assist you in putting your wishes in writing. Information collected for the study before your permission is cancelled will continue to be used in the research.
Whom should you call about this study?
If you have questions about this study or need to report a study related injury, you can call your doctor or the research director for this study: Dr. Haika Mariki (0713 553 881) during normal working hours. If you have questions about your rights as a participant, you may contact, Professor Said Aboud, Director of Publications and Research, Muhimbili University of Health and Allied Sciences, Research and Publication Committee, P. O. Box 65001, Dar es Salaam, Telephone number 2150302-6

What about the costs of this study?
There will be no costs for you if you agree to have your child participate in the study. All study costs will be supported by the research team.

Will you be paid to take part in this study?
There will be no payment to you or your child for participating in the study.
If you agree that your child take part in this study and you sign this consent form, you are not giving up any of your legal rights.

Consent
I have read the above information concerning genetic mutations associated with congenital heart diseases and have been given time to ask questions. I agree to take part in this study and I have been given a copy of this signed consent form.

Signature

_______________________________________________________

Researcher or Designee Signature and Date               Printed Name

_______________________________________________________

Legally Authorized Representative (Parent/legal guardian) and Date               Printed Name
Appendix 2: Consent Form (Kiswahili version)

RIDHAA YA KUSHIRIKI KWENYE UTAFITI HUU

Namba ya Utambulisho……………………

Utafiti: MABADILIKO YA VINASABA KWA WATOTO CHINI YA MIAKA MITANO WENYE UGONJWA WA MOYO KATIKA HOSPITALI YA TAIFA YA MUHIMBILI NA TAASISI YA MOYO YA JAKAYA KIKWETE, DAR ES SALAAM, TANZANIA.

Utangulizi:
Habari! Mimi Naitwa Dk. Haika. Mtoto wako anaombwa kushiriki katika utafiti huu kwa sababu ninafanya uchunguzi wa mabadiliko ya vinasaba kwa watoto chini ya miaka mitano wenyewe ugonjwa wa moyo waliolazwa katika Hospitali ya Taifa Muhimbili.
Uamuzi wako wa kumruhusu au kutomruhusu mtoto wako kushiriki hautakua na athari zozote kwenye ubora wa huduma ya mtoto wako. Tafadhali uliza swali kama kuna kitu usichokielewa kuhusu utafiti huu.

Je dhumuni la utafiti huu ni nini?
Dhumuni la utafiti huu ni kuchunguza mabadiliko ya vinasaba kwa watoto chini ya miaka mitano wenyewe ugonjwa wa moyo waliolazwa katika Hospitali ya Taifa Muhimbili.

Je utafaidika kwa mwanao kushiriki kwenywe utafiti huu?
Mtoto wako atapimwa kwa ridhaaa yake na inawezekana kuwa binafsi asifaidike kwa kuwepo kwenye utafiti huu. Hata hivyo, utafiti unaweza kusaidia watoto wengine katika siku zijazo. Kama ukichagua si kuchukua sehemu ya utafiti motto wako ataendelea kupokea huduma ya kawaida katika hospitali bila kujali.

Je Utafiti huu unahusisha nini?
Uchunguzi wa mtoto wako kama sehemu ya utafiti huu utafanyika wodini pamoja na jengo la moyo, ambapo mtoto atapimwa kipimo cha moyo, na hapo pia nitatoa damu na kuipeleka maabara kwa ajili ya kupima vinasaba.
Je itakuwaje kama hutataka kushiriki kwenye utafiti huu?
Kushiriki kwa mtoto wako kwenye utafiti huu ni hiari kabisa. Mtoto wako atatolewa damu kutoka mkono moja.

Je kuna athari gani za kushiriki katika utafiti huu?
Mtoto wako atatalewa damu kutoka mkono moja.

Kuondoka kwenye utafiti: unaweza kuamua kusitisha kuendelea kushiriki kwenye utafiti huu muda wowote na kwa sababu yoyote. Kama utaamua kuacha kushiriki hautaathiri huduma za afya kwa mtoto wako.

Maelezo mapya: Maelezo mapya kuhusiana na utafiti huu na hasa maelezo mapya kuhusu mtoto wako utajulishwa mara tu yatakapokuwa yamepatikana.

Utunzaji wa siri?
Habari mtakazo toa wewe na mtoto wako pamoja na majibu ya vipimo yatunzwa kwa usiri mkubwa. Habari zihusuzo utafiti zitounzwa kwenye kompyuta zenye ulinzi na rekodi, zilizopo kwenye makarata zitounzwa kwenye makabati yanayofungwa. Ni wafanyakazi wanaohakiki na utafiti huu utaamua kuwa cha kushiriki hautaathiri huduma za afya kwa mtoto.

Ni nani anaweza kutumia au kuona taarifa zako za afya?
Kwa kuweka sahihi kwenye formu hii umeruhusu utafiti kutumia taarifa za afya za mtoto wako na kuwapatikana kuona taarifa za utafiti kutolinzi na rekodi, zilizopo kwenye makarata. Na taarifa hizi zitounzwa siku zote.

Ruhusa ya kutumia taarifa za afya za mtoto wako itaisha wakati utafiti utapokamilika. Unaweza kuomba taarifa za mtoto wako wakati wowote.

Inawezekana mahakama au afisa wa serikali akaamuria kuonyeshwa kwa taarifa za utafiti ikiwa ni pamoja na taarifa za mtoto wako

Je itatokea nini kama utaamua kutotoa ruhusa ya kutumia na kushiriki kwenye taarifa zako za afya?
Kama hautaruhusu taarifa ya afya ya mtoto wako zitumike, hautaweza kushiriki kwenye utafiti huu.

**Je utampigia nani kuhusu utafiti huu?**
Kama una maswali kuhusu utafiti au ukiwa na haja ya kuripoti athari zitokanazo na utafiti, unaweza kumpigia daktari wako au mwendesha utafiti huu: Dr Haika Mariki (0713 553 881) muda wa masaa ya kazi. Kama una swali kuhusu haki zako kama mshiriki unaweza kuwasiliana na Profesa Said Aboud, Mkurugenzi ya Kitengo ya Utatifi, Chuo Kikuu Cha Sayansi na Tiba Cha Muhimbili, S.L.P 65001, Dar es Salaam, Namba ya Simu 2150302-6

**Je kuna gharama juu ya tafuli huu?**
Hakuna gharama kama utakubali mtoto wako ashiriki kwenye utafiti huu. Watafiti na Chuo kikuu cha Muhimbili watasaidia gharama zote za utafiti.

**Je utalipwa kwa kushiriki kwenye utafiti:**
Hapatakuwa na malipo kwako au mtoto wako kwa kushiriki kwenye utafiti
Kama utakubali mtoto wako ashiriki kwenye utafiti huu na ukasaini fomu hii ya ridhaa, haujiondolei haki yoyote ya kisheria.

**Ridhaa**
Nimesoma maelezo hapo juu kuhusu uchunguzi wa mabadiliko ya vinasaba kwa watoto chini ya miaka mitano wenye ugonjwa wa moyo waliolazwa katika Hospitali ya Taifa Muhimbili na nimepewa muda wa kuuliza maswali. Nakubali kushiriki katika utafiti huu na nimepewa fomu ya ridhaa hii iliyoumekwa sahihi

**Sahihi**

__________________________________________________________________________

sahihi ya mtafiti au kaimu na tarehe

Jina kamili

__________________________________________________________________________

sahihi ya mwakilishi wa kisheria

(mzazi/mlezi) na tarehe
Appendix 3: Questionnaire

Study title: GENE EXPRESSION PROFILES ASSOCIATED WITH CONGENITAL HEART DISEASE AMONG CHILDREN AGED 0-59 MONTHS AT MUHIMBILI NATIONAL HOSPITAL AND JAKAYA KIWETE CARDIAC INSTITUTE, DAR ES SALAAM, TANZANIA.

Code No ………

1. Sex …… (F/M)

2. Age ……..

3. Place of residence………………………….

4. Parents’ level of education (please circle correct)
   Mother - No formal education       Primary       Secondary       University
   Father - No formal education       Primary       Secondary       University

5. Parent’s marital status (please circle correct)
   Married       Cohabiting       Divorced

6. Parent’s tribes
   Mother …………………………       Father …………………………

7. Are the parents of the child related? …… (YES/NO)

8. If yes to question above, how?

9. Did you get ill during the pregnancy of this child?
   ……. (YES/NO)
   If yes, what? ………………………………………………………………………………………………………
   ………………………………………………………………………………………………………

10. Did you receive haematenics during pregnancy of this child?………. (YES/NO)
**ECHO findings**

**Results of Baseline Investigations**

<table>
<thead>
<tr>
<th>Test</th>
<th>Result</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIV Test</td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>Sickling Test</td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>Hepatitis B</td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>Hepatitis C</td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>Creatinine</td>
<td>Normal</td>
<td>Above normal</td>
</tr>
<tr>
<td>BUN</td>
<td>Normal</td>
<td>Above normal</td>
</tr>
</tbody>
</table>

**Full Blood Picture:**

<table>
<thead>
<tr>
<th>Component</th>
<th>Value</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBC</td>
<td>...</td>
<td>RBC</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>Abs...</td>
<td>%...</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>Abs...</td>
<td>%...</td>
</tr>
<tr>
<td>Monocytes</td>
<td>Abs...</td>
<td>%...</td>
</tr>
<tr>
<td>Platelets</td>
<td>...</td>
<td>MCH</td>
</tr>
</tbody>
</table>
Appendix 4: Dodoso

Utafiti: MABADILIKO YA VINASABA KWA WATOTO CHINI YA MIAKA MITANO WENYE UGONJWA WA MOYO WALIO LAZWA KATIKA HOSPITALI YA TAIFA YA MUHIMBILI NA TAASISI YA MOYO YA JAKAYA KIKWETE DAR ES SALAAM, TANZANIA.

Namba ya utambulisho ………………

1. Jinsia …… (Ke/Me)  
2. Umri …………………

3. Mahali pa kuishi …………………

4. `Kiwango cha elimu cha wazazi (zungushia jibu sahihi)
   Mama - Hajasoma Primari Sekondari Chuo kikuu  
   Baba - Hajasoma Primari Sekondari Chuo kikuu

5. Hali ya ndoa ya wazazi (zungushia jibu sahihi)
   Wameoana   Wanaishi tu pamoja   Wameachana

6. Kabila ya wazazi
   Mama ………………………….   Baba …………………………

7. Baba na mama ni ndugu? ………. (NDIO/HAPANA)

8. Kama baba na mama ni ndugu, undugu wao ukoje?
9. Kuna kitu chochote ambacho si cha kawaida, kama ugonjwa, kilitokea wakati wa ujauzito wa mtoto huyu?

………… (NDIO/HAPANA)

Kama ndio, ni nini? ……………………………………………………………………………
………………………………………………………………………………

10. Ulitumia dawa za kuongeza damu (Folic acid) wakati wa ujauzito wa motto huyu?

………………(NDIO/HAPANA)

**Matokeo ya ECHO**

**Majibu ya damu**

HIV Test: Positive □ Negative □  Sickling Test: Positive □ Negative □

Hepatitis B: Positive □ Negative □  Hepatitis C: Positive □ Negative □

Creatinine: Normal □ Above normal □  BUN Normal □ Above normal □

Full Blood Picture:

WBC …..  RBC…..

Neutrophils Abs….. %....... Hgb……..

Lymphocytes Abs….. %....... HCT.......

Monocytes Abs….. %....... MCV....... Platelets...... MCH…….
## Appendix 5: Case Record Form

### Genetic study – RT PCR

<table>
<thead>
<tr>
<th>Principal investigator – Haika Mariki</th>
<th>Code N° …………………</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hospital Reg. Number ............................</td>
<td></td>
</tr>
<tr>
<td>Surname ..........................</td>
<td>Other names ............................</td>
</tr>
<tr>
<td>Postal/residential Address .................</td>
<td>Date of Birth .................</td>
</tr>
<tr>
<td>Sex ......................</td>
<td>Ward ............................</td>
</tr>
</tbody>
</table>

**Clinical notes**

```
……………………………………………………………………………………………
……………………………………………………………………………………………
```

**Echo findings and Confirmed Diagnosis**

```
……………………………………………………………………………………………
……………………………………………………………………………………………
```

**For Lab Only**

```
```

**Date**  
**Responsible person for report, name, signature**