GENETIC VARIANTS FOR THE GENE CODIFICATING FOR LOX-1 (Lectin-like Oxidized low-density lipoprotein receptor) DISTRIBUTION, SIMILARITIES AND DIFFERENCES BETWEEN THE CAUCASIC POPULATION AND SUB SAHARAN AFRICAN POPULATION IN PRIMARY AND SECONDARY PREVENTION IN RELATION TO EXISTING CLASSIC RISK FACTORS FOR ARtheroTROMBOSIS.

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DECLARATION

This work to the best of my knowledge has not been submitted for degree in any other university.

This Dissertation Has Been Submitted For Examination With Approval Of The University of Siena Ethical committee, the director and the supervisors.

Director of the Ph.D School

Prof. Alessandra Renieri;

Supervisor

Prof. Luca Puccetti;
DEDICATION

I wish to dedicate this study to all the volunteers who were very helpful and important for the good outcome of this study and to all the sick in the whole world. To my Mother who gave me the basics of education despite many difficulties. To my entire family, brothers, sisters, nieces, nephews, relatives and friends who have walked with me this far. To the Daughters of Charity of Saint Vincent de Paul Province of Siena who have been my Guardian Angels.
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I thank God first and foremost for His unfailing love to me. I would like to acknowledge all the people who contributed in one way or another in the preparation of this research proposal.

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I thank my family, relatives, friends and the Daughters of Charity of Saint Vincent de Paul Province of Siena for continuous prayers, guidance, support and encouragement when I needed it the most throughout this process. I love you all.
LIST OF ABBREVIATIONS

LOX-1 – lectin-like oxidized LDL
BP- Blood Pressure
SBP- Systolic Blood Pressure
DBP- Diastolic Pressure
CVD- Cardiovascular Disease
CAD- Coronary Artery Disease
MI- Myocardial Infarction
WHO- World Health Organization
AHA – American Heart Association
oxLDL - Oxidized low-density lipoprotein
TNF-α - tumor necrosis factor
SNP- Single Nucleotide Polymorphism
Indels- Insertion/Deletion Variation
TC- Total Cholesterol
TG- Trygliceride
MetS- Metabolic Syndrome
VCAM-1- vascular cell adhesion molecule-1
ICAM-1- intercellular adhesion molecule-1
WC-Waist Circumference
BMI-Body Mass Index
USA-United States Of America
NCEP – National Cholesterol Education Program
HDL-High Density Lipoproteins
LDL-Low Density Lipoproteins
vWF -von Willebrand factor
GP -glycoprotein
ADP -adenosine diphosphate
TxA2 -thromboxane A2
PF 4 -platelet factor 4
PSGL-1 P-selectin glycoprotein ligand-1
ATP -adenosine triphosphate
NOS - nitric oxide synthase

**eNOS** – endothelial nitric oxide synthase

**ROS** – reactive oxygen species

**COX** – cyclooxygenase

**ELISA** – enzyme-linked immunosorbent assay

**UTR** – untranslated region
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ABSTRACT

Atherothrombotic disorders are still the leading cause of mortality and residual disability in western countries and are progressively increasing in developing countries.

Since metabolic, mainly classical risk factors, and inflammatory alterations are deeply involved in the atherothrombotic burden through lipid peroxidation, several mechanisms linking both hypothesis are now recognized as primary triggers of clinical events. In this setting, concerning lipid disorders and diabetes, LOX-1 (Lectin-like Oxidized low-density lipoprotein receptor), mediates the recognition of vascular OX-LDL receptors and makes up a key role in the pathogenesis of atherosclerosis and other cardiovascular and cerebrovascular diseases.

This receptor is up regulated by ox-LDL itself and by angiotensin II, by endothelin cytokines and shear stress, all participants in atherosclerosis.

Single nucleotide polymorphisms (SNPs) in OLR-1 (the gene encoding for LOX-1) have been associated with cardiovascular events, mainly in Caucasian and asian population with contrasting data for afro-americans. Futhermore LOX-1 has been associated with the pharmacological anti-thrombotic drugs such as statins. The present body of evidence suggests LOX-1 as a putative novel target for drug therapy.

The aim of this study is to detect whether the 3’UTR T/C and IVS4-14 A/G polymorphisms could be associated with rising cardiovascular events ratio in sub Saharan African population with respect to classical risk factors and the putative differences with Caucasian population. From our results we found that The 3’UTR/T of Lox-1 gene could not correspond with cardiovascular events with the population considered in our study. (O.R. 0.93 95% C.I 0.66-1.02, p-test confirmation test of Hosmer-Lmeshov 0.069). The vascular events are related with classical risk factors, mainly diabetes and hypertension,
that however are not routinely investigated in these subjects. The extensive evaluation of classical risk factors in the sub Saharan population should be the first step to better manage the primary and secondary prevention of atherothrombotic events.
GENERAL REMINDER

Revealing the complete sequences of four individual diploid genomes has given even more insight about the number of existing forms of genetic variations and their evolutionary background as well as susceptibility to human diseases. Analyses of human genetic variations in phenotypic differences have become one of the central efforts to understand the function of the genes and genetic variants in predisposition to disease development. Cardiovascular disease (CVD) is known to have a multifactorial etiology, with genetic factors playing as important a role as environmental and lifestyle factors. Major advances in the field of molecular genetics have been instrumental in identifying numerous polymorphisms in various genes, including in those of platelet receptors, lipoproteins, and others. Their qualitative and quantitative variations strongly predispose towards the development of CVD. The approach of utilizing genetic polymorphisms, as effective biomarkers, to predict CVD risk in individuals and ethnic groups has however not attained popularity but still needs to be established after widespread trials in different populations.

Aims and Objectives Of The Present Study

Primary objectives
To evaluate the IVS4-14 A/G variants for the gene LOX-1 (ORL-1) and assess the distribution, similarities and differences between the Caucasian population and a Sub Saharan African Population.
Secondary objectives:
To evaluate any association with the increasing incidence of cardiovascular diseases in Africa Sub-Sahara in presence of existing risk factors, (obesity, high blood pressure, dyslipidaemia, diabetes etc).

The study also aims to demonstrate possible differences in prevalence demonstrated by the Caucasian population.

Study Area
The study was carried out at The Azienda Ospedaliera Universitaria Senese Policlinico “Santa Maria Alle Scotte” and in Eldoret Hospital located in Eldoret Municipality, Uasin Gishu County, Kenya.

Study Design
A comparative study was used, recruiting subjects from an Africa Sub-Saharan (Kenya) population and a Caucasian population (Italy) presenting with common risk factors for CAD, atherosclerosis and comparing the Caucasian group consisting of patients with history of atherosclerosis and Cardiovascular or related risk factors in a balanced manner.

For this study traditional risk factors were defined on the questionnaire to evaluate the clinical relevance. Infarct for each data, the distribution and frequencies of the risk factors was compared using standard parametric and non parametric methods.

Data Collection
The data was collected using questionnaires and direct interview with the participants for the African and Caucasian population.
Inclusion Criteria

All patients with a documented history of cardiovascular events and/or coronary artery atherosclerosis.

- Randomly selected patients with a diagnosis Cardio-Cerebro-Vascular conditions. Randomly selected patients with cases of:
- Diabetic patients with fasting glucose $>126$ mg dl$^{-1}$ or postprandial glucose $>200$ mg dl$^{-1}$ will be included in the study
- Patients with lipid abnormality (total cholesterol $>200$ mg dl$^{-1}$, LDL cholesterol $>130$ mg dl$^{-1}$, high-density lipoprotein (HDL)-cholesterol $<40$ mg dl$^{-1}$ or triglycerides $>200$ mg dl$^{-1}$)
- Patients with fasting triglycerides $>400$ mg dl$^{-1}$, Smokers and non-smokers
- Individuals with a body mass index (BMI) $>30$ kgm$^{-2}$ or less,
- Hypertensive (blood pressure $<150/90$ mmHg),

Patients with normal pressure, Volunteers with unavailability of complete medical recordings.

- Patients attending the hospital as Out patients and Inpatients

Data were corrected for main risk factors (obesity, high blood pressure, diabetes etc).

Exclusion Criteria

The individuals who did not give consent and those who demonstrated inability to cooperate for whichever reason for example (dementia) were not included in the study.

Confidentiality

Any information given will remain confidential before, during and after the study and will be used for the purpose of this study. Care was not denied to those who refused to participate in the study.
Quality Assurance

1. The study was conducted by well trained personnel during the recruitment, evaluation, specimen handling and analysis of the results.

2. Written and oral informed consent were obtained from all the participants after explanation in simple words/language and understanding before the study was carried out.

Pilot Study

A pilot study was carried out before the execution of the study. This was to test the overall feasibility and help researcher familiarize with the setup. It was also to assist the researcher to test the adequacy and the appropriateness of the data collection methods and statistic analysis.

Data Processing and Analysis.

Demographic data on the study obtained by direct interviews from the volunteers was entered into the data sheet, collated, cleaned and corrected before analysis. All primary and secondary data was then entered into a computer database from which they were analyzed for presentation in collaboration with the statistics analyst for proper analysis of the data and creation of graphical charts. The results are reported as means ± standard deviation or percentages. The complete description of employed statistical methods is reported in section 4.7.

The Research Ethics Committee of the University Teaching Hospital of Siena approved the study.
Minimization of Bias

Information Bias

A well standardized check list was developed, pretested during the pilot study and validated thereafter by the researcher with the guidance of the supervisor Prof. Luca Puccetti.

All questions were well structured and coded to enhance easy recording and analysis.

Implementation.

The actual study was done using questionnaires, direct interview and examination of the participant. Each check-list was given a serial number to ensure that quality data was collected and to make sure that questions were not repeated in the data analysis.

Timeframe/Schedule

The study was scheduled to be carried out during a whole period of 3 years, for better study, assessment, interpretation and analysis, report writing and presentation of the data.

The following was the timeline for the Research

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CHAPTER ONE

EPIDEMIOLOGY

1.1 International statistics and Epidemiology

Within cardiovascular diseases, coronary artery disease (CAD) is the single most frequent cause of death in both sexes and is responsible for more than half of all cardiovascular events.\textsuperscript{1} Findings from the World Health Organization's Monitor Trends in Cardiovascular Diseases (MONICA) project involving 21 countries showed a 4% fall in CAD death rates. Improvement in the case fatality rate accounted for only one third of the decline. However, two thirds of the decline resulted from a reduction in the number of events. These findings strongly suggest that the largest impact on decreasing the global burden of atherosclerosis will come from prevention of events. The risk-factor burden experienced by blacks differs from that of whites. In 1996, 29\% of worldwide mortality was attributable to cardiovascular disease (CVD), making it the leading cause of death globally\textsuperscript{1} By 2020, it is expected that CAD will be the largest cause of disease burden worldwide\textsuperscript{2} An epidemiological transition is now occurring in the developing world where the major causes of death are changing from infectious to non communicable diseases. This epidemiological transition is due, in part, to improved longevity. As life expectancy increases, the period of exposure to cardiovascular risk factors also increases\textsuperscript{3} Other important contributors to this transition are the so-called “globalization” of dietary habits and urbanization.
1.2 Europe

In most countries in the European Union and in the USA, the age-standardized CAD mortality rates have decreased significantly\(^1,2,3\) In the European Union, CAD is considered the single most common cause of death. One in five to one in seven women die of CAD; in men CAD accounts for one in four to one in six of all deaths. Age-standardized and gender-specific CAD mortality rates have significantly decreased during recent decades in many countries in the north, west and south of Europe. However, the decline was less apparent or absent in central and eastern Europe. Thus, the Russian Federation, Belarus, Ukraine, and Central Asian republics show the highest CAD mortality rates ever seen, significantly higher even than recognized peaks in the USA, Australia, New Zealand, Finland, and Scotland\(^4,5\) Population aging represents a major challenge, thus, even if age-specific mortality rates continue to decline, the absolute number of cardiovascular disease (CVD) deaths will increase. Predictions up to 2030 suggest that even with an annual decline in mortality rates of about 1%, the absolute number of deaths will increase, attributable solely to population aging\(^6\) The frequency of clinical manifestations of atherosclerosis in Great Britain, west of Scotland in particular, is especially high. The same is true of Scandinavia in general and of Finland in particular.

1.3 Asia (India)

The Chennai Urban Population Study (CUPS) 33, a population-based study in Chennai, in South India, showed a prevalence of CAD of 11%, which is 10 times more than what it was in 1970\(^1,2,8\) Clustering of risk factors for CAD such as hyperglycemia, central body obesity, dyslipidemia, and hypertension tends to occur, and interplay of these risk factors could explain the enhanced CAD risk in Indians. India is predicted to bear the greatest CAD burden, according to the estimates from the Global Burden of Disease Study.\(^7\) Of the more than 9 million deaths due to CAD in 1990 in developing countries, 2.4 million (25%)
occurred in India\textsuperscript{7,8}. In the same year, mortality rates in India due to acute myocardial infarction (MI) were 141 per 100,000 in males and 136 per 100,000 in females, which was much higher than in China (66 per 100,000 in males and 69 per 100,000 in females) and Latin American countries (81 per 100,000 in males and 76 per 100,000 in females)\textsuperscript{1,8,9.}

The overall cardiovascular mortality in Indians is predicted to rise by 103\% in men and 90\% in women between 1985 and 2015. High prevalence rates of diabetes and CAD are seen not only in affluent migrant Indians, but also in those living within the subcontinent. Indeed the epidemic of diabetes and CAD is now spreading to the middle- and lower-income groups in India\textsuperscript{2,3}.

1.4 United States of America (USA)

In the United States, approximately 14 million persons experience CAD and its various complications. Congestive heart failure (CHF) that develops because of ischemic cardiomyopathy in hypertensive MI survivors has become the most common discharge diagnosis for patients in American hospitals. Approximately 80 million people, or 36.3\% of the population, have cardiovascular disease.

According to the (AHA) statistics 2011, from 1997 to 2007, the death rate from CVD declined 27.8\%. Mortality data for 2007 show that CVD (I00–I99; Q20–Q28) accounted for 33.6\% (813 804) of all 2 243 712 deaths in 2007, or 1 of every 2.9 deaths in the United States.\textsuperscript{2,3,4}

On the basis of 2007 mortality rate data, more than 2200 Americans die of CVD each day, an average of 1 death every 39 seconds. More than 150 000 Americans killed by CVD (I00–I99) in 2007 were <65 years of age. In 2007, nearly 33\% of deaths due to CVD occurred before the age of 75 years, which is well before the average life expectancy of 77.9 years. Coronary heart disease caused \approx1 of every 6 deaths in the United States in 2007. Approximately every 25 seconds, an American will have a coronary event, and approximately every minute, someone will die of one. Mortality data from 2007 indicate that stroke accounted for \approx1 of every 18 deaths in the United States. On average,
every 40 seconds, someone in the United States has a stroke. From 1997 to 2007, the stroke death rate fell 44.8%, and the actual number of stroke deaths declined 14.7%. Data from the National Health and Nutrition Examination Survey (NHANES) 2005–2008 indicate that 33.5% of US adults ≥20 years of age have hypertension. The prevalence of hypertension is nearly equal between men and women. African American adults have among the highest rates of hypertension in the world, at 44%. Among hypertensive adults, ≈80% are aware of their condition, 71% are using antihypertensive medication, and only 48% of those aware that they have hypertension have their condition controlled. Despite 4 decades of progress, in 2008, among Americans ≥18 years of age, 23.1% of men and 18.3% of women continued to be cigarette smokers. In 2009, 19.5% of students in grades 9 through 12 reported current tobacco use. An estimated 33 600 000 adults ≥20 years of age have total serum cholesterol levels ≥240 mg/dL, with a prevalence of 15.0%.12,5

In 2008, an estimated 18.300.000 Americans had diagnosed diabetes mellitus, representing 8.0% of the adult population. An additional 7 100 000 had undiagnosed diabetes mellitus, and 36.8% had pre diabetes, with abnormal fasting glucose levels. African Americans, Mexican Americans, Hispanic/Latino individuals, and other ethnic minorities bear a strikingly disproportionate burden of diabetes mellitus in the United States Fully 33.7% of US adults are obese (body mass index ≥30 kg/m2). Men and women of all race/ethnic groups in the population are affected by the epidemic of overweight and obesity. On the basis of NHANES 2003–2006 data, the age-adjusted prevalence of metabolic syndrome, a cluster of major cardiovascular risk factors related to overweight/obesity and insulin resistance, is 34% (35.1% among men and 32.6% among women).
1.5 Africa Sub Sahara

The incidence, prevalence, and manifestations of CAD vary significantly with race, as does the response to therapy.\textsuperscript{3,4,5}

At the beginning of the twentieth century, high blood pressure was virtually nonexistent among indigenous Kenyans and Ugandans, but the reason may have been the lack of screening programs and access to care. From about 1975, high blood pressure became established in Cameroon, Côte d'Ivoire, Democratic Republic of Congo, Ghana, Kenya, Nigeria, and Uganda. As in developed countries, consumption of salt and alcohol, psychological stress, obesity, physical inactivity, and other dietary factors are thought to have played an important etiologic role in the genesis of primary hypertension in genetically predisposed individuals. Nevertheless, communities still exist in the Democratic Republic of Congo, Kenya, Nigeria, and the Kalahari Desert in which blood pressure is low and does not seem to rise with age. Rural-to-urban migration coupled with acculturation and modernization trends have some relation to the development of high blood pressure as observed in Kenyan and Ghanaian epidemiologic studies. Blacks appear to have higher morbidity and mortality rates of CAD, even when the statistics are corrected for educational and socioeconomic status.

The risk-factor burden experienced by blacks differs from that of whites.\textsuperscript{2,3,14} The prevalence of hypertension, obesity, dysmetabolic syndrome, and lack of physical activity are much higher in blacks, whereas the prevalence of hypercholesterolemia is lower. Blacks with AMI present for treatment later than patients do on average, are less often subjected to invasive strategies, and experience greater overall mortality. The prevalence and incidence of stroke in Sub-Saharan Africa have increased over the last half century, due principally to increased life expectancy and changes in environmental determinants and risk factors.\textsuperscript{14,15,16} The majority of cerebrovascular accidents (CVAs) occur in young and middle-aged people and are related to hypertension. Hypertension is highly prevalent in Sub-Saharan Africa and is often undetected or poorly controlled. This may be the explanation for the
high proportion of hemorrhagic CVAs, whereas in developed countries most CVAs occur in older people and are thrombotic in etiology. This has been confirmed by clinical, radiological, and postmortem diagnostic methods. Overall, CVAs account for 7 percent of deaths in South Africa.  

So prevalent is hypertension today in Sub-Saharan Africa that hypertensive heart disease might in fact be the most common form of CVD in Africa. Hypertension is a risk factor for both stroke and IHD. Left ventricular hypertrophy, congestive heart failure, and stroke are common in Africans with hypertension. There is little published information on formal programs addressing awareness, treatment, and control. Local, regional, and national surveys are required to provide epidemiological data necessary for informed decision making and policy setting on when and whom to treat in Africa.

In Western societies, such as the United States and the United Kingdom, the prevalence of hypertension and standardized mortality rates from stroke are higher for people of African origin than for whites. The same pattern is emerging in Sub-Saharan Africa.

Hyperlipidemia is uncommon in Africa, being present mainly in patients with metabolic disorders (hypertriglyceridemia) or a family history of hypercholesterolemia. It is, however, present in 10 to 70 percent of patients with antecedent CHD or stroke. A study of black African patients admitted to a coronary care unit in Cape Town, South Africa, with acute ischemic syndromes and myocardial infarction revealed relative hyperlipidemia among the patients but not in the healthy controls.

Diabetes mellitus is a well-established risk factor for CVD. The prevalence of type 2 diabetes in Africa is about 2.5 percent, ranging from 0.8 percent in rural Cameroon to 13.5 percent in Mauritius.
CHAPTER TWO

LITERATURE REVIEW

ATHEROSCLEROSIS

2.1 Definition

Atherosclerosis is a multi-factorial disease that involves complex interactions between genes and environmental factors. Atherosclerosis is a chronic inflammatory disease of large and medium-sized elastic and muscular arteries characterised by the narrowing or occlusion of the arteries by a plaque.\footnote{17} The development of atherosclerosis is a complex process influenced by a network of risk factors, such as hypertension, hyperlipidemia, smoking, diabetes mellitus, obesity, genetic predisposition, sedentary lifestyle, diet and several other conditions.\footnote{18,19}

Atherosclerosis results from excessive inflammatory with the basic abnormality lying in the redox-state of the vascular wall cells and proliferative responses to vascular insults.\footnote{21}

The possible role of oxidative imbalance could contribute to atherogenesis from a differential modulation of the expression of several molecules such as cytokines and adhesion molecules.\footnote{20} and the earliest alterations in the vessel wall are formation of the fatty streak and monocyte adhesion. The generation of oxidized low-density lipoprotein (OxLDL) in subendothelial vasculature represents an early event during atherogenesis.\footnote{22} The scavenger receptor lectin-like oxidized-low-density lipoprotein receptor-1 (LOX-1) mediates the binding and internalization of OxLDL in endothelial cells, and is believed to play an active role in atherogenesis.\footnote{23, 24}

The oxidation of low-density lipoproteins (ox-LDL) is considered to be a crucial step in the atherogenesis.\footnote{18} In vitro studies
have demonstrated that oxidatively modified LDL (ox-LDL) is more important than native-LDL for atherogenesis.\textsuperscript{23-25} This is based on powerful data that ox-LDL modifies vascular endothelial cells, smooth muscle cells, monocyte/macrophages and fibroblasts into pro-atherogenic phenotypes the hallmarks of early atherosclerotic lesions. The endothelium may react to diverse stimuli with a limited repertoire of reparative, but ultimately dysfunctional, responses.\textsuperscript{27,28} LOX-1 has been characterized as the primary receptor for OxLDL on the surface of vascular endothelial cells and is up-regulated in atherosclerotic lesions. Upon recognition of OxLDL, LOX-1 is observed to initiate OxLDL internalization and degradation as well as the induction of a variety of pro-atherogenic cellular responses, including reduction of nitric oxide (NO) release, secretion of monocyte chemoattractant protein-1 (MCP-1), production of reactive oxygen species, expression of matrix metalloproteinase-1 and -3, monocyte adhesion and apoptosis.\textsuperscript{29,30,31}

Plaques may progressively increase in size so that they eventually compromise blood flow, or the plaque can ulcerate or rupture, leading to clot formation.\textsuperscript{32} Either event can cause ischemia in distal regions supplied by that artery. Atherosclerosis is the most common cause of coronary heart disease (CHD) (which includes myocardial infarction and angina pectoris), ischemic cerebrovascular accidents (strokes), and peripheral arterial disease (PAD).\textsuperscript{33}

### 2.2 Research and History of Atherosclerosis

The process of atherosclerosis often starts very early in life, in fact already in the fetus state. In newborns accumulations of lipids, connective tissue and smooth muscle cells under the endothelium have been observed.\textsuperscript{35,36} The term atheroma (Greek ‘athero’ = gruel/paste; ‘sclerosis’ = hardening) was created by the Roman author Celsius two thousand years ago, at that time meaning fatty tumour.\textsuperscript{37}

Interestingly, as early as 1755 the term had been designated by Albrecht von Haller as the degenerative process observed in the intima of arteries.\textsuperscript{38,40}
In 1815 the London surgeon Joseph Hodgson (1788–1869) published the hypothesis that inflammation was the underlying cause of atheromateous arteries. 41,42

Most nineteenth-century pathologists however followed Carl Rokitanski’s (1804–1878) view that atherosclerosis was a degenerative process, with intimal proliferation of connective tissue and calcification, a process that was assigned arteriosclerosis by the French pathologist Jean Lobstein (1777–1835). 44,46

The inflammatory theory of atherosclerosis arose again in 1856 when the prominent German pathologist Rudolf Virchow a leading authority of his day in pathology and the greatest contributor to the notion of thrombosis, designated atheroma as a chronic inflammatory disease of the intima that he called "chronic endarteritis deformans". In 1904 Marchand recognized the association of fatty acid degeneration and vessel stiffening and introduced the term atherosclerosis to indicate this combination. 41,45,47

In 1908 a milestone was made when the Russian scientist Alexander Ignatowski showed that atherosclerosis can be induced in rabbits by feeding them milk and egg yolk. Shortly after this discovery, the ability of pure cholesterol to reproduce experimental atherosclerosis in rabbits was demonstrated. These findings revealed the importance of lipids and cholesterol in the atherosclerotic process. 48,49

In 1911, Marc Ruffer identified degenerative arterial changes in an Egyptian mummy, which in 1962 were confirmed by another research group to be atherosclerotic plaques. These findings show that atherosclerosis already existed in antiquity. 50

The 1970s marked the next large step in the history of atherosclerosis when Brown and Goldstein stated that acetylated low-density lipoprotein (LDL) and not native LDL induced foam cell formation of macrophages. 51 The discovery also in 1970s, that a low density lipoprotein (LDL) receptor mutation is underlying familial hypercholesterolemia has linked lipids and lipoproteins to atherosclerosis and nowadays LDL is viewed as a major cause. 36, 40,52
In the late 1970s Russell Ross published the “response to injury hypothesis of atherosclerosis”. He viewed atherosclerosis as a fibroproliferative process that results from a chronic inflammatory response. He also revealed the additive contribution of the endothelium, mononuclear phagocytes, platelets and smooth muscle cells in atherosclerosis. A decade later, the ability of oxidized LDL (oxLDL) to induce foam cell formation was demonstrated by Daniel Steinberg and his group. The concept that infectious agents have an impact on the process of atherosclerosis is not new, but was already proposed in the late 1800s and early 1900s.

Huchard was the first to suggest the involvement of infectious agents in the process of atherosclerosis when he published the article “Infectious diseases of childhood as potential cause of inflammation” in 1891. Shortly thereafter, Weisel and Klotz found a relation between atherosclerosis and Streptococci infections, typhoid, scarlet fever and measles.

In 1908, Osler wrote in his book “Modern Medicine: its practice and theory” about a potential link between acute infection and atherosclerosis. In the late 1940s, a strong association between marek disease virus (MDV) and atherosclerosis was found, which was also demonstrated in the 1980s by Fabricant with co-workers.

In 1980s, the frequent incidence of restenosis (reoccurrence of stenosis) drew research focus to growth factors and smooth muscle cells (SMC), proliferation. From 1990s onwards, the identification of inflammatory cells and molecules in the atherosclerotic lesion has led to the notion that multiple molecular and cellular immune processes are involved in atherogenesis, and that atherosclerosis can be regarded as a chronic inflammatory disease.

A second revolution occurred at the beginning of the 1990s when mouse models of atherosclerosis, apolipoprotein E (apoE)- and LDL receptor (LDLr) deficient mice, were derived by homologous recombination techniques. In contrast to the previous models, mice lacking functional apoE or LDLr genes were shown to develop widely
distributed arterial lesions that progress from foam cell-rich fatty streaks to fibro-proliferative plaques with lipid/necrotic cores, typical of the spectrum of human lesions. The possibility of abolishing the expression of a single gene of interest, or of over expressing it, in these mouse models opened a new era of atherosclerosis research at a mechanistic level.

2.3 Endothelial Injury

Atherosclerosis is primarily intimal disease. In certain parts of the arterial tree, chronic minimal endothelium injury can result in dysfunctional endothelium characterized by increased uptake of LDL and monocyte recruitment into the vessel wall, which are both pivotal initiating events in atherosclerosis. A healthy endothelium tends to favor vasodilation, antithrombosis, fibrinolysis, and monocyte disadhesion. Mechanisms of gene activation in the endothelium’s expression of humoral mediators are very important. Among those mediators are cytokines (which are multipotent intercellular mediators), growth factors, vasodilatory factors. The best characterized and most powerful of the endothelium-derived relaxing factor, is believed to be nitric oxide.

Endothelial structure and function is critical to maintaining blood flow and vascular integrity. The artery wall comprises three concentric layers:

1) an inner (Luminal layer), the tunica intima, constitutes a monolayer of endothelial cells exposed to the vessel lumen which includes the endothelial surface,

2) a middle layer, the tunica media, is composed of concentric layers of smooth muscle cells embedded within an extracellular matrix (ECM) of fibril-forming collagens (type I, III and V), elastic fibres and dermatan and chondroitin sulphate proteoglycans.
the outer layer (external) or tunica adventitia which is separated from the tunica media by the external elastic lamina (EEL), it is composed of vascular fibroblasts embedded within a collagen rich ECM.

The intima consists of the vascular endothelium and a thin layer of collagen and elastin fibers that anchor it to the internal elastic lamina; endothelial cells and SMCs are the principal cellular components of human intima. Amount of smooth muscles and fibro-elastic tissue in the intima, are a function of age. After birth, the intima, which is not well developed in fetal coronary arteries, progressively thickens, so that it is as thick as the media by late adolescence and thicker than the media after adolescence. It is typically during the middle age that the intima may become markedly thickened by atheroclerosis. Often in old age the coronary arteries are tortuous and have an increased luminal diameter, thinned media and increased calcification.

Systemic factors that can induce such injury include hypercholestrolemia, especially minimally modified LDL, and active and passive cigarret smoking, which may lead to endothelial dysfunction and through an increased production of super peroxide radicals by endothelium, resulting inactivation of endothelium-derived relaxing factor/nitric oxide, as well as enhancement of lipoprotein oxidation. Abnormal vasoconstriction is now recognised as one, of the earliest manifestations endothelia dysfunction. In addition, lysolectithin, which is formed by peroxidation of LDL particles, may play a role in the development of abnormal arterial vasomotion. Other major risk factors for CHD, like hypercholesterolema, minimally modified LDLs or oxLDLs, hypertention, active and passive cigarette smoking, diet high saturated fat, physical inactivity, advanced glycosylated end products in diabetes mellitus, obesity/insuline resistance, increasing age, male sex, family history of premature CHD, circulating vasoactive amines, immunocomplexes and certain viral infections, are among additional nonlocal factors that have been associated with endothelial dysfuction. The biologic basis of the site of predilection for atheroma ( e.g, variation in endothelia gene xpression) is beginning to emerge.
Local factors also play a role in atherogenesis, because lesions develop preferentially at specific sites for example where arteries are poorly supported, subject to repetitive bending, dilated, or, when relatively narrow, subject to rapid and variable blood flow.\textsuperscript{51} Not only fluid shear stress but also transmural pressure and pulsatile stretch are important mechanical factors in the shear stress signal throughout vascular cells leads to changes in structure, metabolism, and gene expression. \textsuperscript{44,45}
CHAPTER THREE

LOX-1 GENE AND ROLE PLAYED IN
ATHEROGENESIS

3.1 Introduction

Oxidized LDL (oxLDL) is involved in the very early critical steps of atherogenesis, such as endothelial injury, expression of adhesion molecules, and leukocyte recruitment and retention, as well as the formation of foam cells and thrombi (for a review see\textsuperscript{63}). Macrophages internalize and degrade oxLDL mainly through scavenger receptors, and one crucial step in the initiation and progression of atherosclerosis is the unregulated uptake of oxLDL through these receptors, leading to foam cell formation\textsuperscript{63}. Another receptor, named lectin-like oxLDL receptor-1 (LOX-1), is expressed in all cell types involved in the atherosclerotic lesion, i.e. endothelial cells (ECs), macrophages, and smooth muscle cells (SMCs)\textsuperscript{64}. LOX-1, a membrane glycoprotein that binds oxLDL and acetylated LDL (AcLDL) but not native LDL\textsuperscript{65, 66}, differs from the macrophage scavenger receptors because it contributes to the atherosclerotic process mainly through receptor-mediated signaling mechanisms\textsuperscript{67}.

However, LOX-1 is a multi-ligand receptor that can also recognize multiple classes of ligands, such as activated platelets, neutrophils, apoptotic/aged cells and bacteria\textsuperscript{68-71}, implying versatile physiological functions. This review specifically focuses on the structure, regulation and ligands of LOX-1 and highlights the mechanisms by which LOX-1 activation contributes to atherosclerosis.

The activation of LOX-1 triggers intracellular signaling, production of superoxide radicals and gene expression induced by the redox-sensitive transcription factor NF- B which plays a key role in these
processes. In 2000, the same research group that revealed the presence of LOX-1 in vascular tissue discovered the soluble form of this membrane receptor, named soluble LOX-1 (sLOX-1), showing that it derived from membrane-bound LOX-1 itself.

The crucial role of LOX-1 in the atherosclerotic process highlighted this receptor as a logical and attractive candidate for therapeutic intervention to limit vascular damage and its long-term consequences, although several key questions have not yet been answered. Here we provide a critical review of the recent literature on this receptor and likely future directions.
Fig. 1. Schematic model of the human OLR1 gene.

The OLR1 gene spans more than 7000 base pairs (bp) and consists of 6 exons (as indicated) interrupted by 5 introns. The upper names indicate the domain structure of the LOX-1 protein encoded by OLR1 mRNA: 5’UTR: 5’-untranslated region; CD: cytoplasmic domain; TM: trans-membrane domain; Neck: neck domain; 3’UTR: 3’-untranslated region; CTLD: C type lectin-like domain.
3.2 LOX-1 Gene: Structure, Polymorphism and Alternative Transcripts

3.2.1 Gene Structure

LOX-1 is encoded by the oxLDL receptor 1 (OLR1) gene, located in the natural killer gene com-plex (NKC) on chromosome 12 at p12-p13, which also contains several other families of lectin-like genes, including the CD94 and NKG2 NK receptor genes. The region telomeric of CD94 contains in addition to the LOX-1 gene, the novel human DECTIN-1 and the CLEC-1 and CLEC-2 genes within about 100 kb. Sequence similarities and chromosomal arrangement suggest that these genes form a separate subfamily of lectin-like genes within the NKC encoding receptors with important immune and/or scavenger functions in monocytes, dendritic cells and EC.

The OLR1 gene spans more than 7000 base pairs (bp), and consists of 6 exons interrupted by 5 introns; exons 1–5 range from 102 to 246 bp, whereas exon 6 is relatively long, being 1722 bp (Fig. 1). Exon 1 encodes the 5′-untranslated region (UTR) and cytoplasmic domain, exon 2 encodes the remainder of the cytoplasmic domain and the transmembrane domain, exon 3 encodes the neck domain, and exons 4, 5 and 6 encode the lectin-like domain and the 3′-UTR of LOX-1 protein (as better described in the section “protein structure of LOX-1”). The 5′ promoter/ enhancer region (about 2500 bp) of the human OLR1 gene was also studied, and TATA and CAAT boxes were found in the proximal part of the 5′-flanking region. Computer-based analysis identified a wide variety of potential transcription factor-binding sites, including sites for the STAT family and NF-interleukin (IL)-6.

The transcriptional repressor octamer-1-binding site within the promoter region plays an important role in the human LOX-1 promoter trans-activation in response to oxLDL. Furthermore, the promoter region between nucleotides-2131 and -2247, which includes an active NF- B-
binding site, is required for angiotensin (Ang)-induced transactivation of the human LOX-1 promoter\textsuperscript{81}.

### 3.2.2 Gene Polymorphisms and Splicing Variants

Some studies have shown common genetic variation in the OLR1 gene to be associated with the risk of coronary artery disease (CAD). Three common LOX-1 single nucleotide polymorphisms (SNPs) have been identified in intron 4 (G A), intron 5 (T G) and 3’UTR (T C)\textsuperscript{82}. Contemporaneously, the involvement of LOX-1 in atherosclerosis and acute myocardial infarction (AMI) was confirmed by defining OLR1 genetic variation in an association study of intragenic SNPs\textsuperscript{83}. Seven SNPs were identified of which six were within intron 4, intron 5, and the 3’ UTR. These six polymorphisms were in complete linkage disequilibrium behaving as a single SNP block, including exon 5.

It was observed that the LOX-1/3’UTR SNP genotype and allele frequencies differed significantly between the control and the AMI groups in which the subjects with the T/T or C/T genotype were at higher risk of developing AMI\textsuperscript{83}. Despite the positive results on OLR1 polymorphisms in CAD or AMI subjects\textsuperscript{83-85}, other similar studies were unable to confirm these putative genetic risks\textsuperscript{85-87}. In particular, regarding a functional SNP, the G C transition at position 501 in exon 4 which produces a single amino acid change (K167N) in the ligand-binding domain and affects markedly LOX-1 receptor activity\textsuperscript{88}, different conclusions have been reported\textsuperscript{83-87}.

Furthermore, it has been explored whether the SNPs could give rise to a functional product by examining the existence of messenger RNA (mRNA) isoforms as a consequence of alternative splicing\textsuperscript{89}. A reproducible pattern of alternative splicing in the OLR1 gene around exon 5, with 2 resulting OLR1 transcripts in the RNA fractions, was identified: one of these products corresponded to the full-length transcript, while the other (named LOXIN) lacked exon 5. The LOXIN spliced mRNA has a stop codon in the open reading frame that leads to a premature termination of the translation product and generates a protein
that lacks 2/3 of the lectin-like domain and is unable to bind oxLDL.\textsuperscript{89}

Experiments both in vivo and in vitro indicate that the splice variant LOXIN is expressed at a similar level to the full-length receptor LOX-1, and their relative transcription is modulated by the presence of SNPs.\textsuperscript{89} Macrophages from subjects carrying the “non-risk” haplotype expressed more LOXIN and so fewer cells underwent apoptosis on oxLDL induction.\textsuperscript{89} Therefore, higher levels of LOXIN protect cells from LOX-1-induced apoptosis. Also LOXIN tends to dimerize, therefore its protective role may be due to inactivation of the LOX-1 receptor via the formation of a heterodimer.\textsuperscript{89} Molecular LOX-1/LOXIN interaction and the formation of non-functional heterooligomers have been recently confirmed, suggesting hetero-oligomerization between naturally occurring isoforms of LOX-1 to represent a general model for the regulation of LOX-1 function by its variants.

It is difficult to draw definite conclusions from these conflicting studies; multiple large, well-matched cohorts of cases and controls will be required to achieve valid progress in the genetic analysis of atherosclerosis and other complex human diseases indicating mostly the need for caution in the interpretation of genetic associations.

\textbf{3.2.3 Protein Structure of LOX-1}

LOX-1 has a molecular weight of 50 kDa and is a type membrane protein belonging to the C-type lectin family.\textsuperscript{73} LOX-1 is synthesized as a 40-kDa pre-cursor protein, subsequently glycosylated at four potential N-linked glycosylation sites in the extracellular C-terminal domain, and processed into a 48-kDa mature form within 40 min.\textsuperscript{91} LOX-1 consists of four domains: a short N-terminal cytoplasmic domain, a single transmembrane domain, a connecting stalk region (neck) domain, and a lectin-like extracellular domain at the C-terminus which binds oxLDL.\textsuperscript{73} (Fig. 2). The extracellular part of LOX-1 comprises an 82-residue neck and a ligand-binding domain. A coiled-coil structure is located in the C-terminal part of the neck, and is in dynamic equilibrium among multiple conformational states. This chimeric structural property of the neck
region may enable LOX-1 to cluster at the cell surface, where it exists as a homodimer to recognize oxLDL\textsuperscript{91,93}. One-third of the N-terminal neck is less structured than the remainder of the protein and is highly sensitive to cleavage by a variety of proteases\textsuperscript{93}. Both positively charged and non-charged hydrophilic residues are involved in ligand binding, suggesting that ligand recognition of LOX-1 depends on more complex conformational interactions, rather than merely electrostatic interaction between positively charged residues and negatively charged ligands\textsuperscript{94}. LOX-1 also exists as a soluble form (sLOX-1), corresponding to its extracellular domain only\textsuperscript{74}. Two soluble forms of similar molecular weight (approximately 35 kDa) were identified in the supernatant of bovine aortic EC\textsuperscript{74}). In tumor necrosis factor-(TNF-\textsuperscript{ })-activated cells, cell-surface expression of LOX-1 precedes sLOX-1 release\textsuperscript{74} which is inhibited by serine protease inhibitors, particularly by PMSF\textsuperscript{74}. Nevertheless, it has been recently shown that overexpression of ADAM71, a member of the disintegrin and metalloproteinase family, enhanced the cleavage of sLOX-1 while its inhibition suppressed it, suggesting the effect of PMSF on sLOX-1 production to be indirect\textsuperscript{95}. Purification and sequencing of bovine sLOX-1 identified the two cleavage sites between Arg(86)-Ser(87) and Lys(89)-Ser(90), which were located in the membrane proximal extracellular domain of LOX-1\textsuperscript{74}.

It has been further shown that IL-18 stimulated the shedding of LOX-1 and subsequent release of sLOX-1\textsuperscript{95}. A circulating soluble form of LOX-1, corresponding solely to its extracellular domain, has been identified in human serum, as we will discuss in detail afterwards.
Fig. 2. Structure of the full-length LOX-1 and its soluble form.

The extracellular region of the primary structure of LOX-1 consists of a C-type lectin-like domain at the C-terminus, followed by a connecting stalk region (neck domain). The full-length LOX-1, in addition to the extracellular domain, displays a single transmembrane spanning region and a short N-terminal cytosolic tail. As illustrated, LOX-1 exists as a disulfide-linked homodimer at cysteine 140 on the cell surface. The circulating form derives from enzymatic cleavage at two potential sites of the full-length cell-surface receptor, between Arg(86)-Ser(87) or Lys(89)-Ser(90) residues located in the membrane proximal extracellular domain, and consists only of a part of the extracellular domain of the receptor (a portion of the neck and the entire C-terminal domain). The ligands of LOX-1 are shown on the right side of the figure.
3.3 Regulation of LOX-1 Expression

The LOX-1 promoter is constitutively active only at a low level though its expression can be induced by oxLDL, fluid shear stress, Ang, proinflammatory cytokines, lipopolysaccharide, phorbol 12-myristate 13-acetate (PMA), oxidants, fluid shear stress, heparin-binding epidermal growth factor (EGF)-like growth factor (HB-EGF) and others (Table 1).

In ECs, glycoxidized LDL and oxLDL significantly increase LOX-1 expression and the production of sLOX-1, although the effect of glycoxidized LDL is greater than that of oxLDL. In addition in the same cells, advanced glycation endproducts (AGEs), C reactive protein (CRP), high glucose and IL-6 induce expression of LOX-1. Furthermore, lysophosphatidylcholine, the main phospholipid component of oxLDL, as well as superoxide anions, hydrogen peroxide and homocysteine up-regulate the expression of LOX-1 mRNA. Also endothelin-1 (ET-1) stimulates endothelial LOX-1 expression in a receptor-mediated manner. Trans-forming growth factor, which plays a crucial role in vascular remodeling and the pathogenesis of atherosclerosis, induces the expression of LOX-1 in both EC and SMCs. In this latter cell type, the mediators of the inflammatory acute phase response (IL-1, IL-1 and TNF-) regulate the expression of LOX-1. In addition, in SMCs, LOX-1 acts as a receptor for remnant-like lipoprotein particles (RLPs) that up-regulate its expression and induce cell migration, suggesting a proatherogenic role, especially in the settings of post-prandial hyperlipidemia, diabetes and metabolic syndrome.

Drome. LOX-1 expression in cardiac myocytes, as well as in vessel walls of failing rat hearts in vivo, is induced by neurohormonal factors activated in heart failure such as norepinephrine and ET-1. In this regard, since increased protease activity is a key feature of plaque instability that may achieve an enhancement of sLOX-1 release, cardiac myocytes, may be another source of sLOX-1.

In macrophages, the expression of LOX-1 can be up-regulated by
TNF- and high glucose concentrations\textsuperscript{112, 113}.

In summary, a number of proinflammatory and proatherothrombotic factors are therefore able to regulate the expression of this receptor, suggesting LOX-1 to have an important role in amplifying local inflammatory responses during atherosclerotic development.

**Table 1.**

**Stimuli that regulate the expression of LOX-1**

<table>
<thead>
<tr>
<th>Stimuli</th>
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<tbody>
<tr>
<td>Lipopolysaccharide\textsuperscript{99}</td>
</tr>
<tr>
<td>Tumor necrosis factor\textsuperscript{65, 80, 98}</td>
</tr>
<tr>
<td>Phorbol 12-myristate 13-acetate\textsuperscript{65}</td>
</tr>
<tr>
<td>Fluid shear stress\textsuperscript{98, 99}</td>
</tr>
<tr>
<td>OxLDL\textsuperscript{96, 97}</td>
</tr>
<tr>
<td>Lysophosphatidylcholine\textsuperscript{97, 106}</td>
</tr>
<tr>
<td>Angiotensin\textsuperscript{81}</td>
</tr>
<tr>
<td>Transforming growth factor\textsuperscript{98}</td>
</tr>
<tr>
<td>High glucose concentration\textsuperscript{93-95, 103}</td>
</tr>
<tr>
<td>Norepinephrine\textsuperscript{111}</td>
</tr>
<tr>
<td>Endothelin-1\textsuperscript{111}</td>
</tr>
<tr>
<td>Oxidant species\textsuperscript{100}</td>
</tr>
<tr>
<td>Homocysteine\textsuperscript{100}</td>
</tr>
<tr>
<td>C reactive protein\textsuperscript{104, 105}</td>
</tr>
<tr>
<td>HB-EGF\textsuperscript{101}</td>
</tr>
<tr>
<td>IL-6\textsuperscript{104, 105}, IL-1\textsuperscript{80}, IL-1\textsuperscript{80}</td>
</tr>
<tr>
<td>Remnant-like lipoprotein particles\textsuperscript{110}</td>
</tr>
<tr>
<td>Advanced glycation endproducts\textsuperscript{103}</td>
</tr>
<tr>
<td>Glycoxidized LDL\textsuperscript{102}</td>
</tr>
</tbody>
</table>
3.4 LOX-1 as a Multi-Ligand Receptor with High Specificity for OxLDL: is it a Multifunctional Environmental Sensor or an Endocytic Receptor?

LOX-1 has interesting specificity because it binds oxLDL and with lower affinity, AcLDL, while it does not bind native LDL. In spite of this specificity, LOX-1 recognizes multiple ligands including hypo-chlorite-modified high density lipoprotein, aged red blood cells, apoptotic cells, leukocytes, activated platelets, bacteria, phosphatidylserine and AGEs suggesting a versatility of function for LOX-1 under pathophysiological conditions in vivo (Fig. 2). AGEs are well-known ligands for receptor for AGEs (RAGE) but also for other scavenger receptors, including LOX-1, whose binding was effectively suppressed by an anti-LOX-1 antibody. Moreover, LOX-1 has been identified as an additional receptor for endocytic uptake of heat shock protein 140 (HSP140), chaperoned peptides and CRP. As LOX-1 recognizes and binds activated platelets, exposure of LOX-1 on the activated platelet surface assists thrombosis as demonstrated by the accumulation of LOX-1 protein at sites of thrombi in atherosclerotic plaques. Its broad range of ligands in part overlaps with that of other scavenger receptors and toll-like receptors, and implicates LOX-1 in innate immunity, although its precise role in this immunological context has yet to be determined. The negative charge on ligands is crucial to recognition by LOX-1. In fact, substitution of basic with neutral amino acids greatly decreases the binding capacity of LOX-1. The crystal structure of LOX-1 revealed the ligand-binding interface to be hydrophobic except for the basic spine, which is composed of arginine residues. Experiments with mutated LOX-1 revealed that the substitution of a single amino acid in the basic spine, resulted in the greatest loss of affinity for AcLDL. The ligand-binding domain of LOX-1 has a homodimeric structure with an intermolecular disul-fide bond. A single amino acid substitution in the interface caused a severe reduction in binding activity, suggesting the correct arrangement of the dimer to be crucial.
for binding to oxLDL. Site-directed mutagene-sis indicated that cysteine 140 has a key role in the formation of disulfide-linked human LOX-1 dimers.\textsuperscript{92} LOX-1 assembles on the cell surface as at least a hex-amer comprising three homodimeric LOX-1 mole-cules in binding to oxLDL.\textsuperscript{92} Comparing the size of the dimer surface of LOX-1 (70 Å) with the diameter of LDL (250 Å), it seems plausible that LOX-1 binds to oxLDL as an assembly protein.\textsuperscript{66} Furthermore, amphipathic -helices on LDL can act as multiple binding sites for LOX-1 assembly on the cell surface.\textsuperscript{66} It is reasonable to think that circulating plasma sLOX-1 is still able to bind oxLDL; however, since the amount of sLOX-1 in circulation is very low, it does not affect oxLDL plasma levels and so could not act as a decoy for oxLDL. Macrophages use scavenger receptors to internal-ize and degrade modified LDL, producing foam cells. LOX-1 was detected on human and mouse macro-phages, but not on monocytes\textsuperscript{112, 124} and the expression of LOX-1 can be induced in human monocytes after macrophage-like differentiati-on in vitro\textsuperscript{124}. In macro-phages, LOX-1 expression appears to be regulated differently from CD36 and SR-A expression\textsuperscript{125}. Based upon studies with gene-knockout (KO) mice, CD36 and SR-A play crucial roles in macrophage apoptosis and determining the complexity of atherosclerotic lesions\textsuperscript{126}. LOX-1 may play significant roles when macrophages are stimulated by atherogenic lipids or proinflamma-tory stimuli in atherosclerotic plaques.\textsuperscript{127}

Recently, using LOX-1 KO mice, it has been shown that there was no difference in uptake or degra-dation of oxLDL in macrophages from wild-type and LOX-1 KO mice and no difference in the rate of clearance of oxLDL from plasma in vivo.\textsuperscript{127} However, when expression of LOX-1 was induced with lysophosphati-dylcholine, oxLDL uptake and degradation increased 2-fold in wild-type macrophages but did not change in LOX-1 KO macrophages, suggesting that in pro-inflammatory conditions in which macrophage LOX-1 expression can increase, the oxLDL uptake could sub-stantially increase.\textsuperscript{127} Recently it has been shown that LOX-1 is constitutively internalized from the plasma membrane and thus likely to mediate oxLDL traffick-ing in vascular
Given the many disparities between studies in vitro and in vivo, it remains likely that this receptor is a multifunctional sensor but also an endocytic receptor. However, how it can facilitate intracellular signaling during trafficking of the receptor-ligand complex along the endocytic pathway is not known. Alternatively, it may be that there is diversity of function depending on cell and tissue type, and availability of ligands.

Table 2.

**Cellular effects of ligand-LOX-1 interaction on atherogenesis**

<table>
<thead>
<tr>
<th>Endothelial cells</th>
<th>Alteration of vascular tone $^{126}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Increased intracellular oxidative stress $^{134}$ Induction of apoptosis $^{96}$</td>
</tr>
<tr>
<td></td>
<td>Induction of proliferation and angiogenesis by increasing VEGF expression $^{131, 132, 135}$</td>
</tr>
<tr>
<td></td>
<td>Increased expression of adhesion molecules (VCAM-1, ICAM-1, Selectins) $^{142}$</td>
</tr>
<tr>
<td></td>
<td>Increased expression of monocyte chemoattractant protein-1 $^{142}$</td>
</tr>
<tr>
<td></td>
<td>Induction of plasminogen activator inhibitor-1 $^{140}$</td>
</tr>
<tr>
<td></td>
<td>Reduction of endothelial nitric oxide synthase $^{133, 144}$</td>
</tr>
<tr>
<td></td>
<td>Release of matrix metalloproteinases $^{135, 144}$</td>
</tr>
<tr>
<td>Smooth muscle cells</td>
<td>Induction of apoptosis $^{147}$</td>
</tr>
<tr>
<td>Monocytes</td>
<td>Induction of monocyte adhesion and activation $^{195}$</td>
</tr>
<tr>
<td></td>
<td>Increased oxLDL uptake and foam cell formation $^{11}$</td>
</tr>
</tbody>
</table>

*Vascular endothelial growth factor; Vascular cell adhesion molecule-1; Intercellular cell adhesion molecule-1.*
3.5 Signal Transduction Pathways Triggered by Ligand-LOX-1 Interaction

LOX-1 is expressed in most cell types relevant to the development of atherosclerotic plaques (i.e., ECs, SMCs, macrophages), and the interaction of LOX-1 with its ligands modifies the cell phenotype in a pro-atherogenic sense, so that the cells become dysfunctional and more prone to death\textsuperscript{129} (Table 2).

The identification of LOX-1 as the major receptor for oxLDL in ECs has provided a new clue about the mechanisms by which oxLDL are involved in vessel wall injury.

Most information about LOX-1 signal transduction pathways on EC comes from the activation or inhibition of LOX-1 by oxLDL and LOX-1 antibody respectively. OxLDL has dual effects on cultured cells depending on its concentration and the exposure time: at a lower concentration (from 5 to 10 $\text{g/mL}$) and shorter exposure time it induces proliferation, whereas at a higher concentration (from 50 to 300 $\text{g/mL}$) and longer exposure time it induces apoptosis in ECs, macrophages, and SMCs\textsuperscript{130}, and even necrosis\textsuperscript{131, 132} (Fig. 3). Low concentrations of oxLDL cause the activation of protein kinaseC- (PKC-)\textsuperscript{133}, generate low levels of reactive oxygen species (ROS) and promote angiogenesis through an increase in vascular endothelial growth factor (VEGF) via LOX-1-mediated redox-sensitive signaling pathways\textsuperscript{134, 135}. OxLDL induces ROS production via NADPH oxidase\textsuperscript{136, 137} and downstream alterations include activation of p38 mitogen-activated protein kinase (p38MAPK), a component of oxidant stress-sensitive signaling pathways, phosphatidylinositol-tol-3-kinase, and extracellular-signal-regulated kinase (ERK1/2), which leads to activation of the transcription factor NF- B\textsuperscript{96, 138, 139} (Fig. 3). OxLDL also represses DNA binding of the transcriptional regulator nuclear factor 1, diminishes mRNA levels of monoxygenase cytochrome P450 and decreases production of endothelial-derived hyperpolarization factor, a key regulator of vascular tone\textsuperscript{136, 137} (Fig. 3). Furthermore, oxLDL induces the endothelial expression of plasmin-ogen activator inhibitor-1, implicated in
atherothrombosis, through ERK1/2 activation. Furthermore, the binding of oxLDL to LOX-1 decreases intracellular NO levels through increased production of O2, which could inactivate NO itself.\textsuperscript{134, 141}

OxLDL treatment enhances the expression of vascular cell adhesion molecule 1 (VCAM-1), intercellular cell adhesion molecule 1 (ICAM-1), monocyte chemoattractant protein 1 (MCP-1) and selectins, leading to monocyte attachment and activation.\textsuperscript{142} The notion that oxLDL mediates these effects via LOX-1 is borne out by the finding that antisense LOX-1 mRNA decreased LOX-1 expression and subsequent adhesion molecule expression.\textsuperscript{81} However, recently it has been shown that LOX-1 is involved in redox-sensitive, Akt/eNOS and Ca2 signaling pathways caused by the attachment of monocyte to EC and the blockade of LOX-1 by antibody or small interfering RNA inhibited the monocyte adhesion-triggered signaling pathway in ECs independent of the oxLDL-LOX-1 axis.\textsuperscript{143}

The binding of oxLDL to LOX-1 induces a reduction in endothelial nitric oxide synthase (eNOS) expression, increases levels of matrix metalloproteinase (MMP) 1, MMP 3 and MMP 9 and collagenase activity, but does not increase the expression of tissue inhibitors of MMPs.\textsuperscript{133, 144} Recently, a study provided evidence that in ECs, the membrane type 1-MMP (MT1-MMP), which is involved in atherogenesis, colocalizes and forms a complex with LOX-1\textsuperscript{145}. Since the blockade of LOX-1 or MT1-MMP prevented the oxLDL-induced RhoA-dependent downregulation of eNOS protein expression, Rac1-mediated NADPH oxidase activity, and generation of ROS, the LOX-1-MT1-MMP axis may play a crucial role in the small GTPase RhoA and Rac1 activation signaling pathways.\textsuperscript{145} Recently, from a cDNA microarray analysis performed in a EC line over-expressing LOX-1 and treated with oxLDL, LOX-1-dependent changes in gene expression associated with inflammation, and the induction of mRNA expression for a number of cytokines, including IL-8, cell adhesion, and signal transduction, were confirmed.\textsuperscript{146} In addition, a promoter analysis of the genes that changed following
oxLDL-mediated LOX-1 activation identified EGR1 and CREB as potential novel transcription factors that function downstream of LOX-1.146

In SMCs, oxLDL at high concentrations induces apoptosis by increasing the Bax/Bcl-2 ratio147, and in EC, oxLDL decreases the expression of the antiapoptotic protein Bcl-2, induces the release of cytochrome C and Smac from mitochondria into the cytoplasmic compartment, and activates caspase-987 (Fig. 3). Decreased eNOS and Bcl-2 expression, together with induction of MMP collagenase activity, resulted in EC injury, dysfunction, and apoptosis (Fig. 3). The pro-apoptotic effects of oxLDL are mediated by its receptor LOX-1, because pretreatment of ECs with anti-sense-LOX-1, but not sense-LOX-1, blocked these effects148.

Other evidence has shown that CRP also binds to endothelial LOX-1 resulting in the adhesion of monocyte to ECs and increases oxLDL uptake, so contributing to endothelial dysfunction105,149.

In cardiac myocytes, activation of LOX-1 by oxLDL induced apoptosis via activation of p38MAPK, which could be blocked by an antioxidant catalase111.

In chondrocytes, the binding of oxLDL with LOX-1 markedly increased VEGF mRNA expression and protein release through activation of peroxisome proliferator-activated receptor- (PPAR-), suggesting that LOX-1 signal transduction pathways also lead to PPAR-activation.89
oxLDL-LOX-1 interaction may lead to endothelial dysfunction and/or apoptosis. Low concentrations of oxLDL induce the generation of ROS by activation of NADPH oxidase and PKC. ROS activate the ERK1/2-MAPK and PI3K pathways, leading to NF-κB’s nuclear translocation. Consequently, a reduction in eNOS expression and NO’s release, an increase in adhesion molecule (VCAM-1, ICAM-1 and selectins) expression, and enhanced release of MCP-1 and MMPs, lead to endothelial dysfunction. The activation of LOX-1 could also decrease cytochrome P450 (CYP450) protein levels resulting in diminished levels of endothelial-derived hyperpolarization factor (EDHF). High concentrations of oxLDL cause diminished Akt gene transcription and an enhanced bax/bcl-2 ratio leading to apoptosis via the release of cytochrome c and Smac from mitochondria to the cytoplasmic compartment, and through activation of caspase-9.
3.6 Lessons from Animal Studies

Several animal models have been developed to dissect the contribution of LOX-1 to the pathogenesis of vasculopathy in pro-atherogenic settings including hypertension, hyperlipidemia, diabetes and mainly atherosclerosis. Its expression is dramatically up-regulated in the aorta in hypertensive rats, suggesting a potential role for LOX-1 in the pathogenesis of hyper-tension. In addition, LOX-1 overexpression in hypertensive animal models is localized to vascular ECs, which is compatible with the hypothesis that LOX-1 is related to endothelial dysfunction in hyper-tension.

In rats with streptozotocin-induced diabetes, LOX-1 expression was markedly up-regulated in ECs, especially in bifurcations of artery branches from the aorta. In rats with ischemia-reperfusion (I/R) injury, LOX-1 expression was up-regulated, which through p38MAPK activation increased the expression of MMP-1 and adhesion molecules. Furthermore, increased LOX-1 expression in I/R was associated with lipid peroxidation and apoptosis, a large infarcted area, and a loss of left ventricular function.

The generation of transgenic and KO mice has elucidated the pathogenic role of LOX-1 in atHERO-sclerosis. LOX-1 KO mice fed a high-cholesterol diet show decreased oxLDL-binding to the aortic endothelium and a preservation of endothelium-dependent vaso-relaxation after treatment with oxLDL. The cross-breeding of LOX-1 KO mice with LDL receptor- deficient (LDLr KO) mice led to a reduction in atherogenesis compared with LDLr KO mice. Proinflammatory signals, the expression of NF- B and inflammatory markers were increased in LDLr KO mice, but not in double KO mice. Furthermore, LOX-1 KO mice had greater levels of eNOS while in the LDLr KO mice, there was a marked reduction in eNOS expression and its restoration with LOX-1 deletion. Thus, the deletion of LOX-1 sustains endothelial function leading to a reduction in atherogenesis in association with a reduction in proinflammatory and pro-oxidant signals. Conversely, LOX-1 transgenic mice overexpressing LOX-1 but lacking apoE displayed
accelerated intramyocardial vasculopathy, and a 10-fold increase in atheroma-like lesion area compared with non-transgenic littermates after 3-weeks on a high-fat diet.\textsuperscript{156} Wild-type mice subjected to I/R developed a marked decrease in left ventricular systolic pressure and an increase in left ventricular end-diastolic pres-sure following I/R, and the degree of change was much less in the LOX-1 KO mice, indicating preser-vation of left ventricular function with LOX-1 dele-tion.\textsuperscript{157} There was evidence for marked oxidative stress (NADPH oxidase expression, malondialdehyde and 8-isoprostane) and increase of collagen deposition, fibronectin and osteopontin expression following I/R in wild-type mice, but much less so in LOX-1 KO mice.\textsuperscript{157}

Furthermore, I/R in LOX-1 KO mice resulted in a significant decrease in myocardial injury as well as in the accumulation of inflammatory cells in I/R myo-cardium and lipid peroxidation.\textsuperscript{158} The phosphorylation of p38MAPK and PKB/Akt-1, as well as inducible NOS, was enhanced during I/R in wild-type mice, but much less so in LOX-1 KO mice. These findings provide convincing evidence that LOX-1 is a key modulator of cardiac remodeling which starts immediately following I/R, and its effect is mediated by pro-oxidant signals.\textsuperscript{158}

Many studies have investigated the relationship between LOX-1 expression and plaque instability.

LOX-1 is extensively expressed in the new blood vessels in the core of human carotid advanced athero-sclerotic lesions and colocalized with apoptotic cells, which are present mostly in the rupture-prone regions of atherosclerotic plaques.\textsuperscript{6,159}

In hypercholesterolemic rabbits, LOX-1 expression was more prominent in atherosclerotic plaques with a thinner fibromuscular cap and was localized to the macrophage-rich lipid core.\textsuperscript{160} Moreover, in the same areas, LOX-1 expression was positively correlated with tissue factor expression and apoptosis, suggesting the involvement of LOX-1 in the destabilization and rupture of atherosclerotic lesions and the subsequent formation of thrombi.\textsuperscript{161} Since LOX-1 could be impli-cated in vascular cell dysfunction related to plaque instability, it has been
investigated as a potential target for an imaging tracer of atherosclerosis. In a recent study\textsuperscript{162}, AMI-prone Watanabe heritable hyperlipidemic rabbits and control rabbits were injected intravenously with (99m)Tc-LOX-1-mAb. Imaging clearly visualized the atherosclerotic aortas; the level of (99m)Tc-LOX-1-mAb accumulation in unstable plaques was higher than that in neointimal lesions or other, more stable lesions and therefore nuclear imaging of LOX-1 expression with this tracer may be a useful means of identifying atheromas at high risk of rupture. However, in the absence of suitable animal models of vulnerable plaques, we are far from suggesting that LOX-1 is involved in the destabilization of atherosclerotic plaques.

3.7 LOX-1 to sLOX-1

Several clinical studies have investigated the significance of serum sLOX-1 concentrations in several pathological conditions, particularly vascular disease and diabetes.

The first clinical study to highlight sLOX-1 as a marker in cardiovascular disease dates back to 2005.\textsuperscript{102} In this study, serum sLOX-1 levels were elevated in acute syndrome coronary (ACS). Serum sLOX-1 levels did not show any correlation with troponin-T or creatine phosphokinase levels, suggesting that sLOX-1 is not a marker for cardiac necrosis or injury.\textsuperscript{163} A clinical study carried out on patients with ACS reported an association of serum sLOX-1 levels with the severity of CAD and urinary 8-isoprostane levels and an inverse correlation with extracellular superoxide dismutase levels suggesting that increased serum sLOX-1 levels reflect enhanced oxidative stress in vascular walls.\textsuperscript{164}

In type 2 diabetic patients, serum sLOX-1 levels were higher compared with controls.\textsuperscript{103} Serum sLOX-1 decreased after improvements in glycemic control and the magnitude of this reduction correlated with the improvement in hemoglobin A1c and AGEs but not with the reduction in oxLDL.\textsuperscript{103} Recently, we reported that sLOX-1 levels were increased in CAD patients and associated with the severity
of CAD and inflammatory markers.\textsuperscript{165} Other recent data showed that obese women have higher sLOX-1 levels, which may reflect increased LOX-1 expression in adipose tissue.\textsuperscript{166}

Recently, a likely relationship between LOX-1 gene polymorphisms and circulating sLOX-1 levels has been reported. The 3’UTR-T allele was associated with lower sLOX-1 levels in healthy older subjects suggesting LOX-1 gene variation to be important in the regulation of sLOX-1 levels in plasma.\textsuperscript{167}

Since association studies aiming to link metabolic and cardiovascular disease with sLOX-1 are far from conclusive due to over or misinterpretation of associations and supposed correlations, the clinical relevance of these observations needs to be assessed in extended clinical trials. Platelets share a relevant role in atherothrombosis and ox-LDL has been described as platelet agonists.\textsuperscript{180} Since platelets express both the ligands CD36 and LOX-1, a specific role in the pharmacogenetic modulation of statins antiplatelet action has been described for LOX-1 mutations. Furthermore such a pharmacogenetic influence was observed also in terms of clinical efficacy on statins in the prevention of acute coronary syndromes in hypercholesterolemic subjects beyond LDL levels\textsuperscript{181}.

### 3.8 Drug Modulation of LOX-1 Expression

Several drugs commonly used in the treatment of type 2 diabetes, hypertension and hypercholesterolemia, appear to inhibit LOX-1 expression.\textsuperscript{168}

In hypertensive rats, and in cultured glomerular cells, not only antihypertensive drugs but also antioxidants suppressed LOX-1 overexpression.\textsuperscript{169} PPAR activators inhibited TNF-\alpha and PMA-induced LOX-1 mRNA overexpression in cultured ECs, but PPAR activators did not. In addition, thiazolidinedi-one pretreatment suppressed the renal LOX-1 expression induced by intraperitoneal TNF-\alpha in mice\textsuperscript{91}, thus, the anti-atherogenic effect of PPAR ligands appears to be at least partially mediated through LOX-1. Furthermore, in cultured ECs the PPAR
ligand pioglitazone inhibited LOX-1 expression and monocyte adhesion in a fashion similar to an antisense probe to LOX-1 mRNA.\textsuperscript{170} It is not excluded that the inhibitory effect of PPAR ligands on LOX-1 expression may be exerted through their antioxidant effect.\textsuperscript{152} In SMCs, LOX-1 expression induced by IL-1 was decreased by the PPAR activators as well.\textsuperscript{80} Treatment with losartan, an antagonist of Ang, attenuated aortic intimal proliferation in rabbits on a high-cholesterol diet and markedly decreased LOX-1 expression.\textsuperscript{171} Reinioside C, the main component of Polygala fallax Hemsl, which has putative antihyper-lipidemic properties, inhibited oxLDL-induced LOX-1 mRNA and protein expression.\textsuperscript{111} Mulberry leaf aqueous extracts had potential antioxidative effects, and inhibited LOX-1 mRNA and protein expression induced by TNF- and lipopolysaccharide, through inhibition of NF-\textsuperscript{B}.\textsuperscript{173}

Metformin, a hypoglycemic agent, at therapeutically relevant concentrations reduced the expression of both RAGE and LOX-1 in a dose-dependent manner, with an associated reduction in intracellular ROS.\textsuperscript{174}

In aortas of streptozotocin-treated rats, the dietary supplement taurine improved vascular endothelial dysfunction, an effect possibly associated with down regulation of LOX-1 expression via antioxidative activity.\textsuperscript{175}

A number of studies have shown that 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (statins) reduced total LDL cholesterol levels and exerted a cardioprotective effect.

Pretreatment of ECs with simvastatin or atorvas-tatin reduced the oxLDL-induced expression of LOX-1 as well as adhesion molecules. Both statins inhibited the activation of NF-\textsuperscript{B} induced by oxLDL\textsuperscript{142}; the effect of statins on LOX-1 expression is associated with an increase in PKB activity.\textsuperscript{176}

The same treatments reduced the oxLDL-induced downregulation of eNOS expression and activation of MAPK.\textsuperscript{144} The inhibitory effect on LOX-1 and subsequently MAPK activity provides a potential mechanism of beneficial effects of statins beyond the lower-ing of
cholesterol levels.

In the aorta of hypercholesterolemic rabbits, benidipine, a dihydropyridine-type calcium channel blocker, significantly prevented the up-regulation of VCAM-1 mRNA expression and tended to inhibit LOX-1 mRNA expression, while pravastatin significantly prevented the up-regulation of both VCAM-1 and LOX-1 mRNA expression. Moreover, aspirin and salicylate (but not indomethacin) reduced oxLDL-mediated LOX-1 expression in a dose- and time-dependent fashion. Co-treatment with aspirin and pravastatin synergistically reduced platelet LOX-1 expression, by favorably affecting ROS and NO’s release from activated platelets.

Taken together, these studies provide evidence that LOX-1 expression can be modulated by various pharmacological treatments and could thus represent an attractive target for therapeutic intervention to limit vascular injury and its long-term effects, this particular receptor does, but also what it does not do.

Indeed, clinical observations do not yet convincingly support the proposed central role of LOX-1 in experimental atherosclerosis and raise the question of whether findings made in animal models, particularly in KO mouse models, can easily be translated to human disease.

Truly, a limitation that applies to KO studies in general is worth considering. Since KO mice lack the targeted gene product not only in the tissue studied, but globally and throughout development, they have many chances to alter the expression of other functionally related genes. Thus, these studies may reveal more about the ability of mice to compensate developmentally for the absence of a specific protein than about the role of the protein in normal adult tissues. Until these issues are resolved, the potential utility of drugs that perturb LOX-1 activity to prevent vascular disease remains unclear.
3.9 Genetic Variants

In genetic linkage and association studies several cis- and trans-acting DNA variants have been identified that potentially influence expression levels of human genes. Different alleles located of the cis- and trans-acting variants may have various influences on gene expression profile (Figure 4).

Approximately 58% of mammalian genes are known to possess alternative promoters and transcription start sites. Majority of genes harbour at least two up to more than 20 alternative promoters. In a single gene, multiple promoter regions may contain different transcription start sites and are potentially related to tissue-specific gene expression. Genome-wide analyses of human microarray data have provided evidence that alternative promoter are positively associated with differential expression and disease susceptibility. This kind of high complexity of promoter regions in gene expression profiles makes these regulatory units challenging to study in the etiology of human disease.
Fig. 4. Effects of cis- and trans-acting DNA variants on different expression levels of genes. Polymorphic forms of regulators that act in cis (local) (A) or in trans (distal) (B) to the target gene may lead to the lower or higher expression levels of the gene. Modified from (Cheung and Spielman, 2009).

Human genome is estimated to consist of ~3 billion base pairs (bp) and code over 20,000–25,000 distinct protein coding genes. Comparison of two different human genomes has been shown to exhibit high similarity (99.9%) to one another differing only by 0.1% in DNA level. These differences are mostly represented by the natural genetic variations and are used as markers in dissection of the genetic basis of human disease. Genetic variants in the human genome can be divided into two different nucleotide composition classes: single nucleotide variants and structural variants (Table 1). First class includes variants where only a single base in DNA sequence (A, T, G, or C) is altered. Second class, structural variants, occur when one or more base pairs vary compared to other genomes resulting with changes in DNA length caused by the insertion, inversion, deletion or duplication events of DNA segment. Structural changes are generally composed of few bases up to 80 kb in length.
Table 3.

Examples of classes of human genetic variants modified from (Frazer, et al., 2009)

<table>
<thead>
<tr>
<th>Variation type</th>
<th>Example</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Single nucleotide</strong></td>
<td><strong>Example</strong></td>
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<tr>
<td>variants:</td>
<td><strong>Example</strong></td>
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<tr>
<td>Structural</td>
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<td>variants:</td>
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<td>Insertion-deletion</td>
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<td>variant</td>
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</tr>
<tr>
<td></td>
<td>ATTGGCCTTAACCCCCGATTATCAGGAT</td>
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<tr>
<td>Block substitution</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ATTGGCCTTAAC<strong>G</strong>TGATTATCAGGAT</td>
</tr>
<tr>
<td></td>
<td>ATTGGCCTTAACCCCCGATTATCAGGAT</td>
</tr>
<tr>
<td>Inversion variant</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ATTGGCCTTCCGGGGGTATTATCAGGAT</td>
</tr>
<tr>
<td></td>
<td>ATTGGCCTTACGGCCTTAACCCCCGATTATCAGGAT</td>
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<tr>
<td>Copy number variant</td>
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<tr>
<td></td>
<td>AGGAT</td>
</tr>
<tr>
<td></td>
<td>ATTGGCCTTA- - - ACCTCCGATTATCAGGAT</td>
</tr>
</tbody>
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CHAPTER FOUR

METHODOLOGY

4.1 MATERIAL AND METHODS

The population examined was composed of a Sub-Saharan African population (Eldoret Hospital located in Eldoret Municipality, Uasin Gishu County, Kenya) and a Caucasian population referring to the Atherothrombosi Centre at the University Teaching Hospital of Siena. In this case, participants were recruited by random selection from all those who presented themselves voluntarily in our facility.

4.2 Sampling Technique

The sub-Saharan sample consisted in 297 subjects with ages between 18 and above (115 men and 182 women) at the beginning of the study, but the number of complete recordings dropped to 54 at the end of the study due to difficult logistics. During the recruitment we gathered information from all the participants with regard to socio-demographic features, family history for atherosclerosis, Cardio-Vascular disease and other lifestyle factors such as; tobacco use, alcohol consumption, raised blood pressure, raised lipid levels, increased BMI, low fruit/vegetable intake, physical inactivity, and diabetes. All participants gave an informed consent orally before the study.
4.3 Analyzed variables

4.3.1 Clinical variables

4.3.1.1 Blood Pressure

To better assess the blood pressure we used standard desk mercury sphygmomanometers (Wanross®) and stethoscopes (Littman®). Blood Pressure was measured before breakfast, 30 min or more after the last tea intake or caffeine or cigarette smoked, first seated and then on feet. We made sure that the standard blood pressure cuff was of correct size (width of the bladder with cuff is approximately 40% of the right mid upper arm circumference, and bladder length encodes at least 80% of upper arm). A cuff that is too big will usually mask true high blood pressure, while a cuff that is too small may falsely elevate pressure during reading. Normal blood pressure was described as systolic blood pressure less than 140 mmHg and diastolic pressure less than 90 mmHg.

Three measures were taken at 5 minutes of the initial rest and subsequently at 2-minutes intervals, when an increased diastolic (DBP) or systolic blood pressure (SBP) was recorded (mean DBP >90 and/or mean SBP >140 mmHg).

4.3.1.2 Anthropometric Measurements

Anthropometrics data included age in years, height (m$^2$) and weight (kg) measurement which were used to assess the nutritional status of the participants. Body mass index (BMI) is a measure of body fat based on height and weight that applies to both adult men and women (except for pregnant women).

The body mass index was calculated dividing weight in kilograms (kg) by height in meters squared (m$^2$) as recommended by the World Health Organization.

$$BMI = \frac{Weight\ (Kg)}{Height\ (m^2)}$$
Using the BMI categories we classified the participants in:

- Underweight = <18.5
- Normal weight = 18.5-24.9
- Overweight = 25-29.9
- Obesity = BMI > 30

Class I = 30.0-34.9

Class II = 35.0-39.9

Class III = extreme obesity >40.0

"Morbid obesity" means that a person is either 50%-100% over normal weight, more than 100 pounds over normal weight, has a BMI of 40 or higher, or is sufficiently overweight to severely interfere with health or normal function.

4.3.1.3 Waist circumference

Measurements were taken from all the participants using a tape measure as waist circumference is strongly associated with abdominal fat; excess abdominal fat is an independent predictor of disease. The normal values were placed at < 88cm (35 inches) for women and < 102 (40 inches) for men. Waist circumference cutpoints lose their incremental predictive power with BMI of 35 kg/ m²

<table>
<thead>
<tr>
<th>Waist Circumference</th>
<th>Normal values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Men ≤ 102cm</td>
<td></td>
</tr>
<tr>
<td>Women ≤ 88cm</td>
<td></td>
</tr>
</tbody>
</table>

4.3.1.4 Measuring waist circumference

After giving instructions to breath out normally and not to “suck in” the stomach. We placed the tape measure evenly around the bare abdomen at the level of hip bone ensuring that the tape sung but did
not push tightly into the skin. We then read the tape measure and recorded the waist circumference in inches.

4.4 Biochemical variables

4.4.1 Cholesterol

Following the great difficulties encountered during our study, we were not able to perform blood tests or biochemical analysis to determine total cholesterol, HDL-C, LDL-C, and triglycerides, (TG) in the whole cohort of Sub-Saharan patients because such blood tests are rarely taken as they are costly and did not form part of the routine tests requested by medics. However when available the lipid profile was detected by standard nephelometric methods as in our laboratory (Siemens®, Marburg Germany).

4.4.2 Blood glucose

We were able to check blood glucose using the finger-prick stick test from all the subjects in our study after an overnight fasting (12 h or more). We prepared glucose meter, alcohol pads, sterile finger lancets and sterile test strips. Before carrying out a finger prick test we ensured that the finger was cleaned using cotton pad dipped in alcohol, we used a fresh strip for each participant, checked (and possibly reset) the monitor regularly. We then read and registered the data that appeared on the monitor. Though some participants were on drugs for diabetes, they had no laboratory blood sample records or even the finger-prick sample taken in the past, but we found out that the results from the finger-prick glucose samples confirmed the high blood glucose in those participants. We also dictated others to have high blood glucose of which they had no knowledge about. Those whose blood glucose was higher than 110 mg/dL and less than 126 mg/dL were considered to have impaired fasting glucose.
4.4.3 Saliva samples

Saliva samples were also collected in the morning using a tampon then put in a saliva collection tube. We neatly printed the name, date on the peel off label(s) and affixed to the saliva tube(s) after which, the material was sent to the Laboratory and maintained at 4°C until DNA extraction and final analysis.

4.5 Cardiovascular risk factors

a) Subjects with systolic blood pressure levels >140 mmHg or diastolic blood pressure levels >90 mmHg, or both, were considered hypertensive.

b) Individuals with blood sugar levels above 110 and less than 126 mg/dL and those who make use of medicines to lower glucose were considered persons with diabetics, we encouraged them to go for further check-up to determine their status.

c) Subjects with total cholesterol, LDL-c or elevated TG were to be considered dyslipidemic as well as those who used drugs to lower cholesterol but we have no data in regard.

d) We considered as over-weight those with body mass index (BMI) between 25-29.9 and obese those with body mass index >30. Those women with waist circumference > 88cm and men with waist circumference >102 were considered fat.

e) Tobacco use was assessed by clinical history and the those who smoked cigarettes, pipes or locally made tobacco were classified in smokers. We also classified as non-smokers those who have never smoked and ex-smokers those who have stopped smoking completely.

f) Participants who take alcohol including locally made brews were also classified.
g) Those who did not perform physical activities or those that performed physical activity less than three times a week or those who work or perform their duties seated were considered sedentary.

4.6 Genetic Study

Genomic DNA from saliva samples was extracted by the QIAamp Blood Kit (QIAGEN, Hilden, Germany). LOX-1 3’UTR and K167N polymorphisms detection was performed by allelic discrimination assays. Forward primer for 3’UTR was: 5’-GCCTGGCACCTTTATGTCAAC-3’; reverse primer was: 5’-CTTGGGACAAGCTAGGTAATAAATA-3’. 3’UTR/C allele MGB probe was: 5’-FAM-TTTTGATTCTAGCTAGCTACCTG-3’; 3’UTR/T allele was: 5’-VIC-ATTTTTGATTCTAGCTATCTG-3’. K167N forward primer was 5’-GCAACTTGCCATCCAAAGAC-3’; reverse primer was: 5’-CCTATTTTCCTCGGGCTCATT-3’. K167N/G allele MGB probe was 5’-FAM-TTCTCTGGGCTCTTTT-3’; K167N/C allele was: 5’-VIC-CTTCTTGGCTGTTTT-3’. PCR amplification was carried out in a reaction volume of 25 µl containing 900 nM of each primer, 200 nM of each probe, 12.5 µl of TaqMan universal Master Mix (Applied Biosistems) and 50 ng of genomic DNA. PCR was performed on a ABI prism 7000 sequence detection system (Applied Biosistems) using specific conditions. After enzyme activation for 10 min at 95°C, 40 two step cycles were performed; 15 S denaturation at 95°C followed by 1 min annealing/elongation at 60°C for both studied polymorphisms. The allelic specific fluorescence was measured on the ABI prism 7000 sequence system using the related software.

4.7 Statistical Analysis

Data for measurable variables were expressed as the mean value±standard error and analyzed by ANOVA and the Bonferroni/Dann
method. The $X^2$ test of independence was employed to assess that the allele frequencies conformed to Hardy-Weinberg equilibrium proportions and the differences in genotype and allele proportion between symptomatic and asymptomatic patients. Odds Ratios and 95% C.I. for vascular end-points were determined by multivariate logistic regression analysis while simultaneously adjusting for all variables (sex, age, lipid profile, blood pressure, smoking). A p value of $< 0.05$ was accepted as significant; calculations were performed using the SPSS library (SPSS Inc. Chicago, IL-USA). A formal test for interaction was employed to determine the putative interaction for each single variable including genetic profile and treatment. Starting from logistic regression analysis in which $Y$ was the events ratio; the variable $X_1$ and $X_2$ were the presence or not (1 or 0) of LOX-1 and NOS polymorphisms and $X_3$ the combination of both. The simplified formula for calculation was: 

$$Y=\beta_0+\beta_1X_1+\beta_2X_2+\beta_3X_3$$

whereas discrimination analysis by the Hosmer-Lemeshow method 

$$G^2_{HL}= \sum_{j=1}^{10} (O_j - E_j)^2/E_j \sim X^2_8$$

where $n_j$ = number of observations in the $j^{th}$ group, $O_j = \sum i y_{ij}$ = observed number of cases in the $j^{th}$ group, $E_j = \sum i p_{ij}$ = Expected number of cases in the $j^{th}$ group. The calculations were repeated with the combination of each above mentioned measurable variable. (SPSS 2003 module)

4.8 Results

Sub-Saharan population:

1) The main results of our study are that in only 18.1% of patients with an acute vascular event (coronary or cerebral) a complete atherothrombotic risk profile (including lipids, glucose, blood pressure and tobacco attitude) was available with respect to the 100% of the Caucasian population ($p< 0.0001$), whereas glucose profile plus tobacco attitude plus blood pressure was available in 31% of patients ($p< 0.01$).

2) The analysis of available data suggests that alterations in glucose metabolism and blood hypertension are the main determinants of
coronary acute vascular events (OR. 2.19, 95% C.I. 1.57-3.21, Hosmer-Lemeshow confirmed p=0.011 and 0.2.11, 95% C.I. 1.44-3.06, Hosmer-Lemeshow confirmed p=0.013 respectively) (Fig.5).

3) The 3’UTR/T polymorphism for LOX-1, that is expressed in a range between 21 and 46% in patients with acute cardiovascular events and less than 2 classical risk factors for atherothrombosis, O.R. 2.29, 95% C.I. 1.36-4.63, (p< 0.01 Hosmer-Lemeshow confirmed) 182 (Fig.6) was little expressed in the studied population (whole cohort and vascular event-experienced subjects) O.R. 0.93, 95% C.I. 0.66 -1.02 (Hosmer-Lemeshow confirmed p=0.079) (Fig.5).

![Fig. 5. Sub-Saharan Subjects. (n= 54) Multivariate Logistic regression analysis, resampling technique and Hosmer-Lemeshow confirmation for the association between acute atherothrombotic vascular events and evaluated risk factors. Accepted significant p < 0.05 was from Hosmer-Lemeshow confirmation.](image-url)
Fig. 6 Caucasian Subjects. (n= 114) Multivariate Logistic regression analysis, resampling technique and Hosmer-Lemeshow confirmation for the association between acute atherothrombotic vascular events and evaluated risk factors. Accepted significant p < 0.05 was from Hosmer-Lemeshow confirmation.

4.9 Discussion

In order to map genetic variations behind human diseases two main methodological approaches can be used: linkage and association studies. In linkage studies the responsible trait loci are assumed to cosegregate from parents to offspring with polymorphic markers at a specific chromosomal region. This is based on the assumption that two loci are physically closely linked and because of meiotic recombination a marker that is showing segregation with the trait must be nearby in the genome During the evolution cosegregation of two loci separated by longer distances might be broken up by recombination. Linkage mapping is a powerful tool to identify preferentially rare high-risk alleles contributing to the disease susceptibility. To measure the significance of linkage, the logarithm of the odds/odds score is used to
describe the recombination fraction between a genetic marker and disease locus in terms of likelihood ratio. This is based on the null hypothesis assuming no linkage between the marker and disease loci.

Association studies are based on a statistical correlation between a specific genetic variation and a trait variation among sample of individuals. Because of the effect of the locus variant on the trait variant, this approach enables to measure actual causal risk factor (Borecki and Suarez, 2001). Compared to the linkage analysis an association occurs in short physical distances in the genome. To detect a positive association a large number of common (polymorphic) genetic markers or a combination of markers (haplotype) are required where each contribute with the moderate effects to the disease susceptibility (Figure 7 B). The statistical evidence of association between an allele and a phenotype may arise from the potential variant leading directly to the disease phenotype and is correlated with or is in linkage disequilibrium (LD) with the nearby causal allele. LD is termed as the non-random association between the alleles of different loci. Usually association studies are based on analysis of unrelated affected (cases) and unrelated unaffected (control) individuals in the population. For example, if the prevalence of the allele is more frequent in the cases compared to controls it will have high probability of being associated with the diseases susceptibility. In genetic analysis both the linkage as well as the association studies may be used as complementary approaches to each other. To study complex genetic traits classically a candidate gene based association approach is used. This is based on testing the hypothesis that specific gene(s) are associated with the disease risk.
Fig. 7. A study of segregation of the mutation (M) (ancestral) and a polymorphic marker (A/T) with the disease from parents to offspring is called linkage analysis (A).

In this example a recessive mutation is segregating with the polymorphic marker allele.

A. Affected individual in pedigree is marked in gray. Red arrows refer to the recombination events during the segregation. In association analysis, the causal mutation along with genetic markers is segregating through multiple generations among randomly mating individuals within population (B). Different colors indicate to the chromosomal regions segregating during the generations. Modified from (Cardon and Bell, 2001).
GENERAL CONCLUSION and FUTURE PERSPECTIVES

Conclusions

The axis between LOX-1 and its ligands is an etiological factor that contributes to the atherosclerotic process. The experimental evidence gathered thus far demonstrates that ligand-LOX-1 interaction can alter cell phenotype in a pro-atherogenic sense, so that cells become dysfunctional and more prone to cellular damage and even death. Studies with transgenic and KO mouse models and LOX-1 association with the instability of plaques have in part confirmed the pathogenic role of LOX-1 in atherosclerosis. Therefore, although LOX-1 could represent a valid target for therapeutic intervention in cardiovascular disease and stroke, continued studies on transgenic and KO mice are needed to help us understand not only what this particular receptor does, but also what it does not do.

Indeed, clinical observations do not yet convincingly support the proposed central role of LOX-1 in experimental atherosclerosis and raise the question of whether findings made in animal models, particularly in KO mouse models, can easily be translated to human disease.

Truly, a limitation that applies to KO studies in general is worth considering. Since KO mice lack the targeted gene product not only in the tissue studied, but globally and throughout development, they have many chances to alter the expression of other functionally related genes. Thus, these studies may reveal more about the ability of mice to compensate developmentally for the absence of a specific protein than about the role of the protein in normal adult tissues. Until these issues are resolved, the potential utility of drugs that perturb LOX-1 activity to prevent vascular disease remains unclear.

The incidence, prevalence, and manifestations of atherosclerosis and coronary heart disease vary significantly with race, as does the response to therapy. There is little published information on formal programs addressing
awareness, treatment, and control in most African countries. In Western societies, such as the United States and the United Kingdom, the prevalence of hypertension and standardized mortality rates from stroke are higher for people of African origin than for whites. The same pattern is emerging in Sub-Saharan Africa.

The prevalence of hypertension, obesity, dysmetabolic syndrome, and lack of physical activity are much higher in blacks, whereas the prevalence of hypercholesterolemia is lower. Blacks with AMI present for treatment later than patients do on average and sometimes they do not present at all, are less often subjected to invasive strategies, and experience greater overall mortality. The prevalence and incidence of stroke in Sub-Saharan Africa have increased over the last half century, due principally to increased life expectancy and changes in environmental determinants and risk factors.
# APPENDIX 1: QUESTIONNAIRE

<table>
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<th>SERIAL NO.</th>
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<th>OTHERS</th>
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## 1. DEMOGRAPHIC DATA

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<th>Age (years)</th>
<th>20-30</th>
<th>31-40</th>
<th>41-50</th>
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<th>61-70</th>
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<table>
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<th>Weight (kg)</th>
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<th>Height (cm)</th>
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**Ranges (in cm)**

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<th>40-50</th>
<th>51-60</th>
<th>61-70</th>
<th>71-80</th>
<th>81-90</th>
<th>&gt;90</th>
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</table>
BMI

**Ranges**

- **Underweight** <19
- **Normal** 19-24
- **Overweight** 25-29
- **Obese** 30-34
- **Severe Obese** 35+

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### 2. LIFESTYLE

**Tobacco Smoking**

Documented [ ] Not Documented [ ]

If documented, smoking status:

- Never smoked
- Former smoker
- Current smoker

Number of cigarettes per day

Number of years smoked

**Physical Activity**

Inquired [ ] Not Inquired [ ]

If inquired, what type of exercise:

- Vigorous
- Moderate
- Little
- Never
### Diet

Assessment of diet  Documented  
Not Documented  

If documented, diet consumed:-

Fatty food.  
Carbohydrates.  
Proteins.  
Vitamins.  

Consumption of liquor:-

Beer.  
Wine.  
Spirits.  
Local brew.  

### 3. FAMILY HISTORY

Family history  Taken  

Degree of relative affected  
1st  
2nd  
3rd  

1. Hypercholesterolemia  
2. Diabetics  
3. High blood pressure  
4. Stroke  
5. Atherosclerosis  
6. HB levels
4. LIPID PROFILE

History of hypertension  Presence  Absence
Lipid profile  Done  Not done

If done, value of HDL. . . . . . . . . . . . . . . . .
value of LDL. . . . . . . . . . . . . . . . . .
value of IDL . . . . . . . . . . . . . . . . . .
total cholesterol. . . . . . . . . . .
Statins Use. . . . . . . . . . . . . . . . . . . . . .

5  DIABETICS PROFILE

Blood sugar levels. . . . . . . . . . . . . .
Urine sugar. . . . . . . . . . . . . . . . .

APPENDIX 2: INSTRUMENTS USED IN THE STUDY
REFERENCES


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Bai YP, Zhang GG, Shi RZ, Li YJ, Tan GS, Chen J: Inhibitory effect of reinioside C on LOX-1 expression induced by ox-LDL. Zhong Nan Da Xue Xue Bao Yi Xue Ban, 2006; 31: 659-662


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