Identification of the Major Genetic Loci that Control Disease Severity in *Schistosoma mansoni* Infected Patients in Gezira Area, Sudan: A Molecular Epidemiological Study

MOHAMMED OSMAN ABD EL WAHED MADANI

A Thesis
Submitted in Fulfillment of the Requirements for the Degree of Doctor of Philosophy (Ph.D)
in
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National Cancer Institute (NCI),
University of Gezira, Sudan.

Main supervisor: Professor. Nasr Eldin M. A. Elwali

Co-supervisor: Dr. Adil Mergani Babiker Hassan

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<th>Position</th>
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<td>Professor. Nasr Eldin M. A. Elwali</td>
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<th>Name</th>
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<td>Internal examiner</td>
<td>[Signature]</td>
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</tbody>
</table>

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September, 2012
Dedication

To my father, mother, sisters, brothers, my wife and kids
Acknowledgements

I would like to express my special thanks to my supervisor, professor. Nasr Eldin Elwali, Professor of Molecular Biology, University of Khartoum, for his wonderful guidance during my studies. His encouragement and constructive criticisms have provided a great basis for the present thesis. My gratitude goes to my co-supervisor, Dr. Adil Mergani Babker, Associate Professor of Molecular Biology, University of Gezira and our project collaborator Professor Alian Dessein, for giving me the opportunity to work in his laboratory in France, his continuous fruitful discussion, analysis and help during my works in his laboratory help me to finish my work. Thanks, one word for Dr. Christophe Chevillard and I think it is not enough for his understanding, great support and helpful advice during my study. My thanks are extended to his family.

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Identification of the Major Genetic Loci that Control Disease Severity in *Schistosoma mansoni* Infected Patients in Gezira Area, Sudan: A Molecular Epidemiological Study

MOHAMMED OSMAN ABD EL WAHED MADANI
Ph.D In Molecular Biology, September /2012
Department: Molecular Biology
National Cancer Institute (NCI) - University of Gezira

Abstract

Hepatic periportal fibrosis is a severe consequence of *Schistosoma mansoni* infection; the disease affects 5-10% of the infected population in the endemic areas. The aims of the current study were to evaluate the epidemiological factors, and to study the genetic background which may play a role in *S. mansoni* pathogenesis. Nine hundred and forty one subjects were examined by ultrasound using Niamey protocol. DNA from three hundred and seventy two participants, was obtained from peripheral blood. Samples were genotyped for three genes (Interleukin-13, Interleukin-10 and Connective Tissue Growth Factor), by using a predesigned TagMan allele discrimination assay. Electronic mobility shift assay was done to assess the effect of four SNPs on CTGF gene expression. Severe hepatic fibrosis was observed in 13.6% of the study subjects. Central periportal fibrosis was associated with gender ($P = 0.0001; \text{OR} = 2.735$), ascites ($P < 0.000001$), varices ($P < 0.0000001$), splenic volume ($P < 0.0001$), and ethnic group ($P < 0.0001$). In multivariate analysis IL-10-1082 A alleles ($P = 0.044; \text{OR} = 2.8$), and IL-10-819 T ($P = 0.054; \text{OR} = 2.3$), were associated with severe hepatic fibrosis. Close to CTGF gene two SNPs rs9402373 ($P = 0.008; \text{OR} = 5.2$) and rs1256196 ($P = 0.059; \text{OR} = 7.3$) were independently associated with severe hepatic fibrosis, electronic mobility shift assay revealed that these polymorphisms are affecting nuclear factor binding. In conclusion, the current study suggests that gender, genetic, and ethnic background of study subjects are risk factors in fibrosis development. Furthermore, the polymorphisms of IL-10-1082A alleles, rs9402373 and rs1256196 are predictors of the progression of the hepatic fibrosis disease and could be possible targets for treatment and vaccination.
تحديد المواقع الوراثية الرئيسية التي تتحكم في شدة المرض في المرضى المصابين بالمنشقة المعوية في منطقة الجزيرة، السودان: دراسة وراثية جزيئية

محمد عثمان عبد الواحد مدني
دكتوراه الأحياء الجزيئية – سبتمبر / 2012
المعهد القومي للسرطان – جامعة الجزيرة

الخلاصة

تليف الوريد الكبدي البابي هو نتيجة حتمية للإصابة الحادة بالمنشقة المعوية، هذا المرض يصيب حوالي 5-10% من عدد السكان المصابين في المناطق التي يتوطن بها المرض. هدفت الدراسة الحالية إلى تقييم العوامل الوراثية في تطور مرض تليف الوريد ودراسة العوامل الوراثية التي تفاقم الإصابة بالمنشقة المعوية. تم فحص تسعمائة وواحد وأربعون شخصاً بواسطة الموجات فوق الصوتية باستخدام برتوكول (نيامي). تم جمع الدم من الوريد الطرفى لإجراء التحليل الوراثي لثلاثة من المورثات وهي (انترليوكين-13 و انترليوكين-10 وعامل نمو النسيج الضام) بواسطة تقنية التفاعل السلسلى البلمرى (TagMan) المسبقة التحضير. تم إجراء التحليل الوظيفي لأربعة مواقع وراثية في مورث (عامل نمو النسيج الضام) بواسطة تقنية الفحص الإلكتروني للتحول النقلي. وجد 13.6% من الأشخاص الخاضعين للدراسة مصابين بالتليف الكبدي البابي المركزي. التحليل الإحصائي أثبت وجود ارتباط عوامل الجنس (ذكر - أنثى) (P = 0.0001; OR = 2.735) والاستسقاء (P = 0.000000001) والمجموعات القليلة (P = 0.000000001) بالتيتر الكبدي البابي المركزي. في التحليل متعدد المتغيرات (A) في الموقع 1082-10-10 بالتيتر الكبدي البابي، بالقرب من مورث عامل نمو النسيج الضام (P = 0.054) في الموقع 819-10-10، و (P = 0.008; OR = 5.2) في الموقع rs 9402373، و (P = 0.059; OR = 7.3) في الموقع rs 1256196، و (P = 0.008; OR = 5.2) في الموقع rs 9402373، و (P = 0.059; OR = 7.3) في الموقع rs 1256196، و (P = 0.059; OR = 7.3) في الموقع rs 1256196، و (P = 0.059; OR = 7.3) في الموقع rs 1256196، و (P = 0.059; OR = 7.3) في الموقع rs 1256196، و (P = 0.059; OR = 7.3) في الموقع rs 1256196، و (P = 0.059; OR = 7.3) في الموقع rs 1256196. وقد وجد أنهما مرتبطان بصفة مستقلة مع تليف الوريد الكلبي. ويستخدم تقيية الفحص الإلكتروني للتحول النقلي وجد أن هذا التغير الشكلي المتعدد يؤثر على عامل ارتباط البروتينات النووية. وقد خلصت هذه الدراسة إلى أن العوامل المتعلقة بالجنس والخلفية الوراثية للمشاركين في الدراسة تعتبر مهمة في تطور التليف الكبدي البابي. إضافة إلى ذلك الأشخاص المتعدد المتغيرات الوراثية rs 9402373، تمت مؤشرات جيدة لمعرفة تطور المرض ويمكن أن تكون هدفاً لتلقي العلاج والتطعيم.
Table of Contents

<table>
<thead>
<tr>
<th>Title</th>
<th>Pages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Signature</td>
<td>ii</td>
</tr>
<tr>
<td>Dedication</td>
<td>iii</td>
</tr>
<tr>
<td>Acknowledgement</td>
<td>iv</td>
</tr>
<tr>
<td>Abstract (English)</td>
<td>vi</td>
</tr>
<tr>
<td>Abstract (Arabic)</td>
<td>vii</td>
</tr>
<tr>
<td>Table of Contents</td>
<td>viii</td>
</tr>
<tr>
<td>List of Tables</td>
<td>xvi</td>
</tr>
<tr>
<td>List of Figures</td>
<td>xviii</td>
</tr>
<tr>
<td>List of Abbreviations</td>
<td>xx</td>
</tr>
</tbody>
</table>

**Chapter One**

**Introduction**

1.1. Prevalence of schistosomiasis 1
1.2. Schistosomiasis mortality and morbidity 3
1.3. Schistosomiasis in Sudan 4
1.4. Schistosoma life cycle 5
1.5. S. mansoni infection and disease 7
   1.5.1. Acute schistosomiasis 7
   1.5.2. Chronic pathology and morbidity 7
     1.5.2.1. Intestinal schistosomiasis 8
     1.5.2.2. Hepatic schistosomiasis 8
     1.5.2.3. Ectopic schistosomiasis 9
     1.5.2.4. Genital schistosomiasis 9
1.5.2.5. Neuroschistosomiasis

1.6. Schistosomiasis diagnosis

1.6.1. Microscopic examination

1.6.2. Histological techniques

1.6.3. Immunological techniques

1.6.4. Imaging techniques in assessment of S. mansoni morbidity

1.6.4.1. Diagnosis and clinical finding of liver fibrosis by ultrasonography

1.4.4.2. Computerized tomography and magnetic resonance imaging

1.7. Treatment and control of schistosomiasis

1.7.1. Schistosomiasis treatment

1.7.2. Control of schistosomiasis

1.8. Role of Th cells, cytokines, and macrophages in hepatic fibrosis

1.8.1. Granuloma formation in schistosomiasis

1.8.1.1. Cellular components of granuloma

1.8.1.2. Chemokines and granulomatous inflammation in S. mansoni infection

1.8.2. Role of Th1, Th2, and Th17 lymphocytes in hepatic fibrosis

1.8.3. Role of regulatory T cells in hepatic fibrosis

1.8.4. The role of macrophages in hepatic fibrosis

1.8.5. Role of Th2 cytokines in activation of fibroblast cells

1.8.6. The contribution of macrophages and fibroblasts in fibrosis regulation
1.8.7. The role for macrophages in the regression of fibrosis

1.9. Hepatic stellate cells (or Ito cells)

1.10. Extracellular matrix (ECM) in hepatic fibrosis

1.10.1. Role of extracellular matrix and connective tissue cells in hepatic fibrosis

1.10.2. Matrix metalloproteinases (MMP)

1.10.3. Regulation of matrix degradation

1.10.4. Fibrosis resolution

1.11. Periportal fibrosis (PPF)
1.12. Genetic control of infection and disease in human schistosomiasis

1.12.1. Schistosoma mansoni 1 (SM1) locus

1.12.2. Schistosoma mansoni 2 (SM2) locus

1.13. Candidate genes in susceptibility to severe hepatic disease

1.13.1. Connective tissue growth factor (CTGF) gene

1.13.1.1. Connective tissue growth factor (CTGF) gene and protein structure

1.13.1.2. CTGF gene in fibrotic skin disorders

1.13.1.3. CTGF in organ fibrosis
1.13.1.4. CTGF gene in liver fibrosis

9

1.13.2. Interleukin-13 (IL-13) candidate gene for SM1

0

1.13.2.1. IL-13 gene structure

4

1.13.2.2. The function of IL-13 in haematopoietic and non-haematopoietic cells

40

1.13.2.3. IL-13 receptors complex

42

1.13.3. Interleukin-10 (IL-10) gene

44

1.13.3.1. IL-10 cytokine and inflammation

44

1.13.3.2. IL-10 gene organization and sequence

44

1.13.3.3. IL-10 polymorphisms and disease association

46

1.14. Bioinformatics analysis

48

1.14.1. HapMap project

48

1.14.2. Data analysis

49

1.15. Rationale

51

1.15.1. General objectives

51

1.15.2. Specific objectives

51

Chapter Two
**Materials and Methods**

2.1. Study area and population  
2.1.1. Selection of study subjects  
2.1.1.1. Inclusion criteria  
2.1.1.2. Exclusion criteria  
2.2. Clinical evaluation  
2.3. Ultrasound evaluation of liver fibrosis  
2.4. Genetic analysis  
2.4.1. Blood samples  
2.4.2. DNA extraction  
2.4.3. DNA purification and control of quality and quantity  
2.5. Bioinformatics analysis  
2.5.1. Haploview software  
2.5.2. Selection of candidate SNPs  
2.6. Polymerase chain reaction (PCR)  
2.6.1. Principle  
2.6.2. PCR amplification  
2.7. The TaqMan® assay  
2.7.1. The TaqMan® assay method  
2.7.2. Reporter Dyes  
2.7.3. Allelic discrimination assays  
2.7.4. Preparation of the reaction mix
2.7.4.1. TaqMan PDARs for allelic discrimination 70
2.7.4.2. Preparation of amplification reaction 70
2.7.4.3. Preparation of the reaction plate 72
2.7.4.4. Creating a standard curve plate document 72
2.7.4.5. TagMan assays reaction 72
2.7.4.6. Interpretation of results 74

2.8. Functional analysis 74
2.8.1. Preparation of nuclear and cytoplasmic fractions 74
2.8.2. Electronic mobility shift assay (EMSA) analysis 76
2.8.2.1. EMSA and transfer of DNA probes onto nylon membrane 76
2.8.2.2. Detect biotin-labeled DNA by chemiluminescence 77
2.8.4. EMSA probes sequences 77

2.9. Phenotypes used in genetic analysis 77

2.10. Statistical analysis 80
2.10.1. Epidemiological data 80
2.10.2. Mutational analysis 80

Chapter Three

Results

3.1. Study subjects (Epidemiological data) 81
3.1.1. The prevalence of fibrosis grades by age and gender 81
3.1.2. Central fibrosis is associated with most severe hepatic disease 88
3.1.3. Splenomegaly and hepatic disease 92
3.1.4. Disease progression with age and exposure in males and females 98
3.1.5. Severity of hepatic fibrosis is markedly influenced by tribal
3.1.6. Central PPF grade D, E and F were associated with more severe clinical form of diseases

3.1.7. Strong association between central periportal fibrosis and varices

3.1.8. The effect of previous treatment on fibrosis grades

3.1.9. Evidence for association of central periportal fibrosis with activity (occupation)

3.2. Genotyping analysis

3.2.1. Connective tissue growth factor (CTGF) gene
3.2.1.1. Distribution of CTGF gene polymorphisms in study subjects

3.2.1.2. rs1257705, rs125526196, and rs9402373 polymorphisms are also associated with severe HF

3.2.2. Interleukin-13 (IL-13) gene

3.2.2.1. IL-13 polymorphisms were not associated with severe hepatic fibrosis

3.2.2.2. IL-13 polymorphisms were associated with severe hepatic fibrosis

3.2.3. Interleukin-10 (IL-10) gene

3.2.3.1. IL-10 polymorphisms were associated with severe Hepatic fibrosis
3.2.4. Functional analysis

1. 3.2.4.1. rs9402373 and rs12526196 polymorphisms affect nuclear factor binding and may alter gene transcription or transcript stability

21

Chapter Four

Discussion

4.1. Central periportal fibrosis and gender affects

12

3

4.2. Disease progression with age and exposure in males and females

12

4

4.3. Central fibrosis is associated with severe hepatic disease

12

5

4.4. Central periportal fibrosis is associated with activity (occupation)

12

6
4.5. Splenomegaly and hepatic disease

4.6. The association of hepatic disease with tribal effect

4.7. The effect of previous treatment on peripheral fibrosis

4.8. Factors associated with oesophagus varices

4.9. Genetic susceptibility of \textit{S. mansoni} pathology

4.9.1. Interleukin-10 is associated with increasing susceptibility to HF

4.9.2. Interleukin-13 and severe HF

4.9.3. Variants of CTGF are associated with hepatic fibrosis
Conclusions

13

Recommendations

14

References

14

Appendix

Appendix: I Clinical / Epidemiological form: SUDAN / PPF February 2007

17

Appendix: II Schistosomiasis research project Managil Sudan 2007

17

Appendix: III Sudan four 96 wells plates

17

Appendix: IV Sudan 384 wells plates

17
List of Tables

Pages

Table 2.1: Hepatic fibrosis by Niamey image pattern scores 57
Table 2.2: Preparing TaqMan® Universal PCR reaction master mix 71
Table 2.3: PCR times, temperature and cycles 73
Table 2.4: Complementary oligonucleotides sequence which used in
    EMSA analysis. 78
Table 3.1: Frequency data of the study subjects 82
Table 3.2: Association of central periportal fibrosis with gender 86
Table 3.3: Associations between peripheral fibrosis and gender 87
Table 3.4: Significant association between portal vein and central periportal
    fibrosis 89
Table 3.5: Host factors involved in enlargement of splenic vein 90
Table 3.6: Risk factors associated with severe hepatic form of disease
    (Sclerosis of varices) 91
Table 3.7: Correlation between central periportal fibrosis and splenic volume 93
Table 3.8: The contribution of splenomegaly to portal hypertension 94
Table 3.9: The association between splenic volume and splenic vein 97
Table 3.10: The associations between tribal stocks and severe HF
    10
Table 3.11: Central periportal fibrosis is highly associated with ascites
    10
Table 3.12: Evidence of association between sclerosis varcies and central periportal fibrosis

Table 3.13: Strong association of central periportal fibrosis and oesophagus varices

Table 3.14: Association of peripheral fibrosis with gender and previous treatment

Table 3.15: A significant association between activity and central periportal fibrosis

Table 3.16: The association of farmers with central periportal fibrosis

Table 3.17: SNPs in the region flanking CTGF are associated with severe HF in study subjects infected with S. mansoni

Table 3.18: SNPs in the region flanking IL13 are associated with severe HF
in study subjects infected with *S. mansoni*.

Table 3.19: SNPs in the region flanking IL10 are associated with severe HF in study subjects infected with *S. mansoni* using two phenotypes.
List of Figures

Figure 1.1: Global distribution of schistosomiasis (Map courtesy of US centers for disease control and prevention) 2
Figure 1.2: *Schistosoma* life cycle. 6
Figure 1.3: Naïve Th0 cells subclasses 19
Figure 1.4: RegT cells, IL-10, and IL-13α2 controlling tissue fibrosis 21
Figure 1.5: The role of macrophages in collagen production 26
Figure 1.3: Schematic representation severe consequence of HF 32
Figure 1.7: Schematic representation of CTGF gene 36
Figure 1.8: Schematic representation of CTGF protein 38
Figure 1.9: Schematic representation of IL-13 gene 41
Figure 1.10: Overview of the action of IL-13 gene 43
Figure 1.11: Schematic representation of IL-10 gene 45
Figure 2.1: Gezira map illustrate study areas 53
Figure 2.2: The study strategy 55
Figure 2.3: Ultrasound classification of liver damage induced by *S. mansoni* 58
Figure 2.4: The LD block structure observed in the IL-13 gene 63
Figure 2.5: Correlation bins for IL-10 in African population 64
Figure 2.6: Correlation bins for CTGF and flanking regions in the Sudanese sample 65
Figure 2.7: Allelic discrimination assay 6
Figure 2.8: Allelic discrimination plot for rs1295686 marker in IL-13 gene 75
Figure 3.1: The prevalence of peripheral and central fibrosis in study subjects

Figure 3.2: The distribution of central and peripheral fibrosis according to gender

Figure 3.3: The distribution of the main portal vein (mm), splenic vein (mm) and splenic volume (mm$^3$) by central, peripheral fibrosis and gender

Figure 3.4: The distribution of the mean splenic volume according to villages and tribal by age classes

Figure 3.5 A: The progression of severe hepatic fibrosis by age groups and gender B: The progression of peripheral fibrosis according to age groups and gender

Figure 3.6: Distribution of the central periportal fibrosis in the study villages

Figure 3.7: The prevalence of fibrosis grades among major five tribes in study subjects

Figure 3.8: The occurrence of central periportal fibrosis by number according to major five tribes
Figure 3.9: The distribution of severe phenotype PVD and GB among study subjects in five major tribes

<table>
<thead>
<tr>
<th>Tribe</th>
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Figure 3.10: The prevalence of severe form of disease

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</table>

Figure 3.11: EMSA for SNPs rs12526196, rs9402373, rs12527705, and rs1931002

<table>
<thead>
<tr>
<th>SNP</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td></td>
</tr>
</tbody>
</table>

List of abbreviations

AAMs: Alternatively activated macrophages
ARG1: Arginase-1
CAMs: Classical activated macrophages
CEU: Northern and western European ancestry
CHB: Han Chinese individuals in Beijing, China
CI: Confidence interval
CSF-1R  Colony stimulated factor receptor 1
CT      Computerized tomography
CTGF   Connective tissue growth factor
D’      D prime
dNTPs  deoxyribonucleotide triphosphate
DTH    Delayed type hypersensitivity
ECM    Extracellular matrix
EDTA   Ethylene diamine tetra acetic acid
EMSA   Electronic mobility shift assay
F-0    Fibrosis grade zero
F-II   Fibrosis grade two
F-III  Fibrosis grade three
HGF    Hepatocyte growth factor
HSCs   Hepatic stellate cells
IFN-γR1 Interferon-gamma receptor 1
IGFBP  Insulin-like growth factor binding protein
IL-2   Interleukin-2
IRF-1  Interferon regulatory factor-1
JAK    Janus kinase
JPT    Japanese individuals in Tokyo, Japan
LD     Linkage disequilibrium
MAF    Minor allele frequency
MGB    Minor groove binder
MMP    Matrix metalloproteinases
MRI    Magnetic resonance imaging
<table>
<thead>
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<th>Full Form</th>
</tr>
</thead>
<tbody>
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</tr>
<tr>
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<td>Odds ratio</td>
</tr>
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</tr>
<tr>
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</tr>
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</tr>
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<td>ROR</td>
<td>Retinoic acid receptor–related orphan receptor</td>
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<td>SEA</td>
<td>Soluble egg antigens</td>
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<td>SM1</td>
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<td>Schistosoma mansoni 2</td>
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<td>Single-nucleotide polymorphism</td>
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<td>Statistical Package for Social Science</td>
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<td>STAT</td>
<td>Signal transducer and activator protein</td>
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Identification of the Major Genetic Loci that Control Disease Severity in *Schistosoma mansoni* Infected Patients in Gezira Area, Sudan: A Molecular Epidemiological Study

MOHAMMED OSMAN ABD EL WAHED MADANI
Ph.D In Molecular Biology, September /2012
Department: Molecular Biology
National Cancer Institute (NCI) - University of Gezira

Abstract

Hepatic periportal fibrosis is a severe consequence of *Schistosoma mansoni* infection; the disease affects 5-10% of the infected population in the endemic areas. The aims of the current study were to evaluate the epidemiological factors, and to study the genetic background which may play a role in *S. mansoni* pathogenesis. Nine hundred and forty one subjects were examined by ultrasound using Niamey protocol. DNA from three hundred and seventy two participants, was obtained from peripheral blood. Samples were genotyped for three genes (Interleukin-13, Interleukin-10 and Connective Tissue Growth Factor), by using a predesigned TagMan allele discrimination assay. Electronic mobility shift assay was done to assess the effect of four SNPs on CTGF gene expression. Severe hepatic fibrosis was observed in 13.6% of the study subjects. Central periportal fibrosis was associated with gender (P = 0.0001; OR = 2.735), ascites (P < 0.000001), varices (P < 0.0000001), splenic volume (P < 0.0001), and ethnic group (P < 0.0001). In multivariate analysis IL-10-1082 A alleles (P = 0.044; OR = 2.8), and IL-10-819 T (P = 0.054; OR = 2.3), were associated with severe hepatic fibrosis. Close to CTGF gene two SNPs rs9402373 (P = 0.008; OR = 5.2) and rs1256196 (P = 0.059; OR = 7.3) were independently associated with severe hepatic fibrosis, electronic mobility
shift assay revealed that these polymorphisms are affecting nuclear factor binding. In conclusion, the current study suggests that gender, genetic, and ethnic background of study subjects are risk factors in fibrosis development. Furthermore, the polymorphisms of IL-10-1082A alleles, rs9402373 and rs1256196 are predictors of the progression of the hepatic fibrosis disease and could be possible targets for treatment and vaccination.
تحديد المواقع الوراثية الرئيسية التي تتحكم في شدة المرض في المرضى المصابين بالمنشقة المعوية في منطقة الجزيرة، السودان: دراسة وبائية جزيئية

محمد عثمان عبد الواحد
دكتوراه الأحياء الجزيئية – سبتمبر / 2012
المعهد القومي للسرطان – جامعة الجزيرة

الخلاصة

تليف الوريد الكبدي البابي هو نتيجة حتمية للإصابة الحادة بالمنشقة المعوية، هذا المرض يصيب حوالي 5-10% من عدد السكان المصابين في المناطق التي يتوطن بها المرض. هدفت الدراسة الحالية إلى تقييم العوامل الوراثية في تطور مرض تليف الوريد الكبدي البابي، ودراسة العوامل الوراثية التي تفاقم الإصابة بالمنشقة المعوية. تم فحص تسعين وواحد واربعون شخصًا بواسطة الموجات فوق الصوتية باستخدام برتوكول (نيامي). تم جمع الدم من الوريد الطرفي لإجراء التحليل الوراثي لثلاثة من المورثات وهى (انترليوكين 13 و انترليوكين 10 وعامل نمو النسيج الضام) بواسطة تقنية التفاعل السلسلى البلمرى (TagMan) المسبقة التحضير. تم إجراء التحليل الوظيفي لأربعة مواقع وراثية في مورث (عامل نمو النسيج الضام) بواسطة تقنية الفحص الإلكتروني للتحول النقلى. وجد 13.6% من الأشخاص الخاضعين للدراسة مصابين بال틸يف الكبدي البابي المركزي.

التحليل الأحصائي أثبت وجود ارتباط عامل الجنس (ذكر- أنثى) (P = 0.001; OR = 2.752) والاستسقاء (P = 0.00000000001) بالتيتيف الكبدي البابي المركزي. في التحليل متعدد المتغيرات (P = 0.054) IL-10 في الموقع rs 1256196، و (P = 0.008; OR = 5.2) مجموعتين القبلية و (P = 0.059; OR = 7.3) مجموعتين القبلية賞 алкогلاً انتين من المتغيرات الوراثية 94023737 rs1256196 و rs1800896، وقد وجد أنهما مرتبطان بشدة مستقلة مع تليف الوريد البابي المركزي. بالقرب من مورث عامل نمو النسيج الضام في الموقع rs 1800896 و rs 256196، تم استخدام تقنية الفحص الإلكتروني للتحول النقلى وجد أن هذا التغيير الشكلي المتعدد يؤثر على عامل ارتباط البروتينات النووية. وقد خلصت هذه الدراسة إلى أن العوامل المتعلقة بالجنس والخلفية الوراثية للمشاركين في الدراسة تعتبر مهمة في تطور التليف الكبدي البابي. إضافة إلى ذلك، الاستنساخ المتعدد للمورثات (rs94023737) تمثل مؤشرات جيدة لملاءة تطور المرض ويمكن أن تكون هدفاً للفحص والتطعيم.
Chapter one

Introduction

1.1. Prevalence of schistosomiasis

Schistosomiasis (also termed Bilharziasis) is the most common parasitic disease transmitted through contact with fresh water. It is endemic in more than 70 low income countries where it occurs in rural areas and the fringes of cities (Gryseels et al. (2006). Over 650 million people globally are at risk of infection, with more than 200 million people infected (Chitsulo et al., 2000). Of these, 120 million are estimated to have symptoms, with 20 million people experiencing serious consequences (Gryseels et al., 2006). The economic effects and health implications of schistosomiasis are extensive. Higher infection rates frequently occur in children less than 14 years in many endemic areas (WHO, 2007).

The main schistosomes infecting human beings are: S. mansoni, which is transmitted by Biomphalaria snails and causes intestinal and hepatic schistosomiasis in Africa, the Eastern Mediterranean, and South America; S. haematobium, transmitted by Bulinus snails and causing urinary schistosomiasis in Africa and Eastern Mediterranean; and S. japonicum, transmitted by an amphibian snail, Oncomelania, and causing intestinal and hepatosplenic schistosomiasis in China, the Philippines, and Indonesia (Chitsulo et al., 2004) (Figure 1.1).

Seventy million individuals out of 682 million people in Sub-Saharan Africa (SSA) were estimated to have experience haematuria following infection with S. haematobium, and 32 million with dysuria within the last two weeks of infection. Ultrasound detected serious consequences of S. haematobium, major bladder wall pathology and major hydrenephrosis, were predicted at 18 and 10 million, respectively (van der Werf et al., 2003).
Intestinal schistosomiasis from *S. mansoni* causes most of the remaining cases of schistosomiasis in SSA. An estimated 4.4 million people infected with *S. mansoni* have bloody diarrhoea and bowel ulceration, and 8.5 million develop hepatomegaly associated with periportal liver fibrosis, portal hypertension, and haematemesis, with approximately 130,000 deaths (van der Werf *et al.*, 2003; King and Dangerfield-Cha, 2008).

1.2. Schistosomiasis mortality and morbidity

Schistosomiasis-associated mortality is about 280,000 people per year (0.08% of the deaths worldwide). The majority of these mortalities due to *S. mansoni* infections occurring in 52 countries (Pearce and MacDonald, 2002) include both intestinal schistosomiasis and hepatosplenic schistosomiasis.

Mortality due to *S. mansoni* infection can result from haematemesis, and liver failure. The overall schistosomiasis mortality rate in Brazil was 0.30/100,000/year in 1993 (Katz, 1998). A study in Sudan, in a population with 53% prevalence of infection reported a crude mortality rate of schistosomiasis of 51/100,000/year (Kheir *et al.*, 1999). In a highly infected population in Uganda where the prevalence reached 89%, mortality rate was reported 2.600/100,000/year of which 25% was due to *S. mansoni* infection, resulting in highly mortality rate to reach 650/100,000/year (Ongom and Bradley, 1972).

In general, the morbidity related to schistosomiasis is classified into overt or end-organ morbidity (direct), and subtle systemic morbidity (indirect). Consequence of egg deposition is the most severe and accounts for the overt or end-organ morbidity characterized by hepatic fibrosis, portal hypertension and their clinical complications (van der Werf *et al.*, 2003).
1.3. Schistosomiasis in Sudan

Schistosomiasis was firstly reported in the Sudan in 1904 by Balfour, who found that 17% of primary school children in Khartoum province were infected by *S. heamtoubium* (Balfour, 1904).

In 1928, the intermediate host (*Bulinus* and *Biomphalaria*) invaded and colonized in the Gezira Scheme canals. In 1949, 27.5% of the populations were infected with *S. haematobium* or *S. mansoni*. The prevalence of *Bulinus* and *Biomphalaria* the intermediate host of *S. haematobium* or *S. mansoni* respectively, were equally distributed in Gezira canals and their density was closely related to weed growth, but the infection rate of *S. mansoni* in *Biomphalaria* was twenty times higher than *S. haematobium* in *Bulinus* (Greany, 1952 a, 1952 b)

In Gezira Irrigated Scheme, the prevalence of *S. haematobium* was higher than *S. mansoni* in 1947 (Stephenson, 1947). This situation has changed dramatically in the mid of 1970 and *S. mansoni* have become the predominant species, with a prevalence up to 73% (Amin et al., 1982).

A survey published in 1972, reported a prevalence of 80% of *S. mansoni* in Hibeka village among school boys age (6 - 14) years, while the prevalence reach 45.5% among the girls at same range of age, on the other hand, 56.5% among adults in were infected by *S. mansoni* (Ambroise et al., 1972).

In 1980, considerable differences in prevalence have been noted between ethnic groups in the Gezira-Managil area. While nomads and migrants from the west of Sudan suffered from a low rate of urinary Schistosomiasis (2.3% - 5.0%), the Fallata population had infection rates of 30% for *S. haematobium* and 50% for *S. Mansoni* (Bella et al., 1980). Later, in 1999 the prevalence of *S. mansoni* infection in Al-Tawel village Managil area of Gezira was 72.8% in males and 68.3% in females.
(Qurashi et al., 1999). The same group reported a trend association of severe hepatic fibrosis between two ethnic tribes (Rawashda and Tama) living in same village.

1.4. Schistosoma life cycle

The five species that cause schistosomiasis differ in size and shape of eggs and eggs production (S. haematobium, S. mansoni 20 – 300 and S. japonicum, 3500 eggs per worm per day), and location within the human host and consequently cause different signs and symptoms (Jordan and Webbe, 1993).

Humans become infected through contact with fresh water containing free-swimming larval forms of the parasite called cercariae (Figure 2.1). The release of cercariae is most pronounced around noon and start 3-5 hours after the snail exposed to light (Wolmarans et al., 2002). Cercariae are positively phototropic and swim toward the water surface where the possibilities to contact with man or animals is maximum (McKerrow and Salter, 2002). Upon contact with man skin the cercariae adhere and use their head gland that secretes cytolytic substances. It can penetrate the human epidermis in few minutes (Jordan et al., 1993). Now known as schistosomula, they leave the skin via the blood vessels and draining lymphaticst reach the lungs (Jordan and Webbe, 1993). After several days, the Schistosoma male and female worms leave the lungs and arrive in the hepatic portal system, where they mature, pair up, and migrate downstream. The female lives in the gynaecophoric canal of the male worm. Here they start laying eggs after approximately 30 days of mating. The life span of the adult worms ranges from 3 to 5 years, although some live as long as 37 years (Harris et al., 1984).

Many eggs pass through the intestinal or bladder wall and are discharged in the faeces (S. mansoni and S. japonicum) or urine (S. haematobium). The schistosome life cycle is completed when the eggs hatch and release free-swimming
miracidium, which in turn re-infect the receptive freshwater snails. In the snail, the new cercariae develop by asexual reproduction (Jordan et al., 1993).

Figure 1.2: *Schistosoma* life cycle.
1.5. *S. mansoni* infection and disease

Schistosomiasis causes a range of morbidities, which depend on the nature of immune response initiated against the eggs trapped in different organ. In *S. mansoni* infection, two main clinical conditions were recognized in infected patients, acute schistosomiasis and chronic schistosomiasis.

1.5.1. Acute schistosomiasis

Acute schistosomiasis (Katayama fever) is a systemic hypersensitivity reaction against the migrating schistosomulae, occurring a few weeks to months after a primary infection (Lambertucci, 1993a; Rocha *et al.*, 1995a; Rocha *et al.*, 1995b; Bottieau *et al.*, 2006; Ross *et al.*, 2007). *S. mansoni* occurs as an acute infection in individuals who have recently visited an endemic area, with no previous contact with the parasite. The acute phase is characterized by symptoms such as fever, cough, diarrhoea, anorexia, and arthralgias in combination with leukocytosis and eosinophilia, and a high cellular immune response to schistosome antigens especially those from the parasite's eggs (Caldas *et al.*, 2008).

Katayama fever due to *S. mansoni* or *S. haematobium* is rarely seen in chronically exposed populations, however, it can be observed in tourists, travellers, and other people accidentally exposed to transmission (Jelinek *et al.*, 1996; Hatz, 2005; Bottieau *et al.*, 2006).

1.5.2. Chronic pathology and morbidity

The main lesions in established and chronic infection are due not to the adult worms but to eggs that are trapped in tissues during the perivesical or per-intestinal migration or after embolisation in the liver, spleen, lungs, or cerebrospinal system. The eggs secrete proteolytic enzymes that provoke typical eosinophilic inflammatory
and granulomatous reactions, which are progressively replaced by fibrotic deposits (Cheever et al., 2000).

1.5.2.1. Intestinal Schistosomiasis

Schistosomal eggs migrating through the intestinal wall provoke mucosal granulomatous inflammation, pseudopolyposis, microulcerations, and superficial bleeding (Cheever, 1968; Cheever et al., 1978). Most lesions are situated in the large bowel and rectum. The most common symptoms and signs are chronic or intermittent abdominal pain and discomfort, loss of appetite, and diarrhoea with or without blood (Gryseels, 1989).

1.5.2.2. Hepatic schistosomiasis

Hepatic schistosomiasis can be caused by S. mansoni, S. japonicum, and S. makongi. Inflammatory hepatic schistosomiasis is an early reaction to ova trapped in the presinusoidal periportal spaces of the liver. It is the main cause of schistosomal hepatomegaly in children and adolescents (Gryseels and Polderman, 1991; Gryseels, 1992). Typical features of hepatic schistosomiasis include sharp-edged enlargement of the left liver lobe and nodular splenomegaly, that extending few centimeters below the costal arch to below the umbilicus and even into the pelvis (Gryseels and Polderman, 1987). Clinical and epidemiological differentiation from malaria may be difficult (Booth et al., 2004b). Ultrasonography can reveal mild forms of diffuse fibrosis. Although, in many cases, there is no apparent sign of functional disease (Kardorff et al., 1996). Before the advent of modern schistosomicides, advanced schistosomal liver fibrosis with oesophageal bleeding was a common clinical syndrome in Egypt, Sudan, Brazil, China, and the Philippines but much less frequent in most of sub-Saharan Africa (Gryseels and Polderman, 1991; Gryseels, 1992;
Jordan, 2000; WHO, 2002). These regional morbidity patterns have been attributed to ethnic and genetic factors (Jordan and Webbe, 1993).

1.5.2.3. Ectopic schistosomiasis

Pulmonary schistosomiasis is due to portal-caval shunting, allowing ova to leak into the perialveolar capillary beds. The ensuing granulomas can give rise to bronchial symptoms and later to fibrosis complicated by pulmonary hypertension and cor pulmonale. These symptoms can remain occult for many years (Lambertucci, 1993b). In *S. mansoni* infections, portosystemic leaking of immune complexes to the mesangial areas can lead to glomerulonephritis (Barsoum, 2004).

1.5.2.4. Genital schistosomiasis

Genital schistosomiasis, is quite common in some endemic regions, and a regular finding in travelers. It is due to deposition of eggs of *S. haematobium* and *S. mansoni* in the reproductive organs (Feldmeier *et al.*, 1999; Poggensee and Feldmeier, 2001; Kjetland *et al.*, 2008).

1.5.2.5. Neuroschistosomiasis

Neuroschistosomiasis is caused by inflammation around ectopic worms or eggs in the cerebral or spinal venous plexus, which can lead to irreversible fibrotic scars if left untreated (Naus *et al.*, 2003). *S. japonicum* is associated with cerebral granulomatous lesions, which can lead to epileptic, paralytic, and meningoencephalitic symptoms (Ferrari, 2004).

1.6. Schistosomiasis diagnosis

1.6.1. Microscopic examination

The microscopic examination of excreta remains the gold standard test for the diagnosis of schistosomiasis (Feldmeier and Poggensee, 1993). The eggs are easy to detect and identify by the microscope owing to their size and shape, their typical lateral or terminal spine, and the living miracidium (in fresh samples) with mobile
cilia and pulsing excretory cells (de Vlas and Gryseels, 1992; Engels et al., 1996). Urine should be concentrated by sedimentation, centrifugation, or filtration, and samples should be taken around noon or after physical exercise (Feldmeier and Poggensee, 1993). For the intestinal schistosomes, in the field, the faecal thick smear or Kato-Katz method is commonly used, because it allows quantification of the infections by egg counts, usually expressed as per gram faeces (Feldmeier and Poggensee, 1993).

1.6.2. Histological techniques

Laparoscopy and wedge biopsy can reveal the macroscopic and histological appearance of granulomatous inflammation or periportal fibrosis (Homeida et al., 1988b; Hayashi et al., 2000). Liver biopsy still represents an essential procedure for staging liver disease (Consolo et al., 2009). However, despite its importance, liver biopsy presents some limitations: the risk of a disease underestimation is the most significant one, as hepatic lesions are often irregularly located within the liver. Parallel to the limitations of liver biopsy, clinical needs for an early identification of progressive fibrosis require additional non-invasive techniques like diagnostic markers. These markers should be considered in combination with liver function tests, ultrasonography and clinical manifestations (Das and Vasudevan, 2008; Consolo et al., 2009).

1.6.3. Immunological techniques

Antibody-based assays are quite sensitive but cannot distinguish history of exposure from active infection; they can also cross-react with other helminthes and are not easily applicable under field conditions (Feldmeier and Poggensee, 1993; Rabello, 1997; Tsang and Wilkins, 1997). Most routine techniques aim to detect antibody IgG, IgM, or IgE against soluble worm antigen or crude egg antigen by
EIA, indirect haem-agglutination, or immune-fluorescence (Isnard et al., 2011). Most assays have positive results for at least 2 years after cure and in many cases much longer (Wynn, 2003).

1.6.4. Imaging techniques in assessment of S. mansoni morbidity

1.6.4.1. Diagnosis and clinical finding of liver fibrosis by ultrasonography

The first reports on the use of ultrasound in S. mansoni infections were presented by Abdel-Latif (Abdel-Latif et al., 1981). They demonstrated echogenic areas of thickened, fibrosis portal tracts in the liver in infected patients, which were not seen among six control patients with alcoholic or post-hepatic liver diseases. In 25 patients with biopsy proven hepatic fibrosis, the portal and splenic vein diameters are shown to be associated with portal hypertension in 13 cases, whom portal hypertension was confirmed by trans-splenic portal manometry. The presence of oesophageal varices was associated with an increase in the diameter of portal and splenic vein (Abdel-Latif et al., 1981).

Lesions of advanced S. mansoni disease presented few problems to the examiners. However, early hepatic lesions, which are more prevalent among populations in endemic area and important in planning of selective mass treatment schemes, may be difficult to assess by ultrasonography (Doehler-Schwerdtfeger et al., 1989; Abdel-Wahab et al., 1990). In field study among villagers in Sudan, Homeida et al., (1988a) described a mild grade of periportal thickening which was not observed among hospital patients.

A pathologically thickening gallbladder wall was found to be associated with periportal thickening in 60% of S. mansoni infected patients (Cerri et al., 1984). Ali et al. (1990) reported gallbladder wall thickening in 77% of patients with advance schistosomiasis lesions and portal hypertension. The authors suggested that
periportal thickening might extend to gallbladder wall in advanced schistosomiasis, while thickening of the gallbladder neck (but not of the bladder wall) has been reported in mild disease (Doehring-Schwerdtfeger et al., 1990).

Gastrointestinal bleeding from oesophageal varices is considered to be the most serious complication and the usual cause of death in *S. mansoni* infection (El-Rooby, 1985). Ultrasound was used to measure the diameters of the portal vein and its tributaries specially in investigation of portal hypertension (Bolondi et al., 1982; Kane and Katz, 1982). In a comparative study among 164 *S. mansoni* infected patients with cirrhosis who have performed endoscopy and ultrasound examination, Cottone et al. (1983) found that a portal vein diameter of > 1.7 cm and splenic vein diameter >1.2 cm demonstrated by ultrasound are indicating the presence of oesophageal varices in every cases. In another study, oesophageal varices with or without haematemesis, were associated with larger diameters of portal and splenic veins in patients with schistosomiasis infection (Abdel-Latif et al., 1981).

Eltoum et al. (1994) reported that 4% of patients with periportal thickening had haematemesis during three years follow-up. They found that cases with minor periportal thickening had oesophageal varices but no bleeding. Thus, they suggested that the measurement of portal and splenic vein diameters and longitudinal diameter of spleen might have predictive potential for variceal bleeding.

The spleen is enlarged in all patients of periportal thickening (Cerri et al., 1984; Homeida et al., 1988b; Abdel-Wahab et al., 1989), however, in areas endemic for malaria, the assessment of splenic size may not be valuable indicator for schistosomiasis (Booth et al., 2004b).

The use of ultrasonography in the assessment of schistosomiasis morbidity, however, has some limitations. Fibrosis grading is operator-dependent and antra-
observer variations have been reported (Doehring-Schwerdtfeger et al., 1992). Ultrasonography is also not sensitive enough to detect minor lesions in the liver (Hatz et al., 1992).

### 1.6.4.2. Computerized tomography and magnetic resonance imaging

Characteristic changes in computerized tomography (CT) attenuation or magnetic resonance imaging (MRI) signal intensity and typical enhancement patterns can contribute to the diagnosis of hepatosplenic schistosomiasis. The findings are usually in agreement with US. In CT, the periportal echogenic thickening is seen as low-density periportal zones that extended uniformly throughout both liver lobes with markedly enhancement after intravenous contrast injection, to become either homogeneous with or denser than the surrounding liver (Leask and Abraham, 2006).

MRI has been established as a very sensitive image method in various diffuse liver diseases and differs from US in that, it is not a dynamic study and less examiner-dependent. It's multi-planar capability and its sensitivity to small differences in tissue composition are interesting characteristics of this technique (Leask and Abraham, 2006).

### 1.7. Treatment and control of schistosomiasis

#### 1.7.1. Schistosomiasis treatment

Praziquantel (PZQ), a pyrazinoisoquinoline derivative, is a safe and highly effective oral drug that is active against all schistosome species. It is the mainstay of treatment and is playing a critical part of community-based schistosomiasis control programmes (Ross et al., 2001; Utzinger et al., 2005; Xiao, 2005; Zhou et al., 2005; Doenhoff and Pica-Mattoccia, 2006; Utzinger et al., 2007; Doenhoff et al., 2008). Since its discovery in the mid-1970s, its safety and efficacy have ensured its widespread use. It is well absorbed but undergoes extensive first-pass hepatic
clearance. PZQ side effects are mild, and it can be used to treat young children and pregnant women (Tweyongyere et al., 2009). PZQ acts within 1 hour of ingestion. Although its precise action on adult worms is unknown, it appears to cause tetanic contractions and tegumental vacuoles, causing worms to detach from the wall of the vein and die. Schistosome calcium ion channels have been suggested as the molecular target of PZQ, but the evidence remains indirect (Doenhoff et al., 2008).

Standard treatment of chronic Schistosomiasis japonicum is 60 mg/kg of PZQ in China (Hou et al., 2008) in divided doses; for mass chemotherapy a single dose (40 mg/kg) is used (Ross et al., 2002). Treatment failures with this dose have been reported, particularly in areas where schistosomiasis has been recently introduced. Whether this is because the drug works in concert with the host immune response, which has yet to develop, or due to migrating larvae not yet susceptible to PZQ is unknown (Gryseels et al., 2006). There is laboratory evidence that schistosomes subjected to repeated in vivo PZQ treatments for several generations have reduced drug sensitivity than in the original unselected strain (Pica-Mattoccia et al., 2009).

Other drugs that have been used in the treatment of schistosomiasis are Oxamniquine (Vansil) for S. mansoni and Metrifonate (Trichlorfon) for S. haematobium, but both are ineffective against S. japonicum. Potential new targets for drug development against schistosomes include peroxiredoxin-1 (Kumagai et al., 2009), thioredoxin glutathione reductase (Sharma et al., 2009), tyrosine kinases (Ting-An and Hong-Xiang, 2009), tyrosine kinase receptors (Ahier et al., 2008; Ahier et al., 2009), phosphatidylinositol kinases (Bahia et al., 2009), and neuropeptides (McVeigh et al., 2009). The anti-malarial drug, Artemether, has been used as a chemoprophylactic agent in patients who anticipate high levels of exposure to cercariae during seasonal floods (Utzinger et al., 2000).
1.7.2. Control of schistosomiasis

The global strategy for schistosomiasis control is the reduction of morbidity, which is based on the reduction of the worm load. No vaccine is yet available. Attention is focused on detection and treatment and vector control.

Molluscicides are pesticides against the intermediate snail host for schistosomes. Spread of schistosomiasis is prevented by using molluscicides and other biological agents to eliminate fresh water snails (Lardans and Dissous, 1998). The use of molluscicides alone will not be effective. Unfortunately, in previous studies have reported that new dams, irrigation systems and reservoirs as well as the movement, growth and settlement of populations in new areas has reduced the previous effectiveness of schistosomiasis prevention programs (Jassim et al., 1987).

Health education is not effective if it's not associated with an improvement in sanitary conditions in the district. The erection of numerous bridges over canals and the use of water pumps (drinking water, washing areas) reduce contact with potentially contaminated water (Gu et al., 2001; Noya et al., 2006). Water pumps should be of a design that allows inexpensive local repairs to be made.

Mass treatment can be undertaken with praziquantel (treat everyone without screening) (Kheir et al., 2000; Mohammed et al., 2006). Selective treatment of infected people can also be carried out following active screening. Treatment can be restricted to a particular group (e.g. all school children or children from specific school years). Passive screening (all those people who attend a health centre) is also possible. In each case, repeated interventions will be necessary in view of the fact that a proportion of the population is always missed the first time (Mehanna et al., 1997; de Vlas et al., 2004).
It should be noted that artemether has a preventive effect on *S. mansoni* and *S. japonicum*. Taking one tablet every two to three weeks (6 mg/kg) reduces the risk considerably. Naturally, this is a dangerous strategy to apply in a *P. falciparum* area (Utzinger *et al.*, 2000).

1.8. Role of Th cells, cytokines, and macrophages in hepatic fibrosis

1.8.1. Granuloma formation in schistosomiasis

The severity of the disease is a consequence of fibrosis caused by granulomatous reaction around the schistosome eggs that are trapped in the small vessels of the liver. Granuloma formation is a cell-mediated immune response that is dependent on CD4+ T cells (Jankovic *et al.*, 1998; Stadecker *et al.*, 2001). It is classically described as delayed type hypersensitivity (DTH) reaction (Warren *et al.*, 1967; Fidel and Boros, 1991). However, only a small percentage of individuals infected in an area of endemicity develop a severe form of the disease. Study in deficient mice (knockout mice) infected with *S. mansoni* showed that the formation of egg granulomas and the inducing of hepatic fibrosis are dependent on the regulation of cytokines (Wynn, 2004).

1.8.1.1. Cellular components of granuloma

The cellular components of granuloma around the viable egg (containing viable miracidium) include eosinophils, macrophages, lymphocytes, neutrophils, mast cells, and fibroblasts (Asahi *et al.*, 1999). There are differences in cellular composition; hepatic granulomas contains the largest number of T and B lymphocytes, eosinophils, and mast cells, where as ileal granulomas consist mainly of macrophages (Ferguson *et al.*, 2002). There are different patterns of distribution of T and B lymphocytes within granulomas in different tissues (Ferguson *et al.*, 2002).
1.8.1.2. Chemokines and granulomatous inflammation in *S. mansoni* infection

Chemokines are a superfamily of low-molecular-weight cytokines that were initially described for their chemoattractant activity (Souza *et al.*, 2008). Chemokines appear to play a role in the pathogenesis of several inflammatory diseases (Camargos *et al.*, 2002; Maurer and von Stebut, 2004; Charo and Ransohoff, 2006). Study in mice suggested a relevant role for the chemokine CCL3 and the receptor CCR5 in the pathogenesis of experimental *S. mansoni* infection. Absence of CCL3 is associated with decrease in granuloma size, fibrosis and parasite load. In humans, levels of CCL3 in plasma are associated with disease severity and may be useful for diagnostic purposes (Falcao *et al.*, 2002). In contrast, the absence of CCR5 is associated with enhanced lethality, granuloma size and fibrosis (Souza *et al.*, 2011). It was postulated that the balance of chemokine production and chemokine receptor activation are important determinants of the fate of infection in experimental animals and humans (Souza *et al.*, 2008).

1.8.2. Role of Th1, Th2 and Th17 lymphocytes in hepatic fibrosis

T helper lymphocytes are playing essentials role in controlling the net effects of immune response towards *S. mansoni* eggs that are trapped in the hepatic tissue. The subclasses of Th lymphocytes can be identified based on their repertoire of cytokines. Naïve Th0 cells produce primarily IL-2 but might also synthesize cytokines characteristic of effectors T lymphocytes. In contrast to the murine studies, categorically distinct Th cytokine profiles are seldom apparent in human cells, although there remains an inverse relationship between the tendency of T lymphocytes to produce IFN-γ as opposed to IL-4/IL-5 or IL-17. In human subjects Th1 cells primarily produce IFN-γ and TNF-β but not IL-4 and IL-5. Th2 cells more prominently produce IL-4, IL-5, IL-9, and IL-13. Th1 lymphocytes promote cell-
mediated immune responses and are important in antibody-dependent immunity. Th17 cells are more important in the T cell–mediated immune response to extracellular pathogens and likely contribute to autoimmune diseases. Th2 lymphocytes produce IL-4, IL-5, and IL-13, which induce antiparasitic and allergic immune responses (Figure 1.3) (Cancado et al., 1995; Liu et al., 2008).

1.8.3. Role of regulatory T cells in hepatic fibrosis

Nematode infections can induce and expand naturally occurring regulatory T cells (TReg cells) in humans and mice (McInnes et al., 2003; Abath et al., 2006) suggesting a role for these TReg cells in helminth-induced modulation of inflammatory diseases (Belkaid and Rouse, 2005).
Naïve CD4+ T cells can differentiate into several different types of effector and regulatory cells during helminth infection. Specific cytokines and transcription factors contribute to differentiation and expansion of these cell populations, and their differential activation plays a major role in determining whether an immune response will contribute to host protection or pathological inflammation.
TReg cells have been suggested to play an important role in the suppression of the Th1-type inflammatory response to Soluble Eggs Antigen (SEA) (Figure 1.4). Initial reports suggested this effect was mediated through IL-10 (Hesse et al., 2004; Freeman et al., 2005); however, more recent findings indicate that these TReg cells can function through unidentified IL-10-independent mechanisms (McKee and Pearce, 2004; Baumgart et al., 2006; Leask et al., 2006). TReg cells might also be instrumental in controlling Th2-type responses in chronic S. mansoni infection (Kahn et al., 1987; Freeman et al., 2005).

Ova produced by S. mansoni are highly immunogenic and cause Th2 differentiation, granulomatous inflammation, and marked hepatic and intestinal fibrosis (Chiaramonte et al., 2001; de Jesus et al., 2004). In mice, Th2 derived cytokines (IL-4, IL-5, and IL-13) can produce morbidity during the chronic phase of infection (16-20 weeks post-inoculation) but are less rapidly harmful than Th1 (IFN-γ) or Th17 (IL-17) derived cytokines, which can cause fatal disease during the acute phase (7-9 weeks post-inoculation) (Hoffmann et al., 2000; La Flamme et al., 2001; Stadecker et al., 2004; Rutitzky and Stadecker, 2006). A study by Herbert et al. (2004) reported the important part played by an alternative activated macrophages in controlling the intestinal inflammation that develops once the S. mansoni worm begins to lay eggs. Transgenic mice that fail to express IL-4R on macrophages and neutrophils die of severe intestinal inflammation during the acute phase of infection showing the importance of IL-4 in granuloma formation (Herbert et al., 2004; Herbert et al., 2008).
**Figure 1.4: RegT cells, IL-10 and IL-13Rα2 controlling tissue fibrosis.**

**a** IL-10 can directly inhibit collagen synthesis by fibroblasts. IL-10 also inhibits interferon-γ (IFN-γ) production by TH1 cells, while promoting the development of a polarized but controlled TH2 response.

**b** When a highly polarized TH1 response is generated, little IL-13 is produced. Consequently, fibrosis is minimal and decoy-receptor expression remains low.

**c** IFN-γ might decrease production of the IL-13 decoy receptor and unregulated IL-13 effector function. In this case, although IL-13 concentrations might slightly decrease or remain unchanged, more IL-13 is free to bind the signalling receptor (IL-4R and IL-13α1).
Some studies on the two cytokines, IL-10 and TGF-β, indicated that they are both well known for their immunosuppressive properties and each one can independently regulate the differentiation, proliferation and activation of immune and non-immune cells and they are both elevated in humans during *S. mansoni* infection (O'Garra and Vieira, 2004; Kriegel *et al.*, 2006; Wan and Flavell, 2006; Li *et al.*, 2007).

IL-10 has been shown to play a role in controlling excessive Th1 and Th2 response, suppresses macrophages and dendritic cells activation and limits the hepato-toxicity induced by worms ova during acute phase (Hoffmann *et al.*, 2000; Hesse *et al.*, 2004). In contrast, T cells and macrophages produce TGF-β during *S. mansoni* infection. TGF-β is known to suppress Ag-specific immunity and to promote the differentiation and the suppressive functions of CD4+, CD25+ Foxp3+ and Tregs (Kuroda *et al.*, 2001; Ince *et al.*, 2006). The mechanism by which IL-10 and TGF-β limit hepatic inflammation is not clear, and many factors may contribute to it. However, an increase in IL-17 production was reported in murine schistosomiasis which caused death. This finding indicates the role of IL-17 in hepatocellular damage in the absence of IL-10 and TGF-β (Rütitzky and Stadecker, 2006).

IL-13 has been shown to play essential regulatory role in priming Th2 mediated pulmonary granuloma formation in *S. mansoni* infection. In contrast, two studies demonstrated a strong association between high levels of IL-13 production and the development of severe fibrosis and serves to reinforce the importance of this cytokine in human fibrosis. Moreover, increased levels of IL-13 were produced by peripheral blood mononuclear cells (PBMC) stimulated by soluble egg antigens (SEA) but not in those stimulated by soluble worm antigen preparation (SWAP),
indicating that the response was primarily directed towards egg antigens (Chiaramonte et al., 1999b; Chiaramonte et al., 2001).

An important role for IL-13 in the development of liver fibrosis in mice and in humans has been pointed out by a number of investigators (Chiaramonte et al., 1999b; Chiaramonte et al., 2001; Booth et al., 2004a; Ramalingam et al., 2008). The decoy receptor IL-13Ra2, which blocks IL-13 function, has been shown to be curtailing for hepatic fibrosis (Wynn et al., 2004).

1.8.4. The role of macrophages in hepatic fibrosis

Macrophages are typically considered as phagocytic cells, which engulf unicellular pathogens such as bacteria and protozoa. “Classical” macrophage populations are associated with being activated by the development of Th1 immunity and having strong pathogen killing nitric oxide responses. However, it is now apparent that macrophages can also develop into an “alternative” non-phagocytic phenotype that was first defined by Gordon and colleagues in the early 1990s (Stein et al., 1992). Initial studies in mice observed that macrophage treatment with a major Th2 associated cytokine, interleukin (IL)-4, in vitro resulted in increased mannose receptor (MR) expression, cellular responses associated with collagen deposition and reduced anti-microbial (iNOS) production (Gordon, 2003). This led to define these macrophages as “alternatively activated” as compared with “classically” activated macrophages with phago-cytic bacteria/ protozoa killing responses. In line with the initial definition, these macrophages refer to alternatively activated macrophages (AAMs) (which have also been called M2 myeloid cells) (Martinez et al., 2009), as macrophages responding, via IL-4Ra, to the cytokines IL-4/IL-13 (Brombacher et al., 2009).
1.8.5. Role of Th2 cytokines in activation of fibroblast cells

Th2 cytokines (IL-4 and IL-13) are playing an essential role in the stimulation of collagen synthesis in mouse and human fibrosis directly like TGF-β1, (Chiaramonte et al., 1999a; Oriente et al., 2000; Murray et al., 2008). These cytokines promote the fibroblast to develop classical myofibroblast phenotype in human lung fibroblast (Hashimoto et al., 2001). Two previous studies dissecting the individual role of Th2 cytokines has identified IL-13 as a dominant inducer of hepatic fibrosis in mice (Chiaramonte et al., 1999a; Chiaramonte et al., 2001). Recent study indicating that IL-13 is aggravating fibrosis in chronic hepatitis C virus infection (Weng et al., 2009). Thus, IL-13 directly stimulates collagen synthesis in fibroblast, and indirectly activates the Latent form of TGF-β1 (Lee et al., 2001). IL-13 and TGF-β1 have been shown to cooperate in activation of myofibroblasts (Murray et al., 2008).

1.8.6. The contribution of macrophages and fibroblasts in fibrosis regulation

Macrophages contribute to the pathogenesis of fibrosis after being stimulated by IL-4/IL-13 (Hesse et al., 2000; Hesse et al., 2001). Macrophages activation was first described as a Th1 cell-IFN-γ mediated process. However, it's now clear that Macrophages differentiate into at least two functionally distinct populations depending on whether they are exposed to Th1 or Th2 cytokines (Weng et al., 2009).

Th1 cytokines activate nitric oxide synthase-2 (NOS2), expression in classically activated macrophages; on the other hand, Th2 cytokines IL-4 and IL-13 prefer to stimulate arginase-1 (ARG1) activity in alternative activated macrophages (AAM) (Hesse et al., 2000; Weng et al., 2009). In fibroblast, L-Arginine is the substrate for enzymes. However, the addition of L-Arginine to NOS2 produce L-hydroxy-arginine, L-ornithine, and NO, where, the action on ARG-1 promotes the
production of urea and L-ornithine. L-ornithine is the substrate for two other enzymes, ornithine decarboxylase (ODC) and ornithine amino transferase (OAT). The action of ODC and OAT are produce polyamines and L-proline respectively. The polyamines are crucial for cell growth and proline is the substrate for collagen synthesis. The two pathways (ODC and OAT) are thought to be important in repair processes (Hesse et al., 2001).

Collagen synthesis is depending on the availability of L-proline. Thus activation of ARG-1 compared with NOS2 in macrophages or fibroblasts was proposed to be a possible explanation for the potent pro-fibrotic activity of IL-13 (Chiaramonte et al., 1999a) and, anti-fibrotic of IFN-γ (Wynn et al., 1995; Hesse et al., 2000). The Th2 cytokines stimulate macrophages to produce large quantities of proline through an ARG1-depending mechanism (Hesse et al., 2001). Direct activation of the ARG1 pathway in fibroblasts could be an additional mechanism to augment their collagen-producing potential (Figure 1.5).

1.8.7. The role of macrophages in the regression of fibrosis

Macrophages and the interstitial collagenases (MMP1, MMP2, MMP8, MMP9, and MMP13) are the major actors in resolved fibrosis (Duffield et al., 2005). Hepatic macrophages can play a role in the resolution of fibrosis, by recruiting neutrophils, whose collagenase effectively digest ECM components (Harty et al., 2008). Studies on a schistosomiasis model had revealed an important antifibrotic role for MMP13 in the development of infection-induced liver fibrosis (Madala et al., 2010).

To negatively regulate fibrosis, macrophages secrete factors that induce myofibroblast apoptosis, remove cellular debris, engulf and digest ECM components, and stimulate the production of collagen-degrading. Macrophages secretion can
Figure 1.5: The role of macrophages in collagen production
increase MMPs collagen-degrading in other cells types (stellate cells, myofibroblasts, and neutrophils) in order to digest fibrosis (Issa et al., 2003).

1.9. Hepatic stellate cells (or Ito cells)

Hepatic stellate cells (HSC), previously called Ito cells, are the major effectors during hepatic fibrosis, since they are playing a major role in the process of fibrous tissue formation in the liver. They are responsible for the synthesis of components of extra-cellular and several types of collagens (Friedman, 1993, 2000; Parola and Robino, 2001).

Hepatic stellate cells reside in the Disse’s spaces of the liver sinusoids, and they constitute a minor cell type, roughly 5–8% of the total liver cells (Maubach et al., 2006). Following chronic injury, HSCs differentiate into myofibroblast-like cells, which acquire contractile and fibrogenic properties (Nauser and Stites, 2003). For immune-histochemical identification of HSCs, traditional anti-bodies against desmin (Nauser and Stites, 2003), vimentin, and α-smooth muscle actin were used (Stites et al., 1999). Recently, glial fibrillary acidic protein (GFAP), which is traditionally used as a marker for a stromal cells of the brain, was established as a marker for HSCs (Lim et al., 2008).

1.10. Extracellular matrix (ECM) in hepatic fibrosis

Liver fibrosis is characterized by an abnormal hepatic accumulation of extracellular matrix (ECM) that results from both increased deposition and reduced degradation of collagen fibers (Iredale, 2007). Prolonged liver injury results in hepatocytes damage, which triggers the activation of the hepatic stellate cell (HSCs) and the recruitment of inflammatory cells into the liver (Kisseleva and Brenner, 2006). Surrogate markers are gradually being substituted for biomarkers that reflect the complex balance between synthesis and degradation of the extracellular matrix.
Once the hepatic stellate cell is activated, the preceding matrix changes and recurrent injurious stimuli will perpetuate the activated state (Das and Vasudevan, 2008). The ECM directs cellular differentiation, migration, proliferation and fibrogenic activation or deactivation. The metabolism of the extracellular matrix is closely regulated by matrix metalloproteinases (MMP) and their specific tissue inhibitors (TIMP) (Das and Vasudevan, 2008).

1.10.1. Role of extracellular matrix and connective tissue cells in hepatic fibrosis

The ECM provides cells with positional information and a mechanical scaffold for adhesion and migration. It consists of collagens, glycoproteins, proteoglycans, glycosaminoglycans and molecules that are bound specifically by the ECM, such as certain growth factors/cytokines, MMPs and processing enzymes such as tissue transglutaminase and procollagen propeptidases (Schuppan et al., 2001). This finely tuned ecosystem is dysbalanced in chronic fibrogenesis, which can be regarded as a continuous wound-healing process and which results in scar formation. Importantly, the ECM directs cellular differentiation, migration, proliferation, and fibrogenic activation or deactivation. Partially via defined oligo-peptide sequences or structural domains, the ECM transfers specific signals to cells that act in concert with growth factors/cytokines. These signals either confer stress activation, with a resultant fibrogenic response, or stress relaxation, with a fibrolytic response (Schuppan et al., 2001).

In the dynamic process of deposition and resorption in liver fibrosis in experimental *S.mansoni* infection, glycosaminoglycans and collagens are two major ECM components. Fibroblasts are the most important connective tissue cells involved in ECM production in normal livers. In liver fibrosis, a major proportion of
the ECM is probably produced by mesenchymal fibroblast-like subpopulations (Abdel-Aziz et al., 1991; Friedman, 1993).

1.10.2. Matrix metalloproteinases (MMP)

Matrix metalloproteinases (MMP) are predominant proteases that play an essential role in the remodelling of extracellular matrix (ECM) by degrading collagen, and other extracellular filaments. They also regulate cytokines, growth factors and their receptors (Han, 2006; Wynn, 2007). An excess of proteolytic activity in tissues can be destructive and therefore the synthesis and activity of MMPs are tightly regulated at transcriptional, translational, and posttranslational levels (Tallant et al., 2010). In vertebrates, the MMP family consists of more than 20 different proteases that differ in their tissue expression, cellular location and substrate specificity (Tallant et al., 2010). They are synthesized as inactive pro-proteins that later convert to an active enzyme by an endoprotease in response to specific cellular tasks. In addition, the proteolytic activities of MMPs are regulated by tissue inhibitors of metalloproteinases. The ratio of MMP and tissue inhibitors of matrix metalloproteinases expression in tissues is thought to determine the turnover of ECM and can regulate fibrogenesis or fibrolysis (Vaillant et al., 2001; Wynn, 2007).

1.10.3. Regulation of matrix degradation

The decreased activity of ECM-removing MMPs is mainly due to an increased expression of their specific inhibitors TIMPs. The key enzymes in the degradation of fibrillar collagens are matrix metalloproteinase (MMP)-1 in humans and MMP-13 in rodents (Emonard and Grimaud, 1990). However, during fibrogenesis, the expression of MMP-1 or MMP-13 is very limited, whereas that of MMP-2 increases (Milani et al., 1994; Preaux et al., 1999). On the other hand,
fibrotic livers have high expression of the TIMPs, including TIMP-1 and TIMP-2 (Iredale et al., 1992; Murawaki et al., 1992; Kossakowska et al., 1998). Thus, there is a combination of low expression of interstitial collagenases and high TIMPs that prevents the degradation of the fibrillar collagens. Moreover, it has been recently well documented that fibrillar collagens act as a survival factor for activated HSC. An *in vitro* study has shown that HSC are activated on collagen type I, whereas they are quiescent on a basement membrane-like substrate (Matrigel) (Maher and Bissell, 1993).

1.10.4. Fibrosis resolution

Increased collagenolytic activity is a major mechanism of fibrosis resolution (Arthur, 2002), and fibrillar collagens (type I and III) are degraded by interstitial MMPs. According to several animal models of liver fibrosis resolution, the expression of TIMPs rapidly decreases while interstitial MMPs continue to be expressed during the resolution, resulting in increased MMP activity and consequent matrix degradation within the liver (Iredale et al., 1998; Yoshiji et al., 2002; Issa et al., 2004). Together with these changes in MMP activity, the apoptosis of activated HSC has been observed in the resolution process of hepatic fibrosis (Iredale et al., 1998; Issa et al., 2001; Wright et al., 2001).

1.11. Periportal fibrosis (PPF)

Periportal fibrosis of the liver is a serious consequence of *S. mansoni* infection that involves remodelling of the ECM and excessive deposition of collagen along the branches of the portal tract (Booth et al., 2004b). Fibrotic or chronic hepatic schistosomiasis develops years later in the course of infection, generally in young and middle-aged adults with long-standing intense infections and, presumably, some form of immunogenetic predisposition (Homeida et al., 1988a; Dessein et al.,...
The disease results from a massive deposition of different collagen deposits in the periportal spaces, leading to pathognomonic periportal or Symmer’s pipestem fibrosis (Cheever, 1968). This fibrosis leads in turn to progressive occlusion of the portal veins, portal hypertension, splenomegaly, collateral venous circulation, portocaval shunting, and gastrointestinal varices. The liver is not necessarily enlarged but is generally hard and nodular on palpation (Cheever, 1968). In *S. mansoni* infections, the fibrotic process takes about 5–15 years, by that time the infection might no longer be present or detectable (Cheever, 1968; Gryseels and Polderman, 1987; Homeida *et al.*, 1988a). Bleeding from gastro-oesophageal varices is the most serious, commonly fatal, complication of fibrotic hepatic schistosomiasis (Chen, 1993; Lambertucci, 1993b) (Figure 1.6).
**Figure 1.6:** Schematic representation showing severe consequence of hepatic periportal fibrosis due to *S. mansoni*
1.12. Genetic control of infection and disease in human schistosomiasis

1.12.1. Schistosoma mansoni 1 (SM1) locus

SM1 was mapped by linkage analysis in a Brazilian population. Only one region in the chromosome 5 (5q31-q33) has showed suggestive linkages to susceptibility to S. mansoni infection (Marquet et al., 1996; Marquet et al., 1999). SM1 contains a number of genes [Interleukin-4 (IL-4), IL-5, IL-12, IL-13, CSF-1R, and the interferon regulatory factor-1 (IRF-1)] encodes cytokines that may play an important role in the immune response against helminthes (Alain et al., 2008). The existence of SM1 in controlling susceptibility to S. mansoni was confirmed in an independent study on a Senegalese population (Muller-Myhsok et al., 1997). Interestingly, 5q31-q33 locus is controlling the infection level in a third population study in Dogons of Mali by another schistosome S. Haematobium (Kouriba et al., 2005). In addition SM1 is controlling P. falciparum infections (Rihet et al., 1998), regulating the IgE levels, and controlling the bronchial hyper-responsiveness in asthma (van der Pouw Kraan et al., 1999; Graves et al., 2000; Heinzmann et al., 2000; Liu et al., 2000).

1.12.2. Schistosoma mansoni 2 (SM2) locus

Severe disease in S. mansoni infections is the consequence of periportal fibrosis that develops in the hepatic periportal spaces as a consequence of the inflammation caused by eggs and worm products. It was not clear why some subjects are unable to turn over the fibrosis despite it was part of a normal scar processing. The intensity of infection has been incriminated for a long time, but more recently, it was clear that infection levels are only one among others factors that includes duration of infection, age, sex and genetic factors which control disease severity (Qurashi et al., 1999). A study of ultrasound evaluations in an endemic area of the
Sudan revealed an association between severe fibrosis and portal hypertension. Furthermore, the severity of disease was more frequent in certain families while it was less in others, despite the fact that these families shared the same environmental conditions (Qurashi et al., 1999). A major locus that controls severity of disease was mapped in 6q22-q23 (Dessein et al., 1999). This locus is very close to the gene encoding the α-chain of the interferon-gamma (IFN-γ) receptor1. It is not surprising that IFN-γ has anti-fibrogenic properties (Sempowski et al., 1996). A previous study demonstrated that polymorphisms in IFN-γR1 gene affecting protein structure have lethal consequence. These polymorphisms may cause death by certain infectious diseases such as weak pathogen like BCG tuberculosis (Pierre-Audigier et al., 1997).

Recently, susceptibility genes located in 6q22-q23 region was identified and validated in four independent populations (Dessein et al., 2009). Several polymorphisms that are lying close to the CTGF (Connective Tissue Growth Factor) gene are associated with severe hepatic fibrosis in two Chinese samples, in Sudanese and in Brazilians infected with either S. japonicum or S. mansoni. These variants affect nuclear factor binding and may alter gene transcription or transcript stability (Dessein et al., 2009).
1.13. Candidate genes in susceptibility to severe hepatic disease

In candidate gene approach of the linkage, some chromosomal loci e.g. (SM1 and SM2) or gene that are expected to influence the phenotype are studied for linkage. This approach can lead to localization of the phenotype locus. In current study three genes were selected for genetic analysis CTGF, IL-13 and IL-10 genes.

1.13.1. Connective tissue growth factor (CTGF) gene

Connective tissue growth factor (CTGF or CCN2) is the second member of CCN family of sex proteins named for Cyr61, CTGF and Nov (Bork, 1993). First isolated from human umbilical vein endothelial cell culture supernatants using anti-platelet-derived growth factor (PDGF) antibody (Bradham et al., 1991). The CTGF expression was detected in various normal human cells including fibroblasts, chondrocytes and mesangial cell (Ryseck et al., 1991; Igarashi et al., 1993). In some tissues the constitutive expression is low but the expression is elevated due to wound healing reported in certain fibrotic disorders such as atherosclerotic plaques (Oemar et al., 1997), liver cirrhosis (Abou-Shady et al., 2000) and scleroderma (Igarashi et al., 1996).

1.13.1.1. Connective tissue growth factor (CTGF) gene and protein structure

The gene structure comprises five exons and four introns with each exon corresponding to specific motif in the protein. The first exon codes for signalling peptide which directs the protein to its eventual extra cellular location (Planque and Perbal, 2003). The human CTGF gene was mapped to chromosome 6q23.1 (Martinerie et al., 1992) (Figure 1.7). It spans approximately 3 kb (Grotendorst et al., 1996). The 5’untranslated region contains a variety of regulatory elements (e.g. AP1, CaG, TATA, M-CAT, SP1) as well as a unique TGF-response element that might well account for
Figure 1.7: Schematic representation of CTGF gene. The gene of human maps to chromosome 6q23.1, and comprises of five exons and four introns
much of the expression that has been reported for CTGF (Grotendorst et al., 1996; Xin et al., 1996).

CTGF is a 36-38 KD monomeric protein composed of 349 amino acids (Bradham et al., 1991). The four distinct structural modules of the protein suggest multifunctional properties (Figure 1.8). Module 1 is homologous to insulin-like growth factor binding protein (IGFBP). Module 2 comprises a Von Willebrand factor type C (VWF-C)/chordin-like cystein-rich motif, which binds bone morphogenetic proteins and TGF-beta 1 (Bork, 1993; Abreu et al., 2002). Between the N domains and C domain there is a huge region, prone to proteolytic cleavage, separates into two motifs (N-terminus domain and C terminus). Module 3 contains a thrombospondin motif which binds low density lipoprotein receptor-related protein (Gao and Brigstock, 2003), integrin’s and heparin sulphate proteoglycan (Gao and Brigstock, 2004). The module 4, located at the carboxy terminal end, is known as CT module or cysteine knot. This module works as assistant of vascular endothelial growth factor (VEGF) in binding (Inoki et al., 2002) and has been implicated in heparin and integrin binding (Ball et al., 2003) as it also binds fibronectin (Hoshijima et al., 2006).
Figure 1.8: Schematic representation of CTGF protein-insulin growth factor binding Protein (IGFBP), Von Willebrand factor type C (VWF/CR), Thrombospodin (TSP), and carboxy terminal (CT)
1.13.1.2. CTGF gene in fibrotic skin disorders

Over-expression of the CTGF, and TGF-beta 1 were shown in many fibrotic skin diseases such as systemic sclerosis. This process begins as a normal wound healing after tissue injury that follows closely regulated pathway (Igarashi et al., 1995; Igarashi et al., 1996; Querfeld et al., 2000; Denton and Abraham, 2001). However, in pathological conditions, fibrosis is elevated and fibroblast activity continues unabated causing accumulation of ECM and scaring. In skin sclerosis a strong CTGF mRNA was observed in fibroblast of sclerotic lesion when compared with normal skin control (Igarashi et al., 1995).

1.13.1.3. CTGF in organ fibrosis

The CTGF mRNA or protein level were found to be elevated in many organic fibrotic lesions and this over-expressed level of CTGF mRNA was observed in many organs like Kidney (Blom et al., 2001) lungs, pancreas, cardio-vascular and liver (Oemar et al., 1997; Sato et al., 2000; di Mola et al., 2002; Pan et al., 2002; Paradis et al., 2002)

1.13.1.4. CTGF gene in liver fibrosis

CTGF gene expression was found to be elevated in fibrotic livers (Rachfal and Brigstock, 2003). In fibrotic liver CTGF mRNA and the proteins are produced by fibroblasts (Leivonen et al., 2005), HSCs (Paradis et al., 2002), as well as endothelial cells and bile duct epithelial cells (Narkewicz et al., 2005). CTGF expression in HSCs culture is enhanced by TGF-β1, and then CTGF is able to promote HSCs adhesion, proliferation, migration, collagen, and α-SMA production (Kruidering and Evan, 2000; Paradis et al., 2002; Rachfal and Brigstock, 2003).
1.13.2. Interleukin-13 (IL-13) candidate gene for SM1

1.13.2.1. IL-13 gene structure

IL-13 gene was first described in 1989 as P600, a protein preferentially produced by activated mouse Th2 cells (Brown et al., 1989). The cDNA from human IL-13 is 132 amino acids (Figure 1.9). IL-13 belongs to the class of type 1 cytokines and shares the tertiary structure defined by a 4 alpha helical hydrophobic bundle core (Morgan et al., 1992; McKenzie et al., 1993; Minty et al., 1993).

The gene encoding IL-13 is comprised of four exons and three introns and located 12 kilo-base pairs upstream of the gene encoding IL-4 on chromosome 5q31. Both genes are in the same orientation (Smirnov et al., 1995). This chromosomal region contains genes with immunological contribution in many pathological and immunological disorders. These genes encode IL-3, IL-5, IL-9 and GM-CSF (McKenzie et al., 1993; Smirnov et al., 1995). 5q31 region has been linked to athama (Marsh et al., 1994; Marsh et al., 1995) as well as schistosomiasis and malaria infection levels (Marquet et al., 1996; Naka et al., 2009).

1.13.2.2. The function of IL-13 in haematopoietic and non-haematopoietic cells

IL-13 has many diverse functions in wide ranges of different cell types. In human B-cells, the IL-13 action is similar to that of IL-4, including promoting B cell proliferation. IL-13 is inducing class switching to IgG4 and IgE. Also IL-13 is acting as inducer of surface antigens like low affinity IgE receptors CD23 (FcεRII) and MHC class II (Oettgen and Geha, 2001). While in monocytes and macrophages cells, IL-13 elevates the levels of many member of integrin family expression (including CD11b, CD11c, CD18 and CD23) (Zurawski and de Vries, 1994).
Figure 1.9: Schematic representation of IL-13 gene. The gene of humans maps to chromosome 5q31 and comprises of four exons and three introns.
IL-13 is playing a critical role in the inhibition of the production of pro-inflammatory mediators by monocytes and macrophages like prostaglandins (Endo et al., 1996), reactive oxygen and nitrogen intimidators (Doherty et al., 1993; Sozzani et al., 1995), cytokines like IL-6, IL-1, IL-8, TNF-α and IL-12 (de Vries, 1998), by mechanisms that involve the suppression of nuclear factor kappa B (Lentsch et al., 1997). In endothelial cells, IL-13 was reported to promote eosinophil activation, survival and recruitment (Bochner et al., 1995), while in mast cells, IL-13 was shown to promote IgE synthesis (de Vries, 1998). IL-13 also affects the function of some non-haematopoietic cells, like fibroblast, smooth muscle cells and endothelial cells (Roux et al., 1994). IL-13 promotes and induces synthesis of collagen type 1 in human dermal fibroblasts (Roux et al., 1994). The net effects of IL-13 functions is promoting inflammation while contributing to the control of helminthes infections and suppressing inflammations associated with bacterial infections, most viral and allergic disorder like asthma (Zurawski and de Vries, 1994) (Figure 1.10).

**1.13.2.5. IL-13 receptors complex**

The IL-13 receptor complex has been reported to be shared by IL-4 as well as several cytokines. Two other human receptors have been cloned, one of these referred to as IL-13Rα1(Aman et al., 1996), and the other is IL-13Rα2 cloned byCaput et al. (1996). IL-13Rα1 binds IL-13 with subsequent recruitment of IL-4Rα1, to efficiently transducer a signal (Miloux et al., 1997), while IL-13Rα2 can bind IL-13 in the absence of IL-4Rα1, its role in IL-13 signalling is still unclear (Miloux et al., 1997). IL-13Rα1 binds TYKs. This binding results in activation of JAK1 and TYK2 in haematopoietic and non-haematopoietic cells (Keegan et al., 1995; Welham et al., 1995; Roy and Cathcart, 1998).
Figure 1.10: Overview of actions of IL-13 on hematopoietic and nonhematopoietic cells.
1.13.3. Interleukin-10 (IL-10) gene

1.13.3.1. IL-10 cytokine and inflammation

Interleukin-10 (IL-10) is an anti-inflammatory cytokine with a crucial role in prevention of inflammatory and autoimmune pathologies. IL-10 was initially described as a Th2 cytokine (Hawrylowicz, 2005; O'Garra et al., 2008), but further evidence suggested that the production of IL-10 is associated with tolerance or regulatory T cell (Treg) responses (Moore et al., 2001; O'Garra and Vieira, 2004; Roncarolo et al., 2006). It is now known that the expression of IL-10 is not specific to Th2 cells or Treg cells but instead, it is a much more broadly expressed cytokine. IL-10 is expressed by many cells of the adaptive immune system, including Th1, Th2 and Th17 cell subsets, Treg cells, CD8+ T cells and B cells (Moore et al., 2001; O'Garra and Vieira, 2007). It is also expressed by cells of the innate immune system, including dendritic cells, macrophages, mast cells, natural killer cells, eosinophils and neutrophils (Moore et al., 2001). Thus, IL-10 production seems to be associated with many immune cells, confirming its crucial role as a feedback regulator of diverse immune responses, not only Th1 cell responses (O'Garra and Vieira, 2007), but also Th2 cell responses, example: Aspergillus spp (Grunig et al., 1997), allergens (Zuany-Amorim et al., 2002), and schistosoma parasites (Hoffmann et al., 2000). Indeed, Hoffmann et al. (2000) demonstrated that IL-10 significantly suppresses type 1 and type 2 cytokine development in IL-4 and IL-12 deficient mice respectively, thereby impeding the development of severe egg-induced pathology in the single cytokine-deficient animals.

1.13.3.2. IL-10 gene organization and sequence

Interleukin-10 (IL-10) is an 18-kDa weight, located on chromosome 1q32 (Figure 1.11). The gene has five exons and four introns (Kim et al., 1992). The
Figure 1.11: Schematic representation of IL-10 gene. IL-10 gene is located on chromosome 1q32. The gene comprises of five exons and four introns.
promoter spans a region of 5kb upstream of the transcription start point, and is known to contain several polymorphisms (Eskdale and Gallagher, 1995; Kube et al., 1995; Eskdale et al., 1996; Eskdale et al., 1997). At approximately 1.1 and 4kb upstream, there are two microsatellite \((AC)^n\) repeats termed IL-10G and IL-10R, respectively (Eskdale and Gallagher, 1995; Eskdale et al., 1996). Each exists in multiple forms according to the number of repeat units in normal controls, and there may be population differences in allele distribution (Eskdale et al., 1998). In addition, the IL-10 proximal promoter contains three single-nucleotide polymorphisms at positions -819 C/T, which may affect an oestrogen receptor element) (Lazarus et al., 1997), -592 C/A, lying in a region exerting negative regulatory function and -1082 G/A, lying within a putative ETS-like transcription factor-binding site (Kube et al., 1995).

1.13.3.3. IL-10 polymorphisms and disease association

Variation in the IL-10 promoter were first reported by Turner et al. (1997). In Mycobacterium studies, there are conflicting results some investigators reported no association between tuberculosis (TB) and IL-10 promoter SNPs in Spanish population, Gambian population and Chinese population (Bellamy et al., 1998; Lopez-Maderuelo et al., 2003; Tso et al., 2005). On the other hand, another author reported that the -1082A allele was increased among Cambodian pulmonary TB patients (Delgado et al., 2002). This finding was confirmed in Sicilian patients (Scola et al., 2003).

The -592TT genotype was found to be associated with risk of leprosy (Santos et al., 2002). A case control study in Malawi, revealed no association between IL-10 promoter polymorphisms (-1082, -819 and -592) and leprosy susceptibility (Fitness et al., 2004). In a study of Meningococcemia, the -1082AA genotype was more
frequently associated with severe outcome and with lack of survival (Balding *et al.*, 2003).

IL-10 promoter polymorphisms have been shown to be related to several viral infections. Microsatellite-based studies showed that individuals with -592A promoter allele had increased risk of HIV-1 infection, and once infected, progressed to ADIS more rapidly than homozygotes with the 592CC genotype (Shin *et al.*, 2005). Studies of IL-10 polymorphisms have confirmed the association between EBV infection and disease severity, and reported that -1082A was associated with increased risk susceptibility/severity; that it might be due to lower IL-10 production (Helminen *et al.*, 1999; Helminen *et al.*, 2001).

IL-10 polymorphisms may also influence responses to parasitic infections. In Chagas disease, association was found between certain IL-10 microsatellites and infection (Moreno *et al.*, 2004). Evidence was shown that IL10-819C increases the risk of skin lesion in *Leishmania* (Salhi *et al.*, 2008). Functional analysis to investigate potential functional effects of the promoter polymorphisms were done in two polymorphisms, -819 C/T and -592 A/C. IL10-819C/C genotype was associated with increased IL-10 production in LPS- or HA-stimulated culture, thus, -819C/C in the IL10 promoter region is associated with up-regulation of IL-10, modification of NF binding and an increased risk of lesions (Salhi *et al.*, 2008). Another study in *S. Japonicum* illustrated that, IL-10 was associated with protection against central fibrosis, because of its anti-inflammatory and anti-fibrosis effects (Arnaud *et al.*, 2008).
1.14. Bioinformatics

1.14.1. HapMap project

The international HapMap project (http://www.hapmap.org) is collaboration between scientists from United States of America, Canada, China, Japan, Nigeria, and the United Kingdom. The aim of the project is to assist medical genetic research by providing information on the common patterns of human DNA sequencing variation (Consortium, 2003).

HapMap project was initiated in 2002 with the main objective, in phase one, to genotype one million SNPs. All SNPs with minor allele frequency (MAF) greater than 5% across the euchromatic portion of the genome in 270 individuals from four geographically diverse populations were screened. 90 subjects from the Yoruba in Ibadan, Nigeria (abbreviated YRI); 90 subjects from northern and western European ancestry (CEU); 45 unrelated Han Chinese individuals in Beijing, China (CHB); and 45 unrelated Japanese individuals in Tokyo, Japan (JPT) were analyzed for SNPs variation (David et al., 2005). The data generated by the project can be downloaded and analysed by researchers for genetic association studies. In phase II of HapMap project, another 2.1 million SNPs in the same populations were genotyped (Frazer et al., 2007).

The coinheritance of SNP alleles on these haplotypes leads to associations between these alleles in the population (known as linkage disequilibrium, LD). Because the likelihood of recombination between two SNPs increases with distance between them, on average such associations between SNPs decline with short distance. Many empirical studies have shown highly significant levels of LD, and often strong associations between nearby SNPs, in the human genome (Abecasis et al., 2001; Jeffreys et al., 2001; Reich et al., 2001; Dawson et al., 2002; Gabriel et al.,
2002). These strong associations mean that in many chromosome regions there are only a few haplotypes, and these account for most of the variation among people in those regions (Daly et al., 2001; Patil et al., 2001; Gabriel et al., 2002).

The strong associations between SNPs in a region have a practical value: genotyping only a few, carefully chosen SNPs in the region will provide enough information to predict much of the information about the remainder of the common SNPs in that region. As a result, only a few of these ‘tag’ SNPs are required to identify each of the common haplotypes in a region (Daly et al., 2001; Johnson et al., 2001; Carlson et al., 2003; Goldstein et al., 2003).

1.14.2. Data analysis

The project (HapMap) will apply existing and new methods for analysis and display of the data. LD between pairs of markers will be calculated using standard measures such as D prime (Lewontin, 1964), r^2 (Hill and Robertson, 1968). Various methods are being evaluated to define regions of high LD and haplotypes along chromosomes. Existing methods include ‘sliding window’ LD profiles (Dawson, 2000), LD unit maps (Maniatis et al., 2002), haplotype blocks (Daly et al., 2001; Gabriel et al., 2002) and estimates of meiotic recombination rates along chromosomes (Hudson, 1987; Daly et al., 2001; Fearnhead and Donnelly, 2001; McVean et al., 2002).

Carlson et al. (2004) described the pair-wise tagging method used in Haplview. They put a group of associated SNPs with each other in one bin. No need for associated SNPs to be neighboring SNPs, rather than their association must exceed a certain r^2 threshold. In recent study, the tagSNPs were identified in the YRI population using threshold of r^2 ≥0.8. if all SNPs have been placed into bins, a tagSNPs is identifying that will be captured the SNPs in our bin, addition to that if
the SNPs does not exceed the $r^2$ threshold with any other SNP, we can say that this SNP is called a singleton bin. The number of singleton bins depends on the $r^2$ threshold and the population under investigation (Barrett and Cardon, 2006).

The African population represented by YRI, has been recognized by a higher percentage of singleton SNPs, since they have lower levels of LD (Ke et al., 2008). Lowering the $r^2$ threshold results in identifying fewer tagSNPs for genotyping, and this situation can lead to missing some of the singleton SNPs, which they could be possibly captured by another tagSNPs, this causes a weakness in the power of detection of tagSNPs, and they would not be as highly associated with SNPs they captured (de Bakker et al., 2005).
1.15. Rationale

The pathology resulted from the infection with helminthes e.g. *S. mansoni* was predominately caused by immune response against the eggs of *Schistosoma* which had been laid in the portal venous system and subsequently been trapped in the hepatic sinusoids to form granulomatous lesions. However, only a small percentage of the individuals infected (5-10%) in an area of endemicity actually develops a severe form of the disease. A growing range of cytokines and their receptors and inflammatory cell subsets have further expanding the knowledge about the dynamic process. Collectively, these findings have laid the foundation for continued elucidation of underlying mechanisms, and more importantly for the implementation of rationally based approaches to limit fibrosis, accelerate repair and enhance liver regeneration in patients with chronic liver disease. Thus, the aims of this study were.

1.15.1. General objectives:

1. To evaluate the epidemiological factor in the development of *mansoni* disease.

2. To study the major loci (SM1 and SM2) that aggravate *S. mansoni* disease and to search for additional loci that could play a role in *S. mansoni* pathogenesis.

1.15.2. The specific objectives were:

1. To characterize liver and splenic disease due to *S. mansoni* disease.

2. To identify factors that may aggravate *S. mansoni* hepatic disease

3. To evaluate the role of host genetic factors in controlling severe *S. mansoni* hepatic disease.

4. To identify the causative alleles at the principal loci which aggravate disease at that loci and to determine how those alleles aggravate *S. mansoni* hepatic disease.
Chapter two

Materials and Methods

2.1. Study area and population

This study was a population-based case control type. Representative samples of individuals with disease and those at risk (but not infected) controls were selected using Ultrasound. Central periportal fibrosis (thick central fibrosis) refers to grades D, E, and F were infected cases, while peripheral fibrosis (grade B, C, and C+) in addition to grade A (no fibrosis disease despite exposure to pathogens for several years) were controls uninfected. Preparatory visits to 100 (villages and camps) in Gezira State. School teachers, Shiekh, and traditional leaders were asked for participation in the study. Finally, four small villages in Managil area (Wad Elamin; El Awamra, Kitra Hamdan, and El Krimit) in the south-western extension of the Gezira’s Irrigated Scheme were selected for the current study during the period 2006 - 2010. Most of the study subjects were migrants from west Sudan, where S. mansoni is not endemic. They were settled in the villages more than 20 years ago. The tribal composition is as follows: Tama, Messeria, Mararit, Bargo, Gimir, Zagawa, and Kawahlra. The villages were built in the middle of fields where the villagers were employed for agriculture activities. Various canals encircle the villages, within a short distance (10–100 m) from the houses. Informed consents were obtained from the study subjects, who were free to leave or to enter the study at any time (Figure 2.1). This study was approved by medical ethics committees of the University of Gezira.
Figure 2.1: Gezira map illustrating study areas (Blue Nile Health Project. Annual Report. Khartoum, Ministry of Health, 1982).
2.1.1. Selection of study subjects

2.1.1.1. Inclusion criteria

1. No history of anti-schistosomal chemotherapy treatment in the last 3 years.

2. Hepatic fibrosis as assessed by ultrasonography.

2.1.1.2. Exclusion criteria

1. Age less than 16 years.

2. Past history of positive serology test for human immunodeficiency virus.

3. Human T-cell leukaemia virus type 1 or hepatitis virus types B and C, as well as other conditions that could interfere in the immunological evaluation or classification by ultrasound analysis.

2.2. Clinical evaluation

Patients data was collected on a standardized medical record form. Clinicians recorded information about age, sex, tribe, water contact, exposure to pathogen, past history of infection, bleeding e.g. (Appendix-I). The work plan to achieve the objectives of this study was shown in study strategy (Figure2.2).

2.3. Ultrasound evaluation of liver fibrosis

Volunteers were subjected to ultrasound examinations performed by different physicians. The physicians who performed the ultrasound examinations were not aware neither of the infection status of study subjects nor of the results of the clinical examination. Portal vein diameter, thickness of the walls of the gall bladder, spleen size and splenic vein diameters were assessed using a conventional portable ultrasound instrument (ultrasonic diagnostic instrument model EUB-200; Hitachi, Tokyo, Japan) (Appendix II). Image patterns scores were assigned to each
Figure 2.2: The study strategy
cohort member using the Niamey protocol with slide modification (Richter J, 2000; Kariuki et al., 2001). This protocol grades patients on an ordered categorical scale from A through F, depending on the extent of visible fibrotic tissue around the portal branch and throughout the parenchyma. Peripheral fibrosis grades A and B are considered to be normal livers, whereas grades C was modified in current study to two part (C and C+) represent increasingly severe manifestations of the disease.

Central periportal fibrosis like aspects in pattern D (highly echogenic ‘ruff’ around portal bifurcation and main stem), pattern E (highly echogenic ‘patches’ extending from the main portal vein and branches into the parenchyma), and pattern F (highly echogenic ‘bands’ and ‘streaks’), extending from the main portal vein and its bifurcation to the liver surface, where they retract the organ surface (Table 2.1 and Figure 2.3).
### Table 2.1: Hepatic fibrosis by Niamey image pattern scores (Richter et al., 2000)

<table>
<thead>
<tr>
<th>Pattern</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Normal</td>
</tr>
<tr>
<td>B</td>
<td>Diffuse echogenic foci (‘starry sky’); minimal evidence of wall thickening around portal and sub segmental branches</td>
</tr>
<tr>
<td>C</td>
<td>Ring echoes around vessels in cross-section; pipe-stems parallel with portal vessels</td>
</tr>
<tr>
<td>D</td>
<td>Echogenic ruff around portal bifurcation and main stem; thickening of walls of main portal branches</td>
</tr>
<tr>
<td>E</td>
<td>Hyperechoic patches expanding into parenchyma</td>
</tr>
<tr>
<td>F</td>
<td>Echogenic bands and streaks expanding from main portal vein and its bifurcation to liver surface, where they retract organ surface</td>
</tr>
</tbody>
</table>
Figure 2.3: Ultrasound classification of liver damage induced by *S. mansoni* (Richter et al., 2003).

**A**: normal; **B**: starry sky; **C**: rings and pipe-stems (initial fibrosis of segmentary branches) (peripheral fibrosis). **D**: “ruff” around portal bifurcation, also known as central fibrosis; **Dc**: mixed form; **E**: patches, fibrosis around primary and segmentary portal branches; **Ec**: mixed form; **F**: bird claws, corresponding to marked central and peripheral fibrosis. Grades **D**, **Dc**, **E**, **Ec**, and **F** all were known as central periportal fibrosis grades.
2.4. Genetic analysis

2.4.1. Blood samples

5 to 10 ml of venous blood samples were collected from 370 participants recruited in the study, in vacutainer EDTA tubes. The blood samples were kept frozen at –70°C till the time of DNA extraction and genetic analysis which were done in INSERM U 399, Marseille, France.

2.4.2. DNA extraction

DNA extraction was done using salting out method for DNA extraction and was purified by phenol-chloroform method (Sambrook et al., 1989).

5 ml of the blood samples were mixed to 45 ml of red blood cells lysis buffer (20:5 mM TE; 20 mMTris-HCl, pH 7.6, 5 mM disodium ethylene diamine tetraacetate buffer) in falcon tube. The tubes were incubated in ice for 60 min and then spin at 3000 rpm for 20 min. The supernatant was discarded, and then the volume complete to 50 ml with TE 20:5. The previous step was repeated until the pellet became clear from any red blood cells. 5 ml TE buffer 20:5 was added to each tube, and then 20 µl of proteinase k enzyme (100 µg /ml) was added, mixed thoroughly and incubated to digest over night at 37°C for protein digestion.

One ml of 6M NaCl was added to the previous solution, shaken vigorously with hands, then centrifuged at 3000 rpm at 23°C for 20 min. The clear layer of supernatant was transferred to a new 50 ml falcon tube. An equal volume of pure cold ethanol was added and mixed by inversion to precipitate the DNA. The precipitated DNA was fished out using glass stake, dissolved by addition of 1-2 ml of TE buffer 20:1 mM, and finally the DNA was re-suspended under agitation overnight at 37°C.
2.4.3. DNA purification and control of quality and quantity

To the tube containing genomic DNA, an equal volume of Phenol-Chloroform Isoamyl alcohol (PCI) (25: 24: 1) was added to DNA, shaken well and centrifuged for 10 min at 3000 rpm. The upper layer was transferred into a new eppendorff tube and, an equal volume of chloroform was added and shacked well for 10 min and centrifuged for 1.5 min at 3000 rpm, the upper layer was transferred into anew eppendorff tube and 50 µl of 6M NaCl and 1.0 ml of cold ethanol was added and incubated at -20 °C overnight. The tube then was centrifuged for 10 min at 3000 rpm, and the supernatant was discarded and the tube was placed inverted to dry for 30 min. 400µl of distilled water was added to the tube, mixed gently for 5 minutes and kept at -20 °C for genetic analysis. Finally the DNA concentration and its purity were estimated by spectrophotometer at 260 nm (DNA) and 280 nm (protein) then the value of the DNA absorbance at 260 nm was divided by the protein value absorbance at 280 nm. If the result was between 1.7 to 1.8 that means the purity of the DNA was classified as good. In case the value was less than 1.7 that means the DNA was not pure, then DNA samples were subjected to DNA purification. When the value was more than 1.8 the DNA samples were diluted. For genotyping the purified DNA samples were distributed in 96 eppendorff plates for genetic analysis study (Appendix III). For TagMan PCR amplification and post PCR product analysis, the DNA samples were distributed in 384 eppendorff plates (Appendix IV).
2.5. Bioinformatics analysis

2.5.1. Haploview software

In order to identify tagSNPs that could be used to predict other SNPs in the region for association studies in African populations, genotyping data was downloaded from the international Hap Map project website aforementioned. The data used was analysed by Haploview to assess linkage disequilibrium and haplotype blocks across the entire data. This allowed us to identify the tagSNPs for the study. Haploview software accepts input data in a number of formats and offers flexibility in the choice of the measure of LD and calculation of tagSNPs, as well as the option of altering threshold values.

2.5.2. Selection of candidate SNPs

The selection of tagSNPs and genotyping them for the association study provide us with information about the associated genotypes or haplotypes, which they have association with disease. So we need to do further investigations to identify the causal variant by functional analysis. This would lead to identify possibly a SNP of truly functional importance, which would then be a candidate for a functional investigation.

In the present study, we screened the promoter regions of the IL-10 gene (chromosome 1) IL-13 gene ± 10 Kbp (chromosome 5) around the gene and CTGF gene (chromosome 6) for polymorphisms as well as the coding SNPs published in the NCBI dbSNP database (http://www.ncbi.nlm.nih.gov). In order to cover the entire regions of these genes, the SNP browser software (version 3.5, Applied Biosystems, Foster City, CA, USA) was used to select tagging SNPs within the gene regions ± 10 Kbp.
SNPs with a minor allele frequency in Yoruba (African population) 20% were included. The haplotype blocks were defined by the methods of Gabriel et al. (2002). According to the available HapMap data, the SNPs in the IL-13, IL-10 and CTGF gene regions were analysed to obtain two correlation bin for IL-13 gene and IL-10 gene (Figure 2.4 and Figure 2.5), while CTGF gene give seven correlation bins (Figure 2.6).
Figure 2.4: The LD block structure observed in the IL-13 gene (R²) using genotype data from Yoruba in Abidjan western Africa, Nigeria, available at hapmap database. Squares values of IL-13 box indicate the pairwise magnitudes of LD. The darker the color, the higher LD: black-colored squares indicated a very strong LD. The two bins that are highly correlated in the gene ± 10 Kbp.
Figure 2.5: Correlation bins for IL-10 in African population: correlation ($R^2$ values) between SNPs were determined using Haploview software. The darkest colors indicate the strongest correlation. All SNPs strongly correlated with (MAF $\geq 0.2$, $R^2 \geq 0.8$) are clustering in two correlation bins.
Figure 2.6: Correlation bins for CTGF and flanking regions in the Sudanese samples. Correlations (r^2 values) between SNPs were determined using Haploview software. The darkest colors indicate the strongest correlations (bottom). Correlation bins (r^2 > 0.8; middle) were as follows: bin I, SNPs rs6940184 and rs9493149; bin II, SNPs rs125277705 and rs12526196; bin III, SNPs rs6918698 and 3037970; bin IV, SNPs rs2151532 and rs1931002; bin V, SNPs rs9493150, rs11966728, and rs12198610; bin VI, SNPs rs7747601, rs7768619, and rs9402373; and bin VII, SNPs rs12527379 and rs2095252.
2.6. Polymerase Chain Reaction (PCR)

2.6.1. Principle

Polymerase Chain Reaction (PCR) is a technique for amplifying specific nucleic acids in vitro (Mullis et al., 1986). The method was designed to permit selective amplification of specific target DNA sequences within heterogeneous collection of DNA sequences. To permit such selective amplification some prior DNA sequence information from the target sequence is required. This information is used to design oligonucleotide primers (amplifiers), which are specific for the target sequence; these amplifiers usually range from 15-25 nucleotides long. Thermostable DNA polymerase and four-deoxyribonucleotide triphosphate (dNTPs) act on the template DNA (Mullis, 1990).

2.6.2. PCR amplification

PCR was performed in 30 μl volumes which contain 1X PCR Buffer II (Gene Amp® 10X Buffer II [100 mMTris-HCl pH 8.3, 500 mMkCl], Applied Bio system), 1.5 mM MgCl₂ (MgCl₂, Applied Bio system), 0.2 mM each of the dNTP (Gene Amp® dNTPs, Applied Bio system), 1.0 μmol of each sense and antisense primers, 1 U of AmpTag Gold (Applied Bio system), 5.0 μl of the Template DNA; and the reaction volume was completed to 30 μl by double dH₂O.

2.7. The TaqMan® assay

2.7.1. The TaqMan® assay method

The TaqMan® assay used the 5’-3’ exonuclease activity of TaqDNA polymerase to hydrolyse a hybridisation probe bound to the target amplicon (Lawyer et al., 1989; Lyamichev et al., 1993). The technique allows the direct detection of PCR product by the specific release of a fluorescent reporter molecule during the PCR reaction (Lee et al., 1993).
Two template-specific primers define the endpoints of the amplicon and serve as a first level of specificity. A further level of specificity is achieved by the inclusion of a specific probe, which binds internally to the points defined by the primers. The characteristics of the TaqMan® probe include a reporter molecule at the 5’ end, whose emission is quenched by a second molecule at the 3’ end. The spatial proximity of the reporter to the quencher in an intact probe ensures that no net fluorescence is detected.

The release of the fluorescence only occurs if target-specific amplification occurs, obviating the need to confirm the amplicon following amplification (Lakowicz and Keating, 1983).

2.7.2. Reporter Dyes:

The fluorescence reporter dye, e.g. FAM (6-carboxy-fluoroscein), VIC or JOE, is covalently linked to the 5’end of the oligonucleotide probe. Tetrachloro-6-carboxy-fluoroscein (TET) and hexachloro-6-carboxy-fluoroscein (HEX) can also be used as fluorescent reporter dyes in this system. Each of these reporters is quenched by TAMRA (6-carboxy-tetramethyl-rhodamine) (the quencher dye). The probe is chemically phosphorylated at its 3’ end, which prevents probe extension during PCR applications. When the probe is intact (linearised), the proximity of the reporter dye to the quencher dye results in direct suppression of the fluorescence from the reporter dye by Forster-type energy transfer (Lakowicz and Keating, 1983).

During PCR the probe will specifically anneal between the forward primer (primer 1) and the reverse primer (primer 2). The 5’–3’ exonuclease activity of the AmpliTaq DNA polymerase will cleave the hybridised probe between the reporter and the quencher molecules. Once the reporter molecule is cleaved from its spatial bind with the quencher is resulting an increase in fluorescence that is proportional to
the amount of product that has been accumulated. When the PCR is completed, the reporter dye starts to send its emission which is reported as UV values at 260 nm. By using Sequence Detection Systems (SDS) software, the allelic discrimination assay classifies unknown samples by measures the change in fluorescence of the dyes associated with the probes as homozygote or heterozygote.

2.7.3. Allelic discrimination assays

An allelic discrimination assay is a multiplexed (more than one primer/probe pair per reaction) end-point (data is collected at the end of the PCR process) assays that detects variants of a single nucleic acid sequence. The presence of two primer/probe pairs in each reaction allows genotyping of the two possible variants at the single-nucleic polymorphism (SNP) site in a target template sequence. The actual quantity of target sequence is not determined.

For each sample in an allelic discrimination assay, a unique pair of fluorescent dye detectors is used, for example, two TaqMan® MGB (minor groove binder) probes that target an SNP site (Afonina et al., 1997; Kutyavin et al., 1997). One fluorescent dye detector is a perfect match to the wild type (allele 1) and the other fluorescent dye detector is a perfect match to the mutation (allele 2) (Figure 2.7).

The allelic discrimination assay classifies unknown samples as:

- Homozygotes (samples having only allele 1 or allele 2)
- Heterozygotes (samples having both allele 1 and allele 2)
Figure 2.7: Allelic discrimination assay showing the results from matches of mismatches between target and probe sequences in TaqMan® SNP Genotyping Assays as it was described by (Livak et al., 1995).
2.7.4. Preparation of the reaction mix

2.7.4.1. TaqMan Pre-Developed Assay Reagents

TaqMan Pre-Developed Assay Reagents for Allelic Discrimination (PDARs) require only three components:

• Genomic DNA sample

• Allelic Discrimination Assay Mix (10X), specific for each polymorphism

• TaqMan® Universal PCR Master Mix (2X)

Note: Allele 1 and 2 control DNA were included with each assay, allowing each homozygote signal to be generated on each run.

2.7.4.2. Preparation of amplification reaction

The reaction mix was prepared with TaqMan® Universal PCR Master Mix (2µl), No AmpErase® UNG. The volumes were calculated for a standard 384-well reaction plate. Enough reaction mix was prepared for 8 No template control (NTC) and 340 samples/unknowns, plus 10% extra to account for pipetting losses (Table 2.2).
Table 2.2: Preparation of TaqMan® Universal PCR Reaction Master Mix

<table>
<thead>
<tr>
<th>Component Volume</th>
<th>One Reaction Volume (µL)</th>
<th>384 Reactions (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TaqMan® Universal PCR No AmpErase® UNG</td>
<td>2.5</td>
<td>265.0</td>
</tr>
<tr>
<td>SNP Genotyping Assay Mix (20X)</td>
<td>0.25</td>
<td>26.5</td>
</tr>
<tr>
<td>Total</td>
<td>2.75</td>
<td>291.5</td>
</tr>
</tbody>
</table>
2.7.4.3. Preparation of the reaction plate

Three hundred and eighty four wells clear optical reaction plate was covered with ABIPRISM® optical adhesive cover, and then was centrifuged briefly to spin down the contents and to eliminate air bubbles. The reaction plate was kept on -20°C until loading in the 7900HT Fast System.

2.7.4.4. Creating a standard curve plate document

A standard Curve plate documents were created to store real-time data for allelic discrimination assays using SDS software. New plate wizard was opened then selected Allelic Discrimination (AD). After that, 384 wells clear plate was selected. The samples names were entered according to previous setting in the reaction plate. Then, marker names (for example, C_2984390_10) were used. In addition to that, verification of the detector for allele 1 and 2 were selected. The plate document was saved as an SDS 7900HT Document (*.sds).

2.7.4.5. TaqMan assays reaction

TaqMan assays for genotyping of SNPs, shown in figure 1 and 2 were designed by Applied Biosystems. PCRs were performed using 12.5 ng genomic DNA, and an annealing temperature of 60°C for 45 cycles on Perkin Elmer GeneAmp 9700 (Applied Biosystems) thermo-cyclers. Fluorescence was measured with an ABI Prism 7900HT sequence detection system (Applied Biosystems).
Table 2.3: PCR times, temperature and cycles

<table>
<thead>
<tr>
<th>Times and Temperatures</th>
<th>PCR (Each of 40 cycles)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Initial Steps</strong></td>
<td></td>
</tr>
<tr>
<td>AmpliTaq Gold® DNA Polymerase</td>
<td></td>
</tr>
<tr>
<td>Activation</td>
<td>Melt</td>
</tr>
<tr>
<td></td>
<td>Anneal/ Extend</td>
</tr>
<tr>
<td><strong>HOLD</strong></td>
<td>CYCLE</td>
</tr>
<tr>
<td>10 min @ 95°C</td>
<td>15 sec @ 92°C</td>
</tr>
<tr>
<td></td>
<td>1 min @ 60°C</td>
</tr>
</tbody>
</table>
2.7.4.6. Interpretation of results

An increase in fluorescence was detected using a luminescence spectrophotometer Applied Biosystems 7900 DNA sequencer detector system (2.3 version). Which utilises a laser scanning format to detect increases in fluorescence at defined time-points in the thermocycling protocol (Figure 2.8). Detection was achieved using a 384-well plate reader and avoids the complexities of gel electrophoresis followed by ethidium bromide staining or autoradiography.

2.8. Functional analysis

2.8.1. Preparation of nuclear and cytoplasmic fractions

Nuclear and cytoplasmic extracts were prepared using the nuclear extract kit. Briefly, cells were washed with 5 ml ice-cold phosphatase buffer saline (PBS) containing a phosphatase inhibitor cocktail and scraped into a prechilled conical tube. After centrifugation, cells were re-suspended in a hypotonic buffer solution and incubated on ice for 15 min. Cells were then treated with a detergent and centrifuged at 14,000 g for 30 Sec. The supernatant containing the cytoplasmic fraction was transferred to a clean tube and stored at 80°C until required.

The pellet containing the nuclear fraction was re-suspended in total lysis buffer and incubated on ice for 30 minutes with gentle agitation. This solution was then centrifuged at 14,000 g for 10 min, and the supernatant containing the nuclear fraction was transferred to a clean micro-centrifuge tube and stored at 80°C until required.
Figure 2.8: Allelic discrimination plot for rs1295686 marker in IL-13 gene showed allele 1 in Y axis (blue colours) and allele 2 in X axis (red colours) while both (allele 1 and allele 2) in the middle (green colours).
2.8.2. Electronic mobility shift assay (EMSA) analysis

2.8.2.1. EMSA and transfer of DNA probes onto nylon membrane

The electronic mobility shift assay (EMSA) was carried out essentially as per the manufacturer’s instructions (Light Shift Chemoiluminescent EMSA Kit, Pierce, Rochford, IL 61105 USA). The DNA/receptor complex formation reaction was performed with 5 µg of nuclear extract in 10X binding buffer (2 µl), 50% glycerol (1 µl), 100 mM MgCl₂ (1 µl), 11 µg/µl poly (dI•dT) (1 µl), 1% NP-40 (1 µl), 3’-biotinylated DNA probe and made up to 20 µl with nuclease-free water. The reaction was then incubated at room temperature for 20 min then 5 µl of loading buffer were added.

A polyacrylamide gel 6% was prepared in 0.5X trise base, boric acid and EDTA (TBE) buffer, the gel was placed in the electrophoresis unit, the inner chamber was filled with 0.5 X TBE buffer to a height several millimetres above the top of the well, and then the outside of the tank was filled just above the bottom of the wells. Then wells of the gel were flushed, and pre-electrophoresed for 30-60 min with 100 V.

Samples were loaded in the wells of the gel and electrophoresed for two hours with 100 V. The gel was transferred to a nylon membrane. The nylon membrane was soaked in 5X TBE buffer for 10 min. Then the gel was sandwiched between the nylon membranes in a clean tank. The biotinylated DNA probes were transferred to the nylon membrane by capillary action over night. The biotinylated DNA probes were cross-linked to the nylon membrane by ultra violet (UV) illumination for 2.5 min.
2.8.2.2. Detection biotin-labeled DNA by chemiluminescence

A detection system using the chemiluminescence substrate was applied for detection of higher molecular shifts that were formed by DNA/receptors complexes from the EMSAs. To perform the detection, the membrane was immersed in 20 ml of a blocking buffer for 15 min at room temperature (with gentle shaking). The membrane was then subjected to 66.7 µl of Streptavidin-Horseradish Peroxidase Conjugate in 20 ml of the blocking buffer (1:300 dilutions) for 15 min at room temperature. Then the membrane was transferred to a new tube and washed with 20 ml of 1.0X washing buffer (37-50 °C) four to five times. After that, the membrane was transferred to a new tube flowed with adding 30 ml of substrate equilibration buffer and was incubated for 5 min with gentle shaking. The membrane was then removed from the substrate equilibration buffer and placed onto a clean sheet of plastic was placed on a flat surface. Substrate working solution was prepared by adding 6 ml of Luminol/Enhancer solution to 6 ml stable Peroxide solution. To perform the detection, the substrate working solution was poured onto surface of the membrane for 5 min, then the membrane was washed by double distilled water and detection was performed by X-ray film for 2-5 min to visualize the chemiluminescence signal.

2.8.2.3. EMSA probes sequences

EMSA complementary single-stranded oligonucleotides are commercially synthesized to span ~10 bp on either side of the variant nucleotide (Table 2.4).

2.9. Phenotype used in genetic analysis

On the basis of ultrasound measurements, binary phenotype was used to classify the study subjects as affected or non-affected. The criteria for this classification were depending on the fibrosis grades and the evidence of portal-blood
Table 2.4: Complementary oligonucleotides sequence which was used in EMSA analysis.

<table>
<thead>
<tr>
<th>Probe name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs9402373C</td>
<td>5'-GCTCTCAAAACTAAGCCCAACTC-3'</td>
</tr>
<tr>
<td>rs9402373G</td>
<td>5'-GCTCTCAAAAGTAAGCCCAACTC-3'</td>
</tr>
<tr>
<td>rs12527705A</td>
<td>5'-GTAATATAGAAGATGGGTCTA-3'</td>
</tr>
<tr>
<td>rs12527705T</td>
<td>5'-GTAATATAGATGGGTCTA-3'</td>
</tr>
<tr>
<td>rs12526196C</td>
<td>5'-GAATATACAAACGAATATGGGC-3'</td>
</tr>
<tr>
<td>rs12526196T</td>
<td>5'-GAATATACATGAATATGGGC-3'</td>
</tr>
<tr>
<td>rs1931002A</td>
<td>5'-TGGATGATTCAAAACAACCTTG-3'</td>
</tr>
<tr>
<td>rs1931002G</td>
<td>5'-TGGATGATTCCAAACAACCTTG-3'</td>
</tr>
</tbody>
</table>
hypertension. Since an increase in the portal vein diameter (PVD) in schistosomiasis patient is a direct evidence of portal-blood hypertension (Abdel-Latif et al., 1981; Cottone et al., 1983), and it was correlated with occurrence of gastrointestinal bleeding from oesophageal varices (Eltoum et al., 1994; Qurashi et al., 1999).

The patients were assigned affected when they were exhibiting PVD above a given threshold. Since all patients were above 16 years old, four thresholds for PVD (calculating according to the main standard deviation of portal veins diameter in the studies subjects) depending on gender were set. Males with PVD > 14 mm were cases, and < 13 mm were controls. For females with PVD > 13 mm were cases and < 12 mm were controls.

To strength the phenotype for the genetic study since genetic epidemiological study depend on evaluate the diseases and correctly classify observable outcomes or phenotypes. A combine of additional phenotype of gallbladder thickness with PVD was used for genetic analysis. Gallbladder wall thickness, < 4mm were normal and > 4mm were considered as an increase in gallbladder wall thickness. Thus, the controls were those who have normal PVD (<13mm in males and < 12mm in females) and normal gallbladder wall (< 4mm), while cases with enlargement in PVD (> 14mm in males and > 13 in females), and abnormal gallbladder wall thickness (> 4mm) were not normal. Another phenotype had been obtained using a combination between PVD and central periportal fibrosis. Patients (males and females) with lower than this threshold were classified as unknown phenotype.
2.10. Statistical analysis

2.10.1. Epidemiological data

The data set was normalized using Statistical Package for Social Science (SPSS) version 17. In order to evaluate the associations of the various covariates that might influence the development of hepatic fibrosis. Multivariate analyses were conducted using a logistic regression model. Initially, univariate analyses were performed in order to select the variables to be included in the multivariate analyses. Any variable whose univariate test produced a significance level $P$ of $\geq 0.20$ was considered a candidate for the multivariate model. Together with all variables of known biologic importance for fibrosis in schistosomiasis, even if such variables did not achieve the stated significance. All of the variables that achieved a $P$ value of $\geq 0.15$ in the intermediate logistic regression were retained for the final multivariate analysis. Those variables that attained a $P$ value of $\geq 0.05$ were considered significantly associated with fibrosis after adjustment for the other investigated covariates. In the logistic regression, a stepwise procedure was used, commencing with a model containing all of the selected variables and followed by successive backward elimination. The study subjects were classified in six ages groups, starting from 15-24 years to $\geq 65$ years.

2.10.2. Mutational analysis

The genotype frequencies among all the subsets of periportal fibrosis subjects and controls were compared by means of a chi-squared test for 2x2 contingency tables using the SPSS program. All values of $P< 0.05$ were considered significant. Odds ratios (OR) with 95% confidence intervals (CI) were calculated as a measure of the association of each genotype with the periportal fibrosis.
Chapter three

Results

3.1. Study subjects (Epidemiological data)

This study was a population-based case control study, which recruited a representative sample of individuals with schistosomiasis disease, and a representative sample of those at risk but not affected as controls.

The study subjects were at risk to develop periportal fibrosis due to schistosomiasis which infect a large number of the Sudanese farmers living in the S.mansoni endemic area of the Gezira State. The study subjects were exposed to S. mansoni infection. The canals infested water was the main source of water. The hepatic disease was screened in 941 subjects, 548 were males and 393 were females, with a mean age of 42.27 ± 0.555 years living in four villages that were distant from Wad Madani town no more than 83 km. The irrigation canals were inhabited by Biomphalaria pfeierei and used for baths, fishing, swimming, and washing dishes.

3.1.1. The prevalence of fibrosis grades by age and gender

The fibrosis grades were evaluated with ultrasound in 941 subjects. 161 (17%) were pattern A, 379 (40.3%) were pattern B, 230 (24.4%) were pattern C, 87 (9.2%) were pattern D, 25 (2.7%) were pattern E and 16 (1.7%) persons had pattern F (Figure 3.1, Table 3.1). Significant difference was found between gender and disease severity. Males were more affected by central fibrosis grades D, E and F fibrosis than females. The prevalence of these grades were markedly increased in males than females (Figure 3.2a). While in peripheral fibrosis, grade A and B were more frequent in females. However, the severe grades of the peripheral fibrosis grades (C and C+) were more frequent in males than females (Figure 3.2 b).
Table 3.1: Frequency data of the study subjects

<table>
<thead>
<tr>
<th>Frequencies</th>
<th>Numbers</th>
<th>Percentages%</th>
</tr>
</thead>
<tbody>
<tr>
<td>No of examined subjects</td>
<td>941 Subjects</td>
<td>100</td>
</tr>
<tr>
<td>Gender</td>
<td>Males= 549</td>
<td>58.3</td>
</tr>
<tr>
<td>Total = 941</td>
<td>Females= 393</td>
<td>41.7</td>
</tr>
<tr>
<td>Age</td>
<td>Maximum= 105 years</td>
<td></td>
</tr>
<tr>
<td>Total = 926</td>
<td>Minimum = 16 years</td>
<td></td>
</tr>
<tr>
<td>Age classes</td>
<td>15-25 years = 167</td>
<td>18.03</td>
</tr>
<tr>
<td></td>
<td>26-35 years = 236</td>
<td>25.49</td>
</tr>
<tr>
<td></td>
<td>36-45 years = 185</td>
<td>19.98</td>
</tr>
<tr>
<td></td>
<td>46-55 years = 145</td>
<td>15.66</td>
</tr>
<tr>
<td></td>
<td>56-65 years = 96</td>
<td>10.37</td>
</tr>
<tr>
<td></td>
<td>≥ 65 years = 97</td>
<td>10.48</td>
</tr>
<tr>
<td>Major five tribes</td>
<td>Arakain = 175</td>
<td>18.58</td>
</tr>
<tr>
<td></td>
<td>Tama = 147</td>
<td>15.61</td>
</tr>
<tr>
<td></td>
<td>Mararite = 143</td>
<td>15.18</td>
</tr>
<tr>
<td></td>
<td>Brgo = 148</td>
<td>15.71</td>
</tr>
<tr>
<td></td>
<td>Awamara = 98</td>
<td>10.4</td>
</tr>
<tr>
<td>Total = 711</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Others tribes</td>
<td>230</td>
<td>24.52</td>
</tr>
<tr>
<td>Peripheral fibrosis grades</td>
<td>A = 161</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>B = 379</td>
<td>40.3</td>
</tr>
<tr>
<td>Central fibrosis grades</td>
<td>C = 230</td>
<td>24.4</td>
</tr>
<tr>
<td></td>
<td>D = 87</td>
<td>9.2</td>
</tr>
<tr>
<td>Total = 897</td>
<td>E = 25</td>
<td>2.7</td>
</tr>
<tr>
<td></td>
<td>F = 16</td>
<td>1.7</td>
</tr>
</tbody>
</table>
Figure 3.1: The prevalence of peripheral and central fibrosis in study subjects.
Figure 3.2: The distribution of a: Central fibrosis grades D &EF and b: Peripheral fibrosis according to gender
The study showed a significant difference among males and females with central periportal fibrosis ($P = 0.0001$, OR = 2.735). Males were 2.735 times likely having grades E and F than females (Table 3.2). Also, in peripheral fibrosis ($P > 0.001$, OR = 2.695) males were 2.7 times likely having grades C and C+ (the advance grades in peripheral fibrosis) compared with females (Table 3.3).
**Table 3.2:** Association of central periportal fibrosis with gender

<table>
<thead>
<tr>
<th>Covariate in the model</th>
<th>( p \text{ value} )</th>
<th>OR</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (M)</td>
<td>&lt; 0.0001</td>
<td>2.735</td>
<td>1.688</td>
</tr>
</tbody>
</table>

Covariate excluded from the model: Age \( p \text{ value} 0.512 \)
Table: 3.3 Association between peripheral fibrosis and gender

<table>
<thead>
<tr>
<th>Covariate in the model</th>
<th>$P$ value</th>
<th>OR</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td>&lt; 0.000001</td>
<td>2.695</td>
<td>1.654     2.951</td>
</tr>
</tbody>
</table>

Covariate excluded from the model: Age $P$ value 0.953
3.1.2. Central fibrosis is associated with most severe hepatic disease

Central periportal fibrosis was associated with an increase of portal vein (Figure 3.3a) and splenic vein (Figure 3.3b) diameter and this was more marked in subjects with grades E and F than in subjects with grade D. Peripheral fibrosis was also associated with increased PVD and SVD, the largest diameters were observed in grade C+. Current study showed correlation between peripheral fibrosis and central periportal fibrosis. The association of periportal fibrosis with PVD and SVD could be due to its correlation with central peripheral fibrosis and conversely. The data showed that central periportal fibrosis exhibited the strongest association with PVD ($P< 0.00000001$), and SVD ($P< 0.00001$) and that in the presence of central periportal fibrosis, peripheral fibrosis was barely correlated to PVD ($P= 0.044$) but not with SVD (Tables 3.4 and 3.5). This indicates that PVD is strongly associated with central fibrosis and poorly associated with peripheral fibrosis and that SVD is associated with central fibrosis but not peripheral fibrosis. The logistic regression analysis showed that only central periportal fibrosis ($P= 0.001$, OR = 22.36 and CI = 8.41-59.46) was associated with bleeding from varicose portal veins, peripheral fibrosis was not ($P = 0.135$, Table 3.6).
Table 3.4: Significant association between portal vein and central periportal fibrosis

<table>
<thead>
<tr>
<th>Covariate included in the model</th>
<th>$P$ value</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Central fibrosis</td>
<td>&lt; 0.000001</td>
<td>0.525 0.695</td>
</tr>
<tr>
<td>Peripheral fibrosis</td>
<td>0.044</td>
<td>0.005 0.346</td>
</tr>
<tr>
<td>Gender</td>
<td>0.00002</td>
<td>-0.826 -0.305</td>
</tr>
<tr>
<td>Age</td>
<td>0.017</td>
<td>0.002 0.016</td>
</tr>
</tbody>
</table>
**Table: 3.5** Host factors involved in enlargement of spleen vein

<table>
<thead>
<tr>
<th>Covariate included in the model</th>
<th>$P$ value</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Central fibrosis</td>
<td>&lt; 0.00001</td>
<td>0.576</td>
</tr>
<tr>
<td>Age</td>
<td>&lt; 0.001</td>
<td>-0.029</td>
</tr>
<tr>
<td>Gender</td>
<td>0.055</td>
<td>-0.564</td>
</tr>
</tbody>
</table>

Covariate excluded from the model: Peripheral fibrosis $P$ value = 0.843
Table: 3.6 Risk factors associated with severe hepatic form of disease
(Sclerosis of varices)

<table>
<thead>
<tr>
<th>Covariate in the model</th>
<th>$P$ value</th>
<th>OR</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Central periportal fibrosis</td>
<td>0.001</td>
<td>22.36</td>
<td>8.41</td>
</tr>
</tbody>
</table>

Covariate excluded from the model: Peripheral periportal fibrosis $P$ value = 0.135
3.1.3. Splenomegaly and hepatic disease

Splenomegaly was evaluated by measuring the size of the spleen: it was increased in advanced central periportal fibrosis and peripheral fibrosis grades (Figure 3.3 e, and Figure 3.3 f); again increased spleen size was mostly correlated with central periportal fibrosis ($P<0.0001$) in the linear regression analysis where both central periportal fibrosis and peripheral fibrosis were entered in the regression model (Table 3.7). In (Figure 3.4 A) the splenic volume was analyzed by village number and age classes the data showed that the Kirmit village exhibited the larger splenic volume. The comparison between the mean splenic volume by age classes and five major tribes was indicated clear tribes affect. This was already exhibited in cases of Arikian tribe. Which was shown increase in the splenic volume in three age classes 26-35, 36-45 and 46-55 years. This finding indicated that the association of increased splenic volume was most probably due to tribal effect (Figure 3.4 B), as kirmit village is populated by Arikian.

In advanced liver disease, it has been shown that the spleen increase in size as a result of cell proliferation (proliferative splenomegaly) and blood influx (congestive splenomegaly). At the most advanced stages of the disease splenomegaly contribute to portal hypertension by contributing a massive blood flux toward the portal system. The linear regression analysis that tested central peripheral fibrosis and spleen volume as explicative variables for PVD, was strongly in support of this since both central periportal fibrosis and spleen volume were strongly associated ($P=0.00000118$ and 0.002 respectively) with PVD in the model that included these two variables (Table 3.8). Not surprisingly, the same evaluation of peripheral fibrosis performed on SVD showed that only the splenic volume correlates with SVD than central periportal fibrosis ($P<0.0000000001$ and $<0.0001$ respectively) (Table 3.9).
**Table: 3.7 Correlation between central periportal fibrosis and splenic volume**

<table>
<thead>
<tr>
<th>Covariate included in the model</th>
<th>$P$ value</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Central fibrosis</td>
<td>&lt; 0.0001</td>
<td>36.490</td>
</tr>
<tr>
<td>Age</td>
<td>0.055</td>
<td>-5.370</td>
</tr>
</tbody>
</table>

Covariate excluded from the model: Peripheral fibrosis and Gender $P$ 0.132 and 0.742 respectively.
Table 3.8: The contribution of splenomegaly to portal hypertension

<table>
<thead>
<tr>
<th>Covariate included in the model</th>
<th>( P ) value</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Central periportal fibrosis</td>
<td>0.00000118</td>
<td>0.231</td>
</tr>
<tr>
<td>Splenic volume</td>
<td>0.025</td>
<td>0.000</td>
</tr>
</tbody>
</table>

Covariate excluded from the model: Periportal fibrosis, Gender and Age \( P \) value = 0.693, 0.989 and 0.972 respectively.
Figure 3: The distribution of the main portal vein (mm), splenic vein (mm) and splenic volume (mm³) by central fibrosis, peripheral fibrosis and gender.

a: The portal vein diameter (PVD) was increased in grades D central than peripheral fibrosis 0 (A, B, C & C⁻), and more markedly in grades E & F. b: showed increased in PVD in grades C & C⁺ only. Grades C⁺ was exhibited the largest PVD compared with other grades. c: the splenic vein Diameter (SVD) was increased in central fibrosis grades D, E & F. d: SVD was increased only in grade C⁺. The splenic volume was computed as (length spleen, X width X depth) X 0.523. e: The splenic volume was increased markedly in central fibrosis grades D, E, & F. f: While in peripheral fibrosis grades it was showed increased in grades C & C⁺.
Figure 3.4: The distribution of the mean splenic volume according to A: villages by age classes and B: tribes by age classes.
<table>
<thead>
<tr>
<th>Covariate included in the model</th>
<th>$P$ value</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Central fibrosis</td>
<td>&lt; 0.0001</td>
<td>14.699 - 47.477</td>
</tr>
<tr>
<td>Splenic vein</td>
<td>&lt; 0.0000000001</td>
<td>35.373 - 63.585</td>
</tr>
</tbody>
</table>

Covariate excluded from the model: Peripheral fibrosis, Age, Gall bladder and Gender $P$ value = 0.363, 0.345, 0.371 and 0.417 respectively.
3.1.4. Disease progression with age and exposure in males and females

The prevalence of advanced hepatic disease as measured by central periportal fibrosis grades (D, E and F) is shown in age classes in (Figure 3.5 A). Severe central periportal fibrosis was observed in 5% of the 15 to 25 years; which was shown no difference between males and females. The advanced central periportal fibrosis was increased markedly to reach 22% in males in the 36-45 years and then plateau. The curve was decline after 55 years to represent 10% of males in the >65 old males. Advance hepatic disease also peaked (10%) in the same age group 36-45 years in females and declined thereafter (Figure 3.5 A). On the other hand, it was found that the prevalence of peripheral periportal fibrosis in female reached 70% in age groups less than 45 years. The peripheral periportal fibrosis was peaked at age group 56 years and plateau and declined after 65 years. The curve of peripheral periportal fibrosis in males age groups 15 to 25 years was reached more than 60%. Then it was declined to reach the minimum in age group 46–55 years. However, it was increased in second peaks at age 56 to 65 and declined in age group >65 years (Figure 3.5 B).
Figure 3.5 A: The progression of severe hepatic fibrosis by age groups and gender:

- Males are more likely have central fibrosis than females.
- In males stable frequency of central fibrosis was observed in patients at age range of 36-55 years, whereas in females of same age range there was a sharp decrease in central fibrosis.

B: The progression of peripheral fibrosis according to age groups and gender:

- Females were more likely have peripheral fibrosis than males.
- Males were reached the minimum peripheral fibrosis in age group 46-55 years, while in females they reach the maximum peak of peripheral fibrosis in the same age group.
3.1.5. Severity of hepatic fibrosis is markedly influenced by tribal effect

The prevalence of severe hepatic disease has village effect, Krimit village showed higher prevalence of severe hepatic disease, while Al awamara showed lower affect (Figure 3.6). The villages were recorded in five tribes (Arakian, Tama, Bargo, Mararite and Awamara). The largest five tribes (n> 80) were selected for further analysis. There were large differences in the prevalence of severe hepatic fibrosis among the five studied tribes ranging from zero to 25% severe cases (Figures 3.7 and 3.8). The data was showed additional differences, when severe phenotype in combination between portal vein enlargement (portal hypertension) and thickness of gall bladder was analyzed (Figure 3.9). In order to test whether the villages effect could be in part accounted for by the ethnic affect we tested covariates (villages, ethnic groups), age and gender in the logistic regression model. Only gender ($P = 0.0003$) and majors five tribes ($P= 0.00003, 0.0013, 0.005, 0.002, \text{ and } 0.013 \text{ Arakian, Tama, Mararite, Bargo and Awamara respectively}$) showed significant associations with severe hepatic fibrosis (Table 3.10).
Figure 3.6: Distribution of the central periportal fibrosis in the study villages
Figure 3.7: The prevalence of fibrosis grades among the major five tribes of the study subjects.
Figure 3.8: The occurrence of central periportal fibrosis by number according to major five tribes.
Figure 3.9: The distribution of sever phenotype PVD and GB wall thickness [The controls were those who had normal PVD (< 13mm in males and < 12mm in females) and normal gallbladder wall (< 4mm), while cases had enlargement in PVD (> 14mm in males and > 13 in females), and abnormal gallbladder wall thickness (> 4mm). Patients (males and females) with lower than this threshold were classified as unknown phenotype.], among study subject in five major tribes.
Table: 3.10. The associations between tribal stocks and severe HF

<table>
<thead>
<tr>
<th>Covariate in the model</th>
<th>P value</th>
<th>OR</th>
<th>95%CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td>0.0003</td>
<td>2.812</td>
<td>1.61-4.9</td>
</tr>
<tr>
<td>Arakian</td>
<td>0.00003</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tama</td>
<td>0.0013</td>
<td>50.088</td>
<td>6.77-360.48</td>
</tr>
<tr>
<td>Mararite</td>
<td>0.005</td>
<td>18.906</td>
<td>2.47-144.94</td>
</tr>
<tr>
<td>Brgo</td>
<td>0.002</td>
<td>24.824</td>
<td>3.24-190.04</td>
</tr>
<tr>
<td>Awamra</td>
<td>0.013</td>
<td>14.807</td>
<td>1.78-123.09</td>
</tr>
</tbody>
</table>

Covariate excluded from the model Age, Kirimit, Kitra Hamdan, Wad Elamin and Al Awamara $P$ = value 0.518, 0.245, 0.98, 0.081 and 0.906 respectively.
3.1.6. Central PPF grade D, E and F were associated with more severe clinical form of diseases

Varices, ascites, and sclerosis varices were found strongly correlated with severe form of the central fibrosis of grade (D, E and F) (Figure 3.10 A). However, there was no significant correlation with fibrosis grades A and B, and the correlation was weak for grades C and C+ (Figure 3.10 B).

The severe form of diseases (ascites) was associated with central periportal fibrosis in linear regression analysis ($P < 0.000001$) and there was margin association with peripheral fibrosis ($P = 0.055$). There was no association with the other explanatory variables gender and age (Table 3.11).

Sclerosis of varices is another severe form of the disease. It was evaluated by liner regression again central periportal fibrosis which was more strongly associated than peripheral fibrosis ($P < 0.0001$ and 0.065 respectively) (Table 3.12).

3.1.7. Strong association between central periportal fibrosis and varices

Linear regression analysis that tested central periportal fibrosis, peripheral periportal fibrosis, gender and age as explicative variables for varices, central periportal fibrosis was strongly associated with varices ($P < 0.00000001$, CI 0.072, 0.085). Not surprisingly the other explicative variables were not associated (Table 3.13).
Figure 3.10: The prevalence of severe form of disease: varices (are extremely dilated sub-mucosal veins in the lower third of the esophagus), ascites (Ascites is gastroenterological term for an accumulation of fluid in the peritoneal cavity) and sclerosis of varices (Varicose veins are veins that have become enlarged and tortuous) in central periportal and peripheral fibrosis grades.
Table 3.11: Central periportal fibrosis was highly association with ascites

<table>
<thead>
<tr>
<th>Covariate included in the model</th>
<th>$P$ value</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Central periportal fibrosis</td>
<td>&lt; 0.000001</td>
<td>0.027</td>
</tr>
<tr>
<td>Peripheral fibrosis</td>
<td>0.055</td>
<td>0.000</td>
</tr>
</tbody>
</table>

Covariate excluded from the model: Gender and Age $P$ value = 0.968 and 0.998 respectively
Table 3.12: Evidence of association between sclerosis varies and central periportal fibrosis

<table>
<thead>
<tr>
<th>Covariate included in the model</th>
<th>P value</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Central periportal fibrosis</td>
<td>&lt; 0.0001</td>
<td>0.021</td>
</tr>
<tr>
<td>Peripheral fibrosis</td>
<td>0.065</td>
<td>0.000</td>
</tr>
</tbody>
</table>

Covariate excluded from the model: Gender and Age P value = 0.521 and 0.609 respectively
Table 3.13: Strong association of central fibrosis and oesophagus varices

<table>
<thead>
<tr>
<th>Covariate included in the model</th>
<th>P value</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Central periportal fibrosis</td>
<td>&lt; 0.0000001</td>
<td>0.072</td>
</tr>
</tbody>
</table>

Covariate excluded from the model: Peripheral fibrosis, Gender, and Age P value = 0.984, 0.711 and 0.141 respectively.
3.1.8. The effect of previous treatment on fibrosis grades

The current result was showed a strong association between gender \( (P=0.000002, \text{CI} -0.597 - 0.252) \), and previous treatment \( (P= 0.002, \text{CI} 0.098 -0.445) \) with peripheral fibrosis. While age was not significant \( (P= 0.997) \) (Table 3.14).

3.1.9. Evidence for association of central periportal fibrosis with activity (occupation)

The data was showed evidence of association between activity with central periportal fibrosis \( (P= 0.012 \text{ CI} = 0.045 - 0.365) \). The other explanatory factors were not significant (Table 3.15).

Activities were more closely associated with central periportal fibrosis; the activities of study subjects were classified in two classes’ farmers and non-farmers. Farmers were the most frequent contact with contaminated water. Gender (Males) were provided the best association with activity \( (P< 0.000000001, \text{OR} = 6.52, \text{CI} = 3.85 - 11.01) \) and central periportal fibrosis was associated with activity (farmers and non-farmers) \( (P= 0.014, \text{OR} = 1.28, \text{CI} = 1.05–1.55) \), and no other explanatory variables could be entered in the logistic regression equation (Table 3.16).
Table 3.14: The association of peripheral fibrosis with gender and previous treatment

<table>
<thead>
<tr>
<th>Covariate included in the model</th>
<th>P value</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td>0.000002</td>
<td>-0.597</td>
</tr>
<tr>
<td>Treatment</td>
<td>0.002</td>
<td>0.098</td>
</tr>
</tbody>
</table>

Covariate excluded from the model: Age $P$ value = 0.997
**Table 3.15:** A significant association between activity and central periportal fibrosis

<table>
<thead>
<tr>
<th>Covariate included in the model</th>
<th>P value</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Central periportal fibrosis</td>
<td>0.012</td>
<td>0.045</td>
</tr>
</tbody>
</table>

Covariate excluded from the model: Peripheral fibrosis, Gender, and treatment P value = 0.712, 0.994 and 0.997 respectively.
**Table 3.16:** The association of farmers with central periportal fibrosis

<table>
<thead>
<tr>
<th>Covariate in the model</th>
<th>$P$ value</th>
<th>OR</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td>&lt; 0.000000001</td>
<td>6.25</td>
<td>3.85</td>
</tr>
<tr>
<td>Central periportal fibrosis</td>
<td>0.014</td>
<td>1.28</td>
<td>1.05</td>
</tr>
</tbody>
</table>

Covariate excluded from the model: Peripheral fibrosis, treatment, and Age $P$ value = 0.360, 0.303 and 0.403 respectively.
3.2. Genotyping analysis

3.2.1. Connective tissue growth factor (CTGF) gene

3.2.1.1. Distribution of CTGF gene polymorphisms in study subjects

The frequency distribution of the SNPs among controls and cases were increased significantly in rs9402373 CC alleles (90.1%, 98.4% respectively). While, a trend of association were observed in rs1257705 AA alleles (90.1%, 98.4% respectively), and rs12526196TT alleles (90.2%, 98.4% respectively) among controls and cases (Table 3.17). The data show that these alleles were associated with aggravating disease.

3.2.1.2. rs1257705, rs12526196, and rs9402373 polymorphisms are also associated with severe HF

The data suggested a trend of association of rs1256196 (P = 0.066; OR = 6.8) with HF, and indicated a significant association of rs9402373 (P = 0.008; OR = 5.2) with severe hepatic fibrosis. An additional association was found for rs12527705 (P = 0.069; OR = 6.7). Multivariate analysis of these SNPs simultaneously indicated that SNPs rs9402373 (P = 0.008; OR = 5.2) and rs1256196 (P = 0.059; OR = 7.3) were independently associated with severe hepatic fibrosis in the presence of age as a covariate (P = 0.05) (Table 3.17).
HF in *S. mansoni*-endemic region in Sudan was affected by *CTGF* allelic variants. *CTGF* polymorphisms that were associated with HF in Chinese fishermen were genotyped in both samples. Case and control phenotypes are described in Materials and methods. We first tested for associations between the SNPs and HF phenotypes separately (univariate analysis); the SNPs were then tested simultaneously (multivariate analysis), including SNPs rs12526196, rs1257705, and rs9402373. Genotype is the aggravating genotype. In Sudanese farmers (*n* = 214, 62 cases and 152 controls), the covariate was age only.

**Table 3.16:** SNPs in the region flanking *CTGF* gene are associated with severe HF in study subjects infected with *S. mansoni*

<table>
<thead>
<tr>
<th>SNPs</th>
<th>Genotype</th>
<th>Control %</th>
<th>Cases %</th>
<th>OR</th>
<th>95% CI</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Univariate Analysis</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs1257705</td>
<td>AA</td>
<td>90.1</td>
<td>98.4</td>
<td>6.68</td>
<td>0.86-51.7</td>
<td>0.069</td>
</tr>
<tr>
<td>rs12526196</td>
<td>TT</td>
<td>90.2</td>
<td>98.4</td>
<td>6.8</td>
<td>0.89-52.8</td>
<td>0.066</td>
</tr>
<tr>
<td>rs3037970</td>
<td>NS</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs6918698</td>
<td>CC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs9402373</td>
<td>CC</td>
<td>78.1</td>
<td>94.9</td>
<td>5.22</td>
<td>1.53-17.7</td>
<td>0.008</td>
</tr>
<tr>
<td><strong>Multivariate Analysis</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs12526196</td>
<td>TT</td>
<td></td>
<td></td>
<td>7.26</td>
<td>0.93-56.8</td>
<td>0.059</td>
</tr>
<tr>
<td>rs9402373</td>
<td>CC</td>
<td></td>
<td></td>
<td>5.23</td>
<td>1.5-18.0</td>
<td>0.008</td>
</tr>
</tbody>
</table>
3.2.2. Interleukin-13 (IL-13) gene

3.2.2.1. IL-13 polymorphisms

The frequency distribution of the IL-13 polymorphisms among controls and cases were not significant in rs2066960 AA alleles (32.5%, 39.3%) and rs848 TT alleles (25.7%, 30%) respectively. While, a trend of association was observed in rs1800925 TT alleles (42.4%, 52.4%), and rs1295686 AA alleles (44.3%, 55%) among controls and cases respectively (Table 3.18). The data show that those alleles were not significantly associated with aggravating disease.

3.2.2.2. No association between IL-13 polymorphisms and severe hepatic fibrosis

Genotyping data analysis of study subject showed that no association between IL-13 polymorphisms and severe hepatic fibrosis in regression analysis. However, only a trend of association of rs1800925 ($P=0.102$) and rs1295686 ($P=0.143$) was observed (Table 3.18). The polymorphism rs1800925 was located in the promoter region of IL-13 gene.
Table 3.17: SNPs in the region flanking IL13 are associated with severe HF in Study subjects infected with *S. mansoni*

<table>
<thead>
<tr>
<th>SNPs</th>
<th>Position</th>
<th>Genotype</th>
<th>Controls %</th>
<th>Cases</th>
<th>OR</th>
<th>95% CI</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs2066960</td>
<td>132022334</td>
<td>AA</td>
<td>32.5</td>
<td>39.3</td>
<td>0.708</td>
<td>0.373</td>
<td>1.343</td>
</tr>
<tr>
<td>rs1800925</td>
<td>132020708</td>
<td>TT</td>
<td>42.4</td>
<td>52.4</td>
<td>0.273</td>
<td>0.058</td>
<td>1.294</td>
</tr>
<tr>
<td>rs848</td>
<td>132024399</td>
<td>TT</td>
<td>25.7</td>
<td>30</td>
<td>0.943</td>
<td>0.485</td>
<td>1.834</td>
</tr>
<tr>
<td>rs1295686</td>
<td>132023742</td>
<td>AA</td>
<td>44.3</td>
<td>55</td>
<td>0.596</td>
<td>0.299</td>
<td>1.191</td>
</tr>
</tbody>
</table>

Table 3.18: SNPs in the region flanking IL13 are not associated with severe HF in study subjects infected with *S. mansoni*
3.2.3. Interleukin-10 (IL-10) gene

3.2.3.1. IL-10 polymorphisms were associated with severe hepatic fibrosis

First, cross tabulation analysis was done to determine if there a specific genotype is transmitted more frequent in cases than control, it was found that genotype of AA (-1082) and TT (-819) were more frequent in cases than controls. Among SNPs analyzed in the promoter region of IL-10 gene, -1082 and -819 were the most plausible candidates for showing an association with susceptibility to severe form of fibrosis (Table 3.19).

Using PVD and GB wall thickness phenotype, analysis of SNPs separately showed significant association of IL-10-1082 rs1800896 (P= 0.044) and IL-10-819 rs1800871 (P= 0.05) with severe form disease. Central periportal fibrosis and PVD had been used. The results obtained were not different significantly from PVD and GB phenotype. Multivariate analysis including these SNPs and four villages as co-variable indicated that rs1800896 (P= 0.042) and rs1800871 (P= 0.087) were independently associated with severe fibrosis (Table 3.19).
Table 3.19: SNPs in the region flanking IL-10 gene are associated with severe HF in subjects infected with *S. mansoni* using two phenotypes

<table>
<thead>
<tr>
<th>SNP</th>
<th>Position</th>
<th>Bins</th>
<th>Genotype</th>
<th>Controls</th>
<th>Cases</th>
<th>OR</th>
<th>95% CI</th>
<th>P</th>
<th>OR</th>
<th>95% CI</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>-1082</td>
<td>205013520</td>
<td>II</td>
<td>AA</td>
<td>49</td>
<td>63.3</td>
<td>2.2</td>
<td>1.02-4.6</td>
<td>0.044</td>
<td>2.1</td>
<td>1.03-4.3</td>
<td>0.042</td>
</tr>
<tr>
<td>-819</td>
<td>205013257</td>
<td>I</td>
<td>TT</td>
<td>50.3</td>
<td>64.4</td>
<td>2.1</td>
<td>1.0-4.6</td>
<td>0.054</td>
<td>1.93</td>
<td>1.0-4.1</td>
<td>0.087</td>
</tr>
<tr>
<td>kiramit</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>kitra Hamdan</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wad alamin</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Alawamra</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
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</tr>
</tbody>
</table>

PV and GB Central fibrosis and PV Phenotype
3.2.4. Functional analysis

3.2.4.1. rs9402373 and rs12526196 polymorphisms affect nuclear factor binding and may alter gene transcription or transcript stability

Four polymorphisms were screened for potentiality to interact with nuclear extract from hepacits cells. The data showed that two polymorphisms affecting nuclear factor binding. The first SNP rs9402373C allele was bound to nuclear factor (complex 1) with higher affinity than the C allele. The second SNP rs12526196C allele bind to factors (complex 2). The other allele of rs12526196G allele was not bound to factors (complex 2). No allele specific binding was observed with SNPs rs12527705 and rs1931002 (Figure 3.11 A). These finding indicated that two polymorphisms had functional important in the promoter region of the CTGF gene.

The result of competitive reactions preformed with 100 (5x) or 200 (10x) fmol of unbiotinylated rs9402373G and C probe. The unbiotinylated rs9402373G did not compete with the biotinylated rs9402373C probe for binding. The cold probe (5X) competitive unbiotinylated rs9402373C was competed with biotinylated rs9402373C in binding of nuclear extract. This result in minimized the strength of the band due to compete with the unbiotinylated rs9402373C. Because the unbiotinylated rs9402373C and complex 1 were not visualized by X-ray image. The cold probe (10X) competitive of unbiotinylated rs9402373C was competed with biotinylated rs9402373C in binding of nuclear extract. This result in disappearance of the bound band. The most of unbiotinylated rs9402373C were bound to (complex 1), which were not visualized by X-ray image (Figure 3.11 B).
Figure 3.11: EMSA for SNPs rs12526196, rs9402373, rs12527705, and rs1931002.

It was found that the rs12526196T allele bind nuclear factors (complex 1) with a higher affinity than the C allele. The SNP rs9402373C allele bind nuclear factors (complex 2) that were not bound by the G allele. No allele specific binding was observed with SNPs rs12527705 and rs1931002 (Figure 3.11A).

In (Figure 3.11 B) the result of competitive reactions preformed with 100 (5×) or 200 (10×) fmol of unbiotinylated rs9402373G and C probe. The unbiotinylated rs9402373G did not compete with the biotinylated rs9402373C probe for binding.
Chapter four

Discussion

In regions endemic for \textit{S. mansoni}, 5-30\% of the residents are affected by advanced hepatic disease (PPF and hepatosplenomegaly, HSP) that may progress to complications (portal hypertension, ascites, and haematemesis) (Alain \textit{et al.}, 2008).

The aims of present study were to identify some of the critical factors that may contribute to the development of severe hepatic fibrosis, taking into account the previous factors which had been speculated among the Sudanese (Qurashi \textit{et al.}, 1999) and the Brazilin populations (Dessein \textit{et al.}, 1992) like age, gender, intensity of infection, and genetic background of patients.

The result indicted that the prevalence of severe hepatic fibrosis (HF) was high among study subjects. Males were more affected than females. This result was two time greater than that of a previous study in Gezira State, which reported that fibrosis grade two (FII) or FIII with portal hypertension, affected only 6\% of the population (Qurashi \textit{et al.}, 1999).

4.1. Central periportal fibrosis and gender affects

The current study was indicted a significant difference between males and females for both central and peripheral fibrosis. This finding indicated that the prevalence of central periportal fibrosis and peripheral fibrosis were highly associated with gender. This could be explained by that males were more likely affected by central periportal fibrosis due to high exposure to infected water while females were more frequently affected by mild form of fibrosis (less exposure after puberty) or due to gender related hormone which can increased fibrosis or resolve it in males and females respectively. Previous epidemiological studies support this finding that fibrosis development is gender dependent (Qurashi \textit{et al.}, 1999;
Lambertucci *et al.*, 2001). Non-immune physiological factors, like steroid hormones, could play an important role in the orientation of immune response in females. The ability of these hormones to modulate cytokine production, may lead to slow down fibrosis development. One of these hormones was progesterone which favors the switch of Th2 to determine Th1 response (Choi *et al.*, 2000). Another study, has demonstrated that levels of IL-4, IL-5, and TNF-α in the sera of F-III patients were significantly higher than those found in F-0 individuals, while levels of IL-13 were lower. Levels of IL-4, IL-5, and TNF-α in serum were significantly higher in F-III males than in fibrosis grade zero (F-0) males or F-III females. Conversely, levels of IL-13 were significantly lower in F-III females than in F-0 females and males (Silva-Teixeira *et al.*, 2004). In addition, genetic localization of some cytokine receptor genes such like IL-13 R and IL-2Rγ in the Xq24 and Xq13.1 region of chromosome X (Puck *et al.*, 1993; Guo *et al.*, 1997) may be involved in explanation of this gender variation of HF.

4.2. Disease progression with age and exposure in males and females

Severe central periportal fibrosis was observed in 5% of the 15 to 25 age group and this was not different between males and females probably because males and females have comparable exposure to waters before puberty (Qurashi *et al.*, 1999). Females are less affected than males by severe HF: as discussed before this could be due to a lower exposure of the women after puberty or /and the influence of certain gender related hormones on fibrosis or on the control of infection. The peak of central fibrosis occurred in male as females in the 36-45 age groups suggesting either a change in exposure after that age, better defences against the parasites or the death of the most affected subjects. To the current study knowledge there was no much change in exposure during that age period, furthermore, if sterile immunity
was to play a role we would expect that female would develop more slowly than males sterile immunity since they are less exposed.

Finally advanced central fibrosis is not much reversible especially in subjects who have without doubts continued to be exposed to infection. Then this study favor the latter conclusion that decrease prevalence of severe fibrosis cases is mostly due to death; this conclusion is also supported by close observation which was made during the five years period. Thus, after evaluation of the most subjects with central fibrosis grades D, E, F (severe cases with history of bleeding during this study were followed for endoscope treatment) have died whereas the death in subjects with low central fibrosis represented null. However, the finding that prevalence of severe fibrosis peaks around 40 years is most interesting when put in perspective with observation on HCV infections that show that this age period is a crucial in disease progression (Dessein, A. J. 2011; personal contact). This may suggest that some important changes in fibrosis progression occurs around that age period aggravating the disease and the progression to death (Rahoud et al., 2010).

In a previous study, the highest prevalence of central fibrosis was in the age group 25-30 years in males (Homeida et al., 1988a). While in current study reach the peak ten year later 36-45 years in males: the possible explanation for this result may be due to increase of immunity to disease or regular chemotherapy by the study subjects. The lacking of chemotherapy was found to decrease the prevalence of fibrosis two to three times lower among treated villagers (Homeida et al., 1988c). The genetic background of study subject may affect the susceptibility to disease.

4.3. **Central fibrosis is associated with severe hepatic disease**

Central periportal fibrosis was associated with an increase of portal vein diameter. The association of peripheral fibrosis with PVD and SVD could be due to
its correlation with central periportal fibrosis and conversely. The data show that central periportal fibrosis exhibits the strongest association with PVD and SVD and that in the presence of central periportal fibrosis, peripheral fibrosis was barely correlated to PVD and SVD. The poor association between peripheral fibrosis and enlargement of PVD and SVD may be due to increase in portal vein diameter specially C+ grade, the reasons for this association was the presence of central and peripheral fibrosis in the same patients. This finding was consistent with previous studies which correlated central fibrosis with enlargement in portal and splenic vein diameter (Abdel-Latif et al., 1981; Gerapacher-Lara et al., 1997; Qurashi et al., 1999)

Central periportal fibrosis was associated with bleeding from varices. The mechanism by which central periportal fibrosis cause bleeding is mechanical due to resistance of blood flow in the small portal vessels; this leads to increase portal pressure, thus allowing the blood flow toward collateral branches of the larger veins. This in addition to periportal granulomatous inflammation and deposition of fibrosis cause portal hypertension, followed by splenomegaly and proto-systemic collateral circulation, notably oesophageal varices and their continuous rupture lead to death (Mudawi and Ibrahim, 2007).

4.4. Central periportal fibrosis is associated with activity (occupation)

As a behaviour related disease, the risk of infection with schistosomiasis is associated to age, sex, and occupation of individuals (Gryseels, 1991). The epidemiological features of contamination in two villages in the Brazil were the same, but one village was exhibited severe infection. The result indicted differences in occupation resulting in higher exposure for those working in agriculture (Coutinho et al., 1997). It is widely accepted that morbidity related to schistosomiasis appears
in only a small proportion of the infected populations, mostly those harbouring heavy worm loads (Arap Siongok et al., 1976). However, other factors inherent to the population may be interfering with the results, such as nutritional status, genetic background, etc.

Since the most of study subjects were males, and their main occupation is agriculture. They are more frequently contact with circulating pathogens in the contaminant water. They are increasing the pathogen immunity, which mainly depend on elevated IL-13 cytokine levels. The weak association of central fibrosis and activity (occupation) might be due to increase in clinical immunity, and exacerbate infection immunity by increasing IL-13 level, however high IL-13 level can affect the disease immunity since elevated level of IL-13 enhances fibrosis deposition (Alain et al., 2008).

4.5. Splenomegaly and hepatic disease

Increasing of spleen size was mostly correlated with central periportal fibrosis. Thus the central fibrosis was the main cause of splenomegaly. Similar result have been reported by Homeida et al. (1988a).

Krimit village population exhibited the larger splenic volume, to further confirm this finding; we attempted to compare the prevalence of the main splenic volume by age classes and 5 major tribes. It was found that there was tribal effect in increasing splenic volume, especially by Arikian tribe that shown increase in the splenic volume in three age classes 26-35, 36-45 and 46-55, this finding indicated that the association of increased splenic volume might be due to tribal effect.

In advanced liver disease, it has been shown that the spleen increase in size as a result of cell proliferation (proliferative splenomegaly) and blood influx (congestive splenomegaly). At the most advanced stages of the disease,
splenomegaly contributes to portal hypertension by contributing a massive blood flux towards the portal system. Also we observed in advance stage of disease the enlarged spleen contributed to portal hypertension, to support this finding we run regression analysis we found that the central fibrosis and splenic volume were strongly associated with portal vein. Increasing grade of fibrosis was correlated with increased prevalence of splenomegaly and with the size of spleen as well as increased diameters of portal and splenic veins. (Cerri et al., 1984; Homeida et al., 1988a). This finding was consistent with previous studies in which severe form of disease was correlated with enlargement in portal vein and splenic vein, which lead to create varices and bleeding form oesophageal varices (Homeida et al., 1988b; Abdel-Wahab et al., 1989; Qurashi et al., 1999).

4.6. The association of hepatic disease with tribal effect

The severity of HF likely is influenced by exposure to infections over the many years of exposure during which hepatic fibrosis have developed. It is unlikely, however, that study subjects have experienced very different exposures because they have been living in similar conditions in villages that are close to each other, which are circled by irrigation canals infested by infected snails and have no clean water. Canals are interconnected and close to the villages; the canal snails are easily infected by faeces that are deposited on the banks of the canals or in the water by the teenagers the only source of water for bathing and domestic use including washing, is the canal. Given these remarks it was surprising that we observed marked differences in the prevalence of advanced HF (central fibrosis grades D, E, F) in between villages. However, ethnic origins of villages population were differed suggesting that hepatic disease might be influenced by subject of ethnic origin. This
indicates that the different tribes are differently affected by schistosomiasis. These differences may be related to many tribes specific factors including genetic factors.

Genetic factors are likely important as they were reported that the genetic control of HF in Sudanese population, however, they found a trend of association between the two tribes (Rawashda and Tama), in which the number of samples is very small to detect a clear association (Qurashi et al., 1999). In a previous study done in Bahia (Brazil), ethnic differences were found involved in predisposition to S. mansoni morbidity. It was reported that black individuals were more resistant to disease compared to whites individuals (Cancado et al., 1995).

4.7. The effect of previous treatment on peripheral fibrosis

This study was found evidence of significant association between treatment and peripheral fibrosis. This finding show that previous treatment might reduce the effect of egg inflammation on the peripheral fibrosis more rapidly than central periportal fibrosis, however, the treatment could stop the progression of central periportal fibrosis but it need more time than peripheral fibrosis to reach significant result. When the cause is removed and the inflammation subsides, degradation of excess matrix predominates and tends to result in storing normal liver structure. Reports by the Homeida group, in their studies in the Sudan, young patients with lower PPF grades tend to respond more to anti-schistosomal chemotherapy (Homeida et al., 1991; Homeida et al., 1996; Kheir et al., 2000), this process also occurs in long-standing fibrosis.

This finding was supported by previous study on patients of hepatosplenic schistosomiasis whom were given curative treatment may demonstrate regression of disease months or even years later (Homeida et al., 1991). Data from human and experimental animals studies show that, hepatic fibrosis regresses, accompanied by
reduction in spleen size, a drop in portal pressure, and reduction of oesophageal varices are known to be regressed by previous treatment (El-Zayadi, 2004; Zein et al., 2004; Cameron et al., 2006).

4.8. Factor associated with oesophagus varices

Several factors that could be associated with severe hepatic disease (haematemesis). The only predictor factor was central periportal fibrosis which associated significantly with haematemesis. This finding in line with a previous finding by Eltoum et al. (1994) in which he show that, the major clinical predictors of upper gastrointestinal tract bleeding in patients with schistosomal periportal fibrosis in central Sudan were splenic more than 11 cm, fibrosis above grade I and varices above grade I. Other factors like age, gender, palpable liver and portal vein diameter were not significant predictors of bleeding.

4.9. Genetic susceptibility of S. mansoni pathology

4.9.1. Interleukin-10 is associated with increasing susceptibility to HF

A previous study in subject with acute and chronic schistosomiasis has suggested that IFN-γ and IL-10 cross regulate each other and that IL-10 is beneficial in patients with acute infection or hepato-splenomegaly (Bettelli et al., 2006). Another study did not detect an association of IL-10 and fibrosis in the present or absence of IFN-γ or TNF-α showing that there was a trend of association for lower IL-10 production in both un-stimulated and SEA-stimulated culture of subject with grade FII-FIII (Henri et al., 2002).

In current study, we have been examined for the first time the contribution of three polymorphisms in the promoter region of the IL10 gene for the control of susceptibility to hepatic fibrosis. Number of functional polymorphisms in the promoter of IL 10 gene that may interfere with transcription factors binding sites
have been reported, recent data confirmed that IL10-819 C/T polymorphism was associated with increased expression of IL-10 in individuals carrying CC allele than C/T and T/T genotypes (Salhi et al., 2008). The current finding show that this polymorphism is associated with increasing susceptibility to severe hepatic fibrosis and the polymorphisms at IL-10 -819 C/T and the TT allele was associated with aggravating disease and CC and C/T were associated with protection as they were more frequent in control than cases.

Another polymorphism of IL-10 -1082 A alleles was associated with protection against severe hepatic disease. This finding supported by many studies in which this allele -1082 A was associated with increasing IL-10 transcription activity due to its location in IL-10 promoter, compared to the G allele (Kube et al., 1995; Keijsers et al., 1997; Eskdale et al., 1999), hence, this mutation located in the promoter region a report mention that this allele -1082 lies within a putative ETS transcription factor binding site (Kube et al., 1995).

The mechanism, by which IL-10 could contribute to massive fibrosis, could be due to shift of the immune response towards Th2 and down regulation of IFN-γ directly or indirectly through regulation of IL-12 cytokine. Furthermore, IL-10 controls IL-17 production was reported in murine schistosomiasis and cause death. IL-17 cause hepatocellular damage in the absence of IL-10 and TGF-β (Rutitzky and Stadecker, 2006).

Similar results on the association of IL-10 polymorphisms and infectious disease have been reported on Epstein-Barr Virus (EBV) infection (Helminen et al., 1999; Helminen et al., 2001), in Meningococcemia (Balding et al., 2003), in TB patients (Delgado et al., 2002; Scola et al., 2003) and in Rheumatoid Arthritis patients (Keijsers et al., 1997). In contrast to our finding some authors reported no
association between IL-10 promoter polymorphisms and infection, in Chinese TB patients (Tso et al., 2005), and in Gambian TB patients (Bellamy et al., 1998). A study found that, IL-10 in *S. japonicum* was associated with protection against central fibrosis, because of its anti-inflammatory by controlling the balance between egg destruction and tissue damage, thereby preventing the extensive deposition of fibrotic tissue (Arnaud et al., 2008). Also IL-10 had anti-fibrosis effects enabling it to modulate extracellular matrix deposition by down-regulating collagen production or up-regulating the production of collagenases, such as matrix metalloproteinase 13 (MMP-13), which degrades extracellular matrix (Yamamoto et al., 2001; Mathurin et al., 2002; Horsnell et al., 2011). These results can be explained first by genetic background of Chinese population was different from African population represented by Nigerian in Abidjan or among the Sudanese samples due to more frequency of singletons SNPs in the Sudanese population, second the differences between *S. mansoni* and *S. japonicum* species could possibly interfere with outcome of disease.

### 4.9.2. Interleukin-13 and severe HF

IL-13 has been demonstrated in mouse models to play a fundamental role in the pathogenesis of fibrotic lesions associated with experimental murine schistosomiasis (Chiaramonte et al., 1999a; Chiaramonte et al., 1999b; El-Zayadi, 2004).

IL-13 gene is one of type I cytokines which dominated the immune response in mice and humans chronically infected by *S. mansoni*. Both of IL-4 and IL-13 cytokines have a role in egg granuloma formation in mice; in addition to that, IL-13 was reported to be associated with the development of hepatic fibrosis (Rabello et al., 1997; Chiaramonte et al., 2001). While the pattern of cytokine in human revealed association between low level of INF-\(\gamma\) and high level of TNF-\(\alpha\) and hepatic fibrosis
(Henri et al., 2002; Booth et al., 2004a), it is still not clear which cytokines are involved in the progression of *S. mansoni* pathology.

Studies have pointed out an important role for IL-13 in the development of liver fibrosis (Chiaramonte et al., 2001; El-Zayadi, 2004). IL-4 and IL-13 share the same receptor and many biological activities, but there are differences in the function between these two cytokines. In granuloma formation IL-4 deficient mice partially reducing the granuloma size, while blocking IL-4 receptor and IL-13 indeed fully eliminated granuloma formation and tissue fibrosis in these animals (Chiaramonte et al., 2001). This study showed the central role played by IL-13 in fibrogenesis. Thus, therapeutic approaches aimed at disrupting the IL-13 pathway will be highly effective at preventing fibrotic disease caused by chronic Th2-mediated inflammatory reactions.

Earlier studies on schistosomiasis pathology in mice demonstrated that INF-γ reduces the cellularity of granulomas and down modulates granuloma size (Boros and Lukacs, 1992). Moreover, other studies had shown a correlation between the production of high levels of IL-13 and the development of more severe fibrosis, suggest that this cytokine might also play a significant role in human schistosomiasis fibrosis (de Jesus et al., 2004; Alves Oliveira et al., 2006). Taken together these findings, it’s partially explaining the trend of the association between IL-13 rs1800129 TT allele in the promoter of the IL-13 cytokine and severe hepatic disease in this study population, as revealed by univariate analysis. This finding was limited may be due to small sampling number.

In the present study, two polymorphisms of IL-13 had a trend of association rs1800925 and rs1295686. The polymorphism rs1800925 was located in the promoter region of IL-13 gene and this polymorphism can alter DNA-protein
interaction. In previous study done by Cameron et al. (2006) which support our finding, they showed that the IL13-1112T (rs1800925) allele enhanced IL13 promoter activity in primary human and murine CD4+ Th2 lymphocytes. Increased expression of IL13-1112T in Th2 cells was associated with the creation of a Yin-Yang 1 binding site that overlapped a STAT motif involved in negative regulation of IL13 expression and attenuated STAT6-mediated transcriptional repression. Because IL-13 secretion was increased in IL13-1112TT homozygotes and they proposed the increased expression of IL13-1112T in vivo may underlie its association with susceptibility to allergic inflammation (Cameron et al., 2006).

More recent study done in Malian population revealed additional polymorphism rs7719175, located in the IL13 promoter region was associated with controlling infection levels in S. haematobium. The haplotype rs7719175T-rs1800925C was associated with high infection levels. This was indicated a synergistic role in controlling infection Levels caused by S. haematobium in Malian population (Isnard et al., 2011).

The functional effects of individual polymorphisms may be small; risk for complex diseases is substantially increased by synergism between multiple SNPs arrayed along a single regulatory pathway. Gene-environment interactions in the nucleus may also offer a rationale for the common but disquieting finding that many published associations could not be replicated (Lohmueller et al., 2003; Hall and Blakey, 2005). If the functional outcome of genetic variation contributing to disease risk is determined not only by the genetic but also by the biological context. There are many studies established associations with different functional variants within the same gene or with opposite alleles at the same SNP in different populations (Ober and Hoffjan, 2006). For example, IgE levels are associated with IL13-1112C > T in
some populations (Heinzmann et al., 2003; Hoffjan et al., 2004), and with $IL13$-2044G >A (Heinzmann et al., 2003), or $IL13$-1512A > C (Maier et al., 2006) in others. It is tempting to speculate that these seemingly contradictory results may often represent an outcome of gene environment interactions in the nucleus. Thus, the gene environment interactions in the nucleus, they might be interfere with the outcome of this result and masked the clear association of the IL-13 polymorphisms.

4.9.3. Variants of CTGF are associated with hepatic fibrosis

SNP rs9402373 is associated with hepatic fibrosis in the Sudanese samples, and confirmed in other two different populations (Dessein et al., 2009). This association appear clearly when we used restricted phenotype combining between hepatic central fibrosis, portal hypertension and gall bladder thickness. The other SNP rs1256196 was associated independently with severe hepatic fibrosis in univariate analysis, and subsequently had been confirmed in the Chinese population (Dessein et al., 2009). An additional association was found for rs12527705. In multivariate analysis, there are association of SNPs rs9402373 and rs1256196 with severe hepatic fibrosis in the presence of age as a covariate.

This study indicated that, the allelic variants of rs1256196 and rs9402373 bind differently to the nuclear factors, remarking for possible effect in the site of regulation of the CTGF gene transcription or the stability of transcripts. This was appear obviously when we compare the risk of severe hepatic fibrosis with controls. The risk was increased more than seven times in cases carrying rs1256196 TT alleles than control. While, it was increased more than five times in cases carrying rs9402373 CC alleles. This could be due to increase in CTGF gene transcriptions because these polymorphisms were lie in the promoter regions of the gene. Or they
could offer sustainable action of CTGF proteins, which increased the production of collagen and cause elevated fibrosis.

CTGF plays a key role in the pathogenesis of hepatic fibrosis and stimulates the transformation of resting hepatic stellate cells into myofibroblasts, which leads to the production of more CTGF (Paradis et al., 1999; Hayashi et al., 2002). CTGF also stimulates the production of collagens, fibronectin and laminin, the predominant molecules of the extracellular matrix (ECM) of the liver (Abou-Shady et al., 2000; Rachfal and Brigstock, 2003). These products were participated in deposition of fibrosis in the liver, thus increased the collagens accumulation and aggravated the liver fibrosis.

CTGF not only directly mediates hepatic stellate activation and ECM production but also specifically mediates the profibrogenic activity of TGF-β in vitro and in vivo (Grotendorst, 1997; Paradis et al., 2002). CTGF synergizes with TGF-β to promote a sustained fibrogenic response in vivo (Holmes et al., 2003; Leask and Abraham, 2003). Down regulation of CTGF expression can inhibit both CTGF- and TGF-β-1 mediated ECM production both in vitro and in vivo (Duncan et al., 1999; Wang et al., 2004). So blocking the over-expression of CTGF could be a potential strategy to prevent the accumulation of ECM proteins and thus hepatic fibrosis.

CTGF contributes to fibrosis by acting in synergy with various profibrogenic growth factors (Leask and Abraham, 2006), including platelet-derived growth factor, vascular endothelial growth factor, and the master fibrogenic molecule, TGF-β. CTGF is a TGF-β downstream modulator (Leask and Abraham, 2003, 2006). These factors contributed to increase of CTGF protein, and increased fibrosis accumulation. Thus, they participated in increasing liver fibrosis and severity of hepatic disease.
SNP rs6918698 has been shown to be associated with sclerodermia in Caucasians (Fonseca et al., 2007). Another two investigation groups failed to find association between this polymorphism and sclerodermia (Gourh et al., 2008; Morita et al., 2008). This result reported no association between rs6918698CC and hepatic fibrosis in our Sudanese samples and the same result was obtained in Chinese farmer, but this SNP was associated with HF in Brazilians and in Chinese fishermen (Dessein et al., 2009). The possible explanation for that, the SNP rs6918698 CC allele may be in linkage disequilibrium with another causal SNP. This SNP was in linkage disequilibrium with rs3037970. Thus functional analysis for the rs3037970 may confirm the role of this SNP in fibrosis production. The effect of these SNPs may be masked by other mutations in the pathway of the gene.

CTGF also increases ECMP networking through its binding to fibronectin domains on ECMP (Yoshida and Munakata, 2007; Gressner and Gressner, 2008). The inhibition of CTGF with siRNAs is preventing or reducing rat tissue fibrosis (Li et al., 2006; George and Tsutsumi, 2007). Thus, CTGF is a major factor in fibrosis, and polymorphisms in CTGF could certainly affect HF in Schistosoma infections. In addition to profibrogenic effects, other CTGF properties, such as stimulation of angiogenesis (Shimo et al., 2001; Horsnell et al., 2007), stimulation of cell proliferation (Gao et al., 2004) and survival (Nair et al., 2009), partly mediated through its effect on TGF-β and BPM-7, may also be directly relevant to other aspects of Schistosoma induced pathology.

The current study reported an association between CTGF polymorphisms and IL-10 polymorphisms with severe fibrosis does not rule out the possibility that polymorphisms in other pathways also affect fibrosis progression. In this regard, there are several studies with various experimental models that show that IL-13 has a
major profibrotic effect in mice infected with *S. mansoni*. Several human studies have also shown that polymorphisms in IL-13 or IL-13 receptor chains are associated with aggravated fibrosis, such as in sclerodermia (Granel et al., 2006a; Granel et al., 2006b). In human schistosomiasis, Joseph et al. (2004) have reported an increased production of IL-13 by white blood cells stimulated by *Schistosoma* egg antigens in cultures from subjects with advanced disease. IFN-γ production was associated with HF in *S. mansoni*-infected Sudanese subjects; also polymorphisms in IFN-γ that reduce IFN-γ production were associated with increased HF (Henri et al., 2002; Chevillard et al., 2003).

This study showed that, gender, tribal effect, genetic background, increasing of gallbladder wall > 4mm, enlargement of portal and splenic veins, and splenic volume where the main risk factors controlling severe hepatic disease and bleeding from oesophageal varices in the study subjects.
4.10 Conclusions

1. Central periportal fibrosis is associated with gender and more common in males than females.

2. Central periportal fibrosis is associated with the tribes indicating possible genetic control of susceptibility to severe HF.

3. Two SNPs -1082 and -819 in the IL-10 promoter region are associated with severe form of hepatic disease in Sudanese patients.

4. The allele of IL-10 -1082 AA is associated with the high level production of IL-10

5. IL-10 is playing a major role in aggravating liver disease, and IL-10 -1082 AA can be used as a marker for progression of severe hepatic fibrosis due to *S. mansoni* eggs.

6. CTGF is a major factor in fibrosis, and its polymorphisms could affect hepatic fibrosis in *S. mansoni* infection.

7. rs9402373 and rs12526196 polymorphisms in CTGF gene affect nuclear factor binding and can alter gene transcription or transcript stability. These polymorphisms have been confirmed in three other populations in two Chinese populations (fishermen and farmers) and a Brazilian population.
4.11. Recommendations

1. A case-control study with clear classification of PPF disease and healthy controls, enough sample size (with MAF data and set up test power as 0.8), and proper data presentations are required. Moreover, multiple genetic variations that show biological connections should be considered to be investigated at the same time. More SNPs should be investigated in SM1 and SM2 regions.

2. Identification of susceptibility genes is expected to improve our knowledge on the pathogenesis of infection and advanced fibrosis in schistosomiasis. Moreover, it may lead to opening of new avenue for new therapeutic approaches, preventive treatments (including vaccination) as well as curative treatments. In addition, it may enable setting up a genetic screening procedure in endemic populations that would allow the identification of subjects particularly at risk for this disease resulting in cheaper, more effective, earlier and targeted treatment.

3. During the course of study, high levels of IL-10 and CTGF were associated with development of severe hepatic fibrosis. Thus, a follow-up of those populations should be conducted in order to evaluate the incidence of periportal fibrosis, the factors involving the development of fibrosis, and the cytokine profile associated with regression of fibrosis after treatment.

4. Several candidate genes may be screened to identify their role in hepatic fibrosis such as, TGF-β, IL-17, IL-22AR1, CCR3, CCR5 and ARG-1.
REFERENCES


classes and subtypes in the *Schistosoma mansoni* genome for designing subtype-specific inhibitors. *Biochem Biophys Res Commun* **380**: 525-530.


182


Hesse, M., Cheever, A.W., Jankovic, D., and Wynn, T.A. (2000). NOS-2 mediates the protective anti-inflammatory and antifibrotic effects of the Th1-


(Cambridge: CAB International).


by regulating interferon gamma and the IL-13 decoy receptor. 


Appendix I
Clinical / Epidemiological form: SUDAN / PPF February 2007

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Name: ........................................................................ Male (1) Female (2)
Age: ............years.
Your activity? ........Farmer...House wife. Canal leaners.. and Other............

**Water contacts:**
Were you born in this village? Yes / No ....
When did you settle in the village ............years ago. Where did you come from? .........
**How many years have you been living in the village?** .................years ....
Do you bath in canal? : how often ....... / week
Do you fish in canal? ......how often ............../week
Do you wash clothes in canal? ........./ week
Do you bath at home? .............with water from canal? ..............since what age? .......

**Water Contacts:** ...........................................

**Past medical history:**
Clinical signs of severe schistosomiasis:
- Haemathemesis.......................................Ascites.............................................
- Splenectomy ..................when........ Sclerosis of varices
..............................when........
Treatment for schistosomiasis? How many .......... Date of last treatment: .....
Tabs (Praziquantel) ............or injections (Hycanthone) ? ..........

**Malaria:** How often ........../ year Severe / Mild............................................
**Tuberculosis** ............................... **Hepatitis** .......................................

**Risk factors for hepatitis infections:**
Blood transfusion: yes / no How many times: ..... 
Injections in village ..........how many times............../year.
Injections at hospital .........how many times............../year.
What were the injections for............................................................
Surgical operations at hospital..........................................................
Surgical operations in the village.....................................................
Traditional scars: ...........................................................................

**Family clinical history:**
Any one in the family with haematemesis or / and ascites? ......................
Any one died of severe Schistosomiasis? ...........................................
(........None (0), brother (1), sister (2), father (3), mother (4), child (5))
Other infectious diseases...(Leprae, KA., Tuberculosis(60 inj), Hepatitis./ who
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