# BIOCHEMICAL AND CLINICAL CHARACTERIZATIONOF URBAN BLACK AFRICANS WITH MYOCARDIAL INFARCTION

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A dissertation submitted to the Faculty of Medicine University of the Witwatersrand, Johannesburg, for the Degree of Master of Science.

## *ABSTRACT*

It is widely accepted that the occurrence of myocardial infarction (MI) among Black South Africans is much lower than that of South Africans of European or Asian ancestry. Over the past decade, this disease among Black Africans in urban areas appears to be increasing. The prevalence of MI among Black Africans admitted to Baragwanath Hospital (BGH) over a four year period (1984-1987) was determined by computer identification of all patients demonstrating elevated creatine kinase isoenzymes. Diagnosis of MI was confirmed by reviewing the hospital bedletters. The yearly ratio of hospital admissions for MI to total hospital admissions has increased since 1975 however, it was not possible to demonstrate population incidence due to a lack of demographic data. In conjunction with the hospital survey, selected biochemical risk determinants were measured in 25 black African outpatients with a confirmed diagnosis of MI. These values were compared to those of a normal control group. In an attempt to demonstrate differences in dietary habits between the two groups, the fatty acid content of platelet poor plasma (PPP) and whole washed red blood cells (RBCs) were measured. Total cholesterol (TC) was higher in the patient group (5.82 $\pm$ 0.85 mMol/L vs. 4.99 mMol/L $\pm$

0.77), as was low density lipoprotein cholesterol (LDL-C) (3.86mMol/L ± 0.99 vs. 2.92mMol/L ± 0.93) and apolipoprotein B (apo B) (1.13g/L ± 0.25 vs. 0.86g/L ± 0.24). No differences were seen in the levels of high density lipoprotein cholesterol (HDL-C) (1.09mMol/L ± 0.44 vs. 0.93mMol/L ± 0.35) or apolipoprotein A1 (apo A1) (1.27g/L ± 0.39 vs. 1.35g/L ± 0.55). In the patient group, platelet total saturated fatty acids were increased (45.87% ± 3.2 vs. 42.64% ± 2.9) and platelet total polyunsaturated fatty acids were decreased (34.52% ± 2.3 vs. 37.11% ± 2.8). Eicosapentaenoic acid (EPA) was slightly but significantly decreased in the platelets, PPP and RBCs of the patients. No differences were seen in the total saturated or total polyunsaturated fatty acids of PPP or RBCs indicating similar dietary habits. Serum fibrinogen was significantly higher in the patient group (439g/L ± 127.2 vs. 275 ± 120.4). Elevated levels of TC, LDL-C, apo B, fibrinogen and saturated fatty acids in platelets appear to be significant risk determinants in this group; as do decreased levels of EPA in platelets, plasma and red blood cells. Elevated levels of saturated fatty acids in platelets do not appear to be caused by differences in diet.

This is to certify that the thesis

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presented for the degree of Masters of Science of the University of the Witwatersrand is my own work, and has not been presented for a degree at any other University.

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# CHAPTER 1.

## 1. Aims of the Current Study

It has been well documented and highly publicized that the major cause of death among industrialized urban populations is coronary artery disease (CAD). In the United States, at the beginning of this century, diseases of the vascular system were the major cause of mortality and accounted for 14.2% of all deaths. By 1940, after death rates from most infectious diseases had fallen sharply, coronary artery disease accounted for 37% of all deaths, and by 1960, the incidence of ischemic heart disease had risen to 48% (Norris 1982). Since 1960 the incidence of vascular disease, specifically ischemic heart disease, seems to have leveled out and has in fact declined to some extent in certain populations. The reason for this apparent rapid rise and gradual decline of CAD has become the topic of much discussion. While some feel that it is the result of fluctuating trends in disease classification (Harper 1983), others argue that it is caused by changing trends in diet (Walker 1978). Still others propose that it is directly related to changing patterns of medical care and public awareness of causative factors (Kuller 1986).

In South Africa today, ischemic heart disease is the major cause of death among the Asian and Caucasian populations. Among the black population of South Africa, as among the black population of North America

the progression of CAD over the past 50 years seems to have followed quite a different pattern from that observed in other ethnic populations. Reasons for this are highly speculative.

Becker (1946) was the first to examine the extent and pathological consequence of atheroma in the Black African. Since that time a number of studies have been conducted on the epidemiology and biochemical specifics of coronary artery disease in this ethnic group and all researchers seem to agree on only two points: the incidence of coronary artery disease in the black population is much lower than that of the white population; and the incidence of coronary heart disease among urban blacks is increasing.

The intent of this study is twofold and will be presented in two parts.

Part 1:

To establish the current prevalence of myocardial infarction at Baragwanath Hospital, an attempt will be made to identify all individuals admitted for this disease over a four year period. Inpatient records will be retrieved in order to verify a diagnosis of MI. A clinical profile of these patients will be attempted, depending on the depth

of information available from the charts. The world wide incidence of the progression of coronary artery disease will be reviewed with specific emphasis on black populations. Discussion as to how various socioeconomic and ethnic factors may relate to the current levels of this disease in the Black African population will be offered.

Part 2:

In order to establish the impact of the major known risk factors on the black population, a biochemical profile will be determined in an urban Black African male group with a diagnosis of myocardial infarction. These values will be compared to a matched normal black control group. What is currently known concerning the biochemical mechanisms behind the various risk factors will be reviewed with emphasis on fatty acids and lipids.

# PART I.

## Clinical Characterization and Prevalence of Myocardial Infarction in Urban Black Africans. A Pilot Study.

# CHAPTER 2.

# 2. Clinical Characterization and Prevalence of Black Africans with Myocardial Infarction. A Pilot Study.

## 2.1 Coronary Artery Disease in Different Ethnic Populations. A Review of the Literature.

### 2.1.1 Introduction.

Following the Second World War, the apparent dramatic increase of death from coronary artery disease (CAD) in the USA began to attract the attention of medical health care workers. Beginning in 1948 and extending into the 1970's a multitude of long term prospective studies were initiated in an attempt to pinpoint any factors which might predispose to the occurrence of this disease. Each of these studies were designed and instigated by independent researchers and therefore various aspects and conclusions are not comparable. Certain common results however, have lead to our current understanding of risk factors. A related number of these studies will be briefly reviewed as they apply to the historical progression of the incidence of this disease in black and white populations.

### 2.1.2 Coronary Artery Disease in White and Asian Populations.

Beginning in 1949 a general population sample of 5209 white men and women aged 30 to 62 years were followed up

biannually in Framingham, Massachusetts to determine the relationship of personal attributes and living habits to development of CAD (Gordon and Kannel 1971). Follow-up continued for over 20 years and during that time approximately twenty different variables were measured at each examination. These ranged from serum cholesterol content to electrocardiogram. Due to the size, length of follow-up and quality of data collection, the Framingham Study has been used as a standard model when discussing the progression of and contributing factors for the many manifestations of coronary artery disease. Some of the findings of this study will be briefly reviewed.

One of the initial findings of the Framingham Study was a significant difference in occurrence of CAD between the sexes. The overall disease rate was lower in women than men, and men tended to display more overt manifestations of the disease. Myocardial infarction and sudden death were more common in men while the chief presenting complaint in women was anginal pain (Kannel and Feinleib 1972). The incidence of myocardial infarction increased with age in both sexes so that the risk of infarction in women at age 55-59 was about the same as in men aged 35-39.

Aside from sex and age, of all the variables studied, five were found to make independent contributions to the

progression of CAD. The single most important risk factor after age was hypertension. Cigarette smoking and serum cholesterol came next and displayed approximately equal effects. Impaired glucose tolerance and left ventricular hypertrophy as demonstrated by ECG were important but demonstrated lessor effects. It was furthermore shown that risk factors tend to be additive in their effect so that a coronary risk profile based on a risk factor score could be calculated for any individual. (Kannel 1976).

Another important finding of the Framingham Study was that in the first fourteen years of follow-up 23% of subjects were found to have had a myocardial infarction that went unrecognized by the patients and their doctors. Of these unrecognized infarctions almost half in both sexes produced no symptoms. The remainder were so atypical in symptomatology that neither the patient nor the attending physician had considered the possibility of myocardial infarction. This problem was re-examined after four additional years of follow-up study and once again the incidence was found to be 23%. Thus despite the more wide spread use of electrocardiography and an apparent increase of physician awareness of the problem, the incidence remained the same as it was in 1950 (Margolis et al., 1973, Kannel and Abbott 1984).

While the Framingham Study served as a model for

epidemiologists and has shown the way for accurate assessment of risk factors and the possibility of primary intervention, the findings can not accurately be applied to other, more diverse populations.

The first large scale multinational prospective study to determine varying incidences of CAD between different population groups was the Seven Countries Study (Keys 1980). The study followed sixteen cohorts of men in Finland, Greece, Italy, Japan, the Netherlands, the United States, and Yugoslavia for a ten year period and examined test subjects for the various risk factors which had been pinpointed by previous prospective studies. This study was also one of the first to include an analysis of the chemical composition of the various diets consumed by the populations under observation, and it was shown for the first time that the amount of saturated fat in the diet was perhaps the environmental factor most closely associated with CAD (Keys 1975).

The Keys study demonstrated that among the seven populations, Japan had the overall lowest incidence of CAD. In order to determine if some genetic influence was responsible for this Robertson et al., (1977) examined first and second generation Japanese populations living in Hawaii and California. It was found that the incidence of CAD in Japanese living in Hawaii was twice

as high as Japanese living in Japan and that the incidence increased another 50% in those who migrated to California. This increase correlated positively with increases in serum cholesterol, relative weight, serum triglycerides and systolic blood pressure. Also, the additive risk of smoking was insignificant in Japan but marked among the migrants to Hawaii. When statistical analysis was applied to the four common variables however, there was an over prediction for incidence of CAD in both the Hawaiian and Californian cohort. This suggested that there was still some unknown factor protecting against the occurrence of the disease in the Japanese. Furthermore, the Japanese in California continued to have an incidence of CAD that was four times lower than that of the White Californian population.

Since the initiation of the Framingham Study a multitude of scientific investigation has been undertaken in a search for missing risk factors. These investigations have examined literally every aspect of human physiology in an attempt to explain the world wide differences in the occurrence of CAD. The search has focused primarily on blood lipids and their interaction with other blood components and the vascular endothelium; diet and its effect on the blood lipids; plasma proteins involved in the coagulation process; and blood platelets and their relationship to all of the above. Harper (1983) noted

that since the Framingham Study at least thirty-seven variables have been positively correlated with atherosclerosis. It is important to note however, that correlation analysis implies only that these variables are associated with an increased risk of occurrence of the disease in a population. They have not been established as causative agents of the disease. With this final point in mind it seems that there are at least three points that can be drawn from the various early epidemiological studies. These are: 1) certain factors within a population cause certain members of that population to be at an elevated risk for CAD; 2) these factors vary between populations thus causing a variation in the incidence of CAD; 3) some populations have a greater incidence of CAD than would be expected based on known risk factors while others seem to be protected.

In the following sections, comparisons of the progression of CAD in Black and White American populations and a Black African population will be examined.

2.1.3. Coronary Artery Disease in Black Americans.

The earliest account of heart disease in Black Americans appeared almost 60 years ago in a paper published by Stone and Vanzant (1927). Their report focused on the incidence and etiology of heart disease in blacks and was based primarily on clinical observations and autopsy

findings of patients admitted to a hospital in Galviston, Texas. The conclusions of the study were that heart disease occurred to a much greater extent in the black population and resulted mostly from rheumatic fever and syphilis. It was further observed that hypertensive heart disease occurred more frequently in the black patient as it was noted that "hypertension is usually frequent in persons performing hard work." The authors also reported that most complaints of angina originated from the private (white) rooms while reports of this from the charity (black) wards were rare. These observations were confirmed by Schwab and Schulze (1931) who also alluded to the higher incidence of heart disease yet lower incidence of angina and atherosclerosis in black patients. He also commented on the fact that most blacks do not report to the charity wards until they are in dire need of hospitalization. He offered no explanation for this final observation, however. Finally, Weiss (1939) in an article published in the American Heart Journal explained the lack of angina in the black patient as follows: "We do not find angina pectoris in the Negro because he is not able to give an adequate description of the sensations which the attack produces. Every clinician knows how difficult it is to obtain a good history from a Southern Negro." Furthermore: "more than moronic intelligence is necessary to perceive the sensation of pain."

It seems reasonable to assume that the clinical attitudes of the time were swayed by reports such as these published in reputable scientific journals. Also, it is conceivable that when black patients presented for examination, coronary artery disease would be placed low on the list of diagnostic considerations. The result of this type of preconceived notion was that blacks were much less likely to be hospitalized to rule out myocardial infarction than were whites.

The first important community based comparative study designed to determine the distribution of CAD in a population of Southern American Blacks and Whites was conducted by McDonough et al., (1965) in the rural area of Evans County, Georgia. The Evans County Study determined the relationship between blood pressure, cigarette smoking and serum lipid levels on the incidence and prevalence of CAD. In this study prevalence estimates were determined by presence of angina, or ECG evidence of myocardial infarction. Overall, the rates for CAD were found to be approximately four times higher in white males than in black males and rates between white and black females were about the same. When blacks were compared to "lower class" rural whites no differences were found between either cholesterol or CAD prevalence. The number of whites in this category however, were considerably lower than numbers in the other cohorts. Neither dietary fat intake, cholesterol level, cigarette

smoking nor physical activity could totally explain the differences in CAD prevalence between blacks and whites or high and low social class, indicating the contribution of some other factor(s).

Another longitudinal population study of CAD in blacks, the Charleston, South Carolina Heart Study (Keil et al., 1985) differed from the Evans County Study by the inclusion of a predominately urban cohort. Criteria for CAD were similar in both studies as were the statistical models for determining prevalence and incidence rates. The study populations included a random sampling of blacks and whites above the age of 35 years. As in the Evans County Study, at entry, black and white women had similar prevalence rates. Among the men prevalence estimates were slightly more than twice as high in whites. At the follow-up period fifteen years later, black women and white men had experienced the highest incidence of CAD; 161/1,000 and 188/1,000 respectively. In black men the incidence was 132/1,000.

These two studies demonstrated higher incidence rates for CAD among white men compared to black men and similar rates among black and white women. These findings are surprising given that hypertension is both more prevalent and more severe in adult blacks (Gillium 1982) and obesity is pandemic in black females (McDonough et al.,

1965). This low incidence in black males may be explained by the finding that they have higher high density lipoprotein cholesterol and higher apolipoprotein A-1, along with decreased levels of serum triglycerides and low density lipoprotein cholesterol (Tyroler et al., 1980a) Black females on the other hand, show smaller black-white differences in these levels (Tyroler et al., 1980b). More will be said on the protective and atherogenic properties of lipoproteins and apolipoproteins in the following sections.

2.1.4. Coronary Artery Disease In Black South Africans. The first investigation concerned with heart disease in Black South Africans was conducted by Becker (1946) who performed post mortem studies on 3000 subjects. Becker determined that while atheroma was relatively common in blacks, it was the direct cause of death in only 0.4 percent. He further indicated that the incidence of atheroma up to the age of 45 years in blacks was not far removed from that found in other races.

Soon after Becker's initial study some conflicting reports concerning the extent of atheroma in blacks appeared in the literature. Higginson and Pepler (1954) performed a number of post mortem studies on both black and white patients and reported almost a total lack of atheroma at any age in the aorta and coronary arteries of

the blacks. Conversely, a few years later Laurie and Woods (1958), who performed post mortem examinations on only black subjects from the poor rural areas of Natal, found atherosclerosis and cerebral vascular disease to be common. Furthermore, they reported that black women demonstrated as high an incidence of atherosclerosis and its complications as that commonly found in women of European descent. In response to Laurie's findings Wainwright and Sheff (1961) conducted an independent study in Durban to determine the degree of atheroma present in the three major racial groups found in that area, namely: Africans; Europeans; and Indians. They reported that Europeans had the most extensive amount of atherosclerotic disease while Africans had the least and Indians were intermediate. Age groups were from 40 to 70 years. Wainwright commented that possibly Laurie's findings could have been tainted due to lack of comparative control since they rated tissue originating only from blacks.

The first clinical studies of CAD in African Blacks were conducted in the Cape Town area. Vogelpoel and Schrire (1955) reviewed 5000 electrocardiograms taken over a one year period at Groote Schuur Hospital, a multiracial population of Cape Town. Of the 5000 ECGs requested during this time period twice as many were requested for

whites as non-whites, although more than twice as many of the latter attended the hospital. The adjusted percentage of ECGs demonstrating myocardial infarction was 13.5% 6.8%, and 0.9% for whites, coloureds and blacks respectively. Out of the 5000 ECGs however, only 224 were performed on blacks. At this same time, Bronte-Stewart et al., (1955) conducted a clinical trial which determined the serum cholesterol levels of the three racial groups. They found high, medium and low levels in the white, coloured and black groups respectively and this correlated well with the amount of animal fat in the diet.

In order to observe the changing pattern of cardiac disease in Africans over the preceding fifteen years, Isaacson (1977) reviewed autopsy records from death at Baragwanath Hospital and compared these to the 1954 findings of Higginson and Pepler. The variability seen between the major causes of cardiac disease is considerable over the relatively short time period. The most variation occurs between idiopathic heart failure (33.0% to 17.0%) and myocardial infarction (0.9 % to 11.7%). Idopathic heart failure was (and remains) a poorly defined nutritional disease similar to Beri Beri which results in reversible congestive failure and generalized cardiomegaly. Isaacson commented on the fact that this low mortality from myocardial infarction is

surprising given the extent of obesity, hypertension, diabetes and smoking habit in the black population.

The first study to specifically examine myocardial infarction in blacks of the Johannesburg area was conducted by Seftel et al., (1963). They reviewed clinical records over a ten year period at Baragwanath Hospital (BGH) and uncovered thirty cases of myocardial infarction (27 men and 3 women). In fifteen of these patients, the diagnosis was made post mortem. A history was obtained on twenty-six of these patients, either from the patient or from close relatives, and it was found that most demonstrated one or more of the previously defined risk factors. An analysis of the post mortem data from the fifteen deceased patients showed that in only six out of the fifteen was atherosclerosis the primary cause of occlusion. In the other nine, the occlusion consisted largely of thrombus indicating that increased coagulability or decreased fibrinolytic activity was the primary cause. This differs considerably from patterns found in white populations where the critical lesion is most often caused by atherosclerosis. Several years later Seftel et al., (1970) repeated his original study at the Johannesburg non-European Hospital which caters to a more affluent class of blacks and determined that the incidence of myocardial infarction was approximately twenty times that found at BGH. Seftel et al., suggested that some reasons

for this increase in incidence could be: an increased awareness of CAD among the black community; new diagnostic procedures that were not available during the previous study, and finally, admissions to BGH were not selected whereas those at the non-European Hospital were biased in favor of teaching and research programs.

The most recent study to examine the occurrence and clinical attributes of Black Africans with myocardial infarction at BGH was conducted by diBisceglie et al. (1982). This was a retrospective study in which numbers of individuals admitted to the BGH intensive care unit (ICU) over a six year period were recorded. Clinical information on these patients was acquired from ICU notes and patient bed letters. It was shown that the numbers of Black Africans admitted to ICU for myocardial infarction steadily increased over this six year period. All patients with available laboratory results (50%) had raised serum cardiac enzyme levels. Hypertension was described in 50% of the patients, diabetes mellitus in 11.1% and other forms of vascular disease (peripheral vascular disease and cerebro-vascular disease) in 14.8%. The ratio of males to females was 3:1. An interesting observation in this study was that most of the patients were admitted to ICU two or more days after the onset of symptoms, while in other centers, patients are admitted within a few hours of the onset of pain.

## 2.2. Materials and Methods.

### 2.2.1. Identification of Patients with Elevated Creatine Kinase Isoenzymes.

The Baragwanath Laboratory computer history files were accessed. These were reviewed, and the names and hospital numbers of patients who demonstrated a serum creatine kinase greater than 195 U/L and/or a serum creatine kinase MB greater than 12 percent of total were extracted. Computer data was only available from 1984 onwards. There were, for the years 1984 through 1987, 582 such cases. The inpatient charts for these cases were retrieved manually and reviewed for information which was pertinent to this study.

### 2.2.2. Inclusion Criteria.

In this study, the diagnostic criteria for determining acute myocardial infarction (AMI) was that established by the World Health Organization (1959), and is now referred to as the "two out of three criteria." AMI is assumed if two or more of the following are present: chest pain; elevated cardiac enzymes in circulating plasma; or new Q waves or S-T segment abnormalities on the electrocardiogram. Of the three, the most sensitive and specific diagnostic marker for AMI is an elevation of total creatine kinase, and the creatine kinase isoenzyme MB (CK/CKMB) (Pierce 1986) however, an increased CK/CKMB

may be found in a number of clinical settings not associated with cardiac muscle damage (Chan et al., 1986). These are presence of muscular or skeletal trauma, neuromuscular disease, hypothyroidism, myocarditis or malignancies. A most important consideration in the interpretation of enzyme elevations are the peak intervals following myocardial infarction. CK/CKMB levels peak an average of 18 hours after the onset of AMI and decline rapidly, usually within two to four days after infarction. Thus serial measurement of total CK and of percent CKMB every twelve hours for 46 hours is necessary to ensure accurate assessment of results. Sustained increases without the characteristic temporal pattern are unlikely to be due to AMI and are more likely the result of on going muscle damage. Normal CK/CKMB values in the presence of AMI may also occur although these are most often related to errors in sampling time rather than lack of analytical accuracy (Pierce, 1986; Chan et al., 1986).

From the records reviewed, the patient was deemed to have had an AMI if either upon admission, or sometime during the hospital stay, he or she experienced either chest pain or the presence of S-T segment deviation and/or Q wave abnormalities on the electrocardiogram (Rude 1983), accompanied by an elevated CK/CKMB (Pierce 1986). No inpatient records were available on

approximately ten percent (61) of patients with elevated CK/CKMB. Of these, if a discharge diagnosis of AMI was listed on the computer printout and serial enzymes demonstrated the usual temporal pattern of myocardial infarction (Pierce 1986) they were included in the study. There were eight patients in this category.

2.2.3. Hospital Records Review.

Of the 582 records reviewed, 120 fit the criteria for inclusion. The only information available on all 120 records was sex and age. Of these 120, 85 contained a patient history giving information concerning seven common risk factors. These were history of diabetes, hypertension, peripheral or cerebral vascular disease, gout, obesity, tobacco use and alcohol use. Very few of the records offered information on dietary habits, occupational history or family history of cardiac disease. Most of the records indicated that laboratory tests (blood glucose, uric acid, lipogram, etc.) had been requested however, very few of the records contained the results of these tests. Of the 462 records not included in the study, 55 had to be rejected due to illegible handwriting, missing pages or incomplete history.

## 2.3. Results.

### 2.3.1. Occurrence Of Myocardial Infarction at Baragwanath Hospital From 1984 to 1987.

Figure 2.1 illustrates the occurrence of myocardial infarction at Baragwanath Hospital in Soweto over a four year period. The only demographic data available was a monthly breakdown of total hospital admissions. These admissions included maternity, pediatrics and St. Johns Eye Hospital. Values are reported as a percentage of the total hospital admissions, adjusting possibly for fluctuations in population density. Actual numbers of patients are given for each year.

### 2.3.2. Characteristics of Patients Presenting with a Diagnosis of Myocardial Infarction at Baragwanath Hospital over a Four Year Period.

For the years 1984, 1985, 1986, and 1987 one hundred and twenty patients presented with myocardial infarction at Baragwanath Hospital, and their bed letters were reviewed. As can be seen in figure 2.2 the ages were normally distributed with the majority of patients in both groups occurring in the 50-59 year age group. The mean age (±S.D.) for males was 54 ± 1.4 years and for females was 55 ± 1.4 years, and these were not statistically different. Of these subjects,

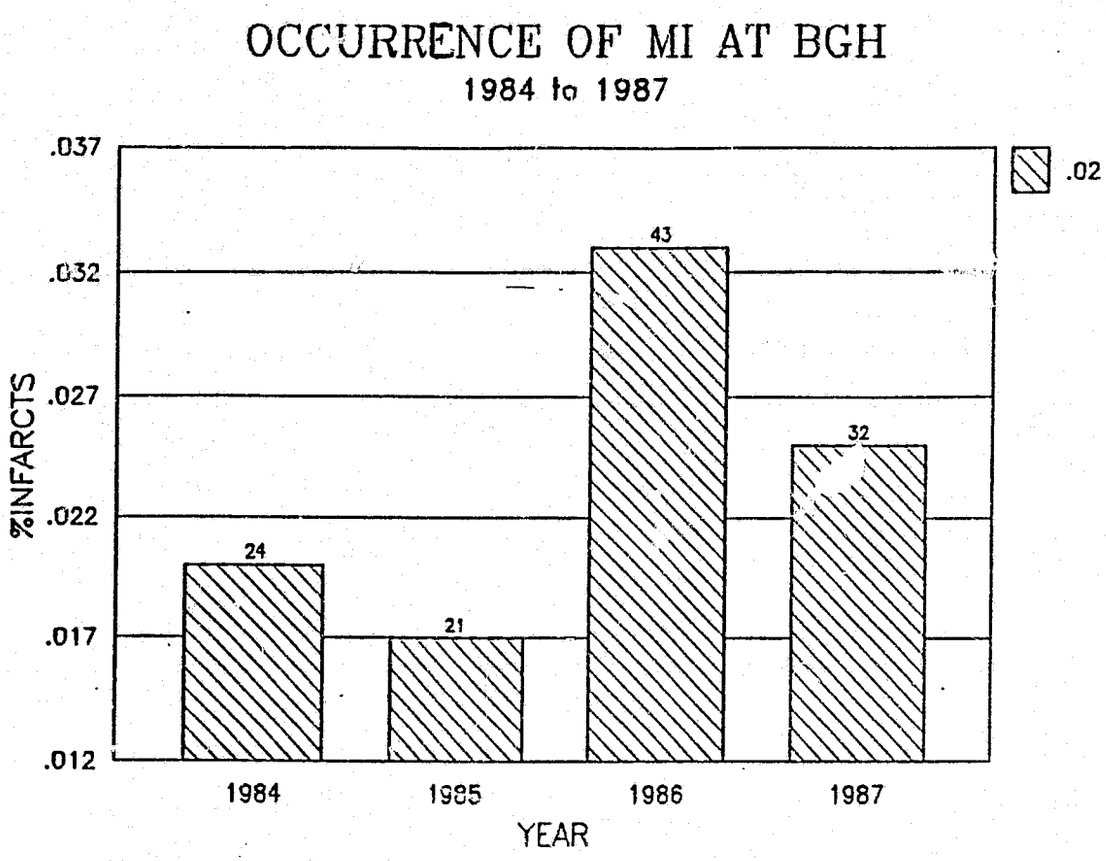



Figure 2.1. Four year occurrence of Myocardial Infarction at Baragwanath Hospital. Levels are given as percent infarctions in total hospital admissions.

eighty-one were male and thirty-nine were female, giving a ratio of two to one.

In eighty-five of the bed letters history was available on seven common risk factors. These are given in table 2.1. A history of diabetes was reported in 18% of the males and 28% of the females, while hypertension was present in 34% of the males and 46% of the females. The possible existence of gout was mentioned in only eleven

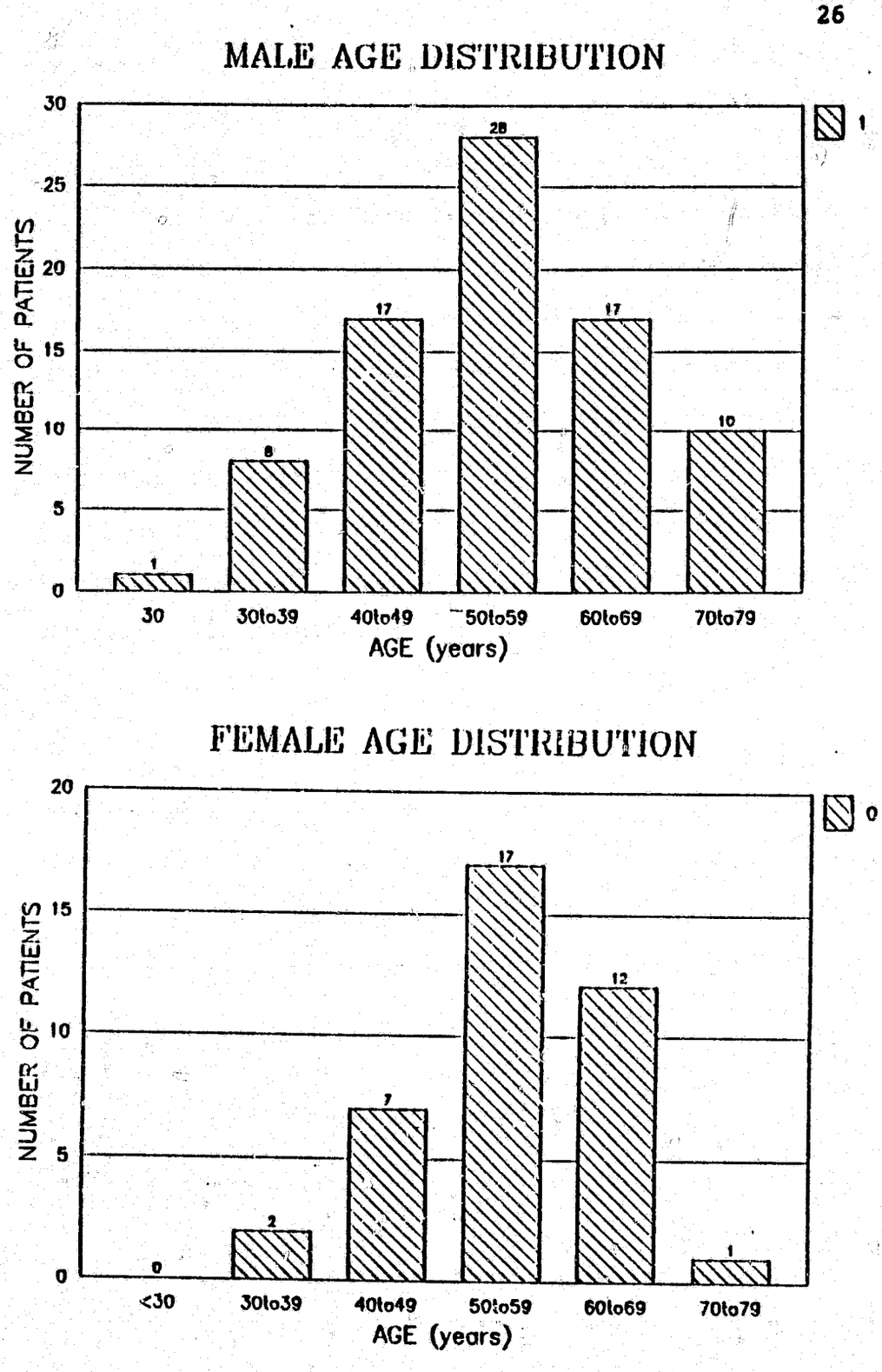



**Figure 2.2. Age distribution of Male and Female Black Africans with History of Myocardial Infarction.**

bed letters (3 female, 8 male) and in these, uric acid levels were ordered. In those bed letters mentioning gout; however, none contained the resulting uric acid values and none made further mention of the condition. The patient was considered to have a history of cerebral vascular disease (CVD) or peripheral vascular disease (PVD) if he or she presented with a neurological deficit, hemiparesis, intermittent claudication or decreased peripheral pulses. History of CVD or PVD were found in 15% of the males and 28% of the females. The degree of obesity, tobacco use, and alcohol consumption were most often indicated in the chart by a series of

Table 2.1 Risk Factor Inventory of Patients with Myocardial Infarction Presenting at Baragwanath Hospital Over a Four Year Period.

| | MALE | FEMALE |
|---|---|---|
| Number Of Subjects | 62 | 23 |
| History of Diabetes | 18% | 28% |
| History of Hypertension | 34% | 46% |
| History of PVD or CVD | 15% | 28% |
| History of Gout | 0% | 0% |
| Obesity | 10% | 55% |
| Tobacco Use | 82% | 55% |
| Alcohol Use | 53% | 5% |
| None Of The Above | 10% | 0% |

"pluses." The patients were included in these categories if they rated a "one-plus" or greater. Obesity was a condition in 10% of the males and 55% of the females. Tobacco was used by 82% of the males and 55% of the females. Alcohol was consumed by 53% of the males and only 5% of the females. No risk factors were found in 10% of the males and all of the females had one or more of the risk factors.

## 2.4. Discussion

The bed letter review of patients presenting at BGH with myocardial infarction over a four year period was a dual purpose pilot study. The first objective was to demonstrate the incidence of MI in an urban black population, and to determine the degree to which this disease has progressed in the population over a given time period. The second objective was to describe the clinical characteristics of this disease in the black population based on information found in patient bed letters.

In the bed letter review it was found that the ratio of males to females was similar to that seen in white populations. The mean age between the two groups however, was identical and in white populations, females tend to demonstrate the disease at a much older age (Gordon and Kannel 1971). In white populations, certain disease states and life styles have been shown to predispose to the occurrence of MI. The finding that black females may experience a MI at an age similar to their male counterparts is most probably related to a disparity in the occurrence of these disease states and life styles between the two groups. It was found in the bed letter review that black females had higher levels of diabetes, hypertension and other forms of vascular disease, and this was most probably related to the

finding that five times more of the females were considered to be obese. It is not known to what extent these findings are representative of the population at large since ten percent of the records could not be located and ten percent of those which were located, had to be rejected due to poor quality.

It was not possible, based on the findings of this study to determine actual disease incidence in the population of Soweto. The first reason for this is the inability to identify the population at risk due to a lack of appropriate demographic data. The second reason is that the numbers detected in this study must represent a severe underestimation of the disease occurrence. Reasons for his are that individuals with MI and normal CK/CKMB, and individuals with MI whose records were lost or inadequately maintained would not be detected.

In order to give some idea of the progression of MI at BGH over a meaningful time period, in figure 2.3 the findings of the current study are compared to the findings of diBisceglie et al., (1982). In this earlier study, numbers of individuals with MI admitted to BGH intensive care unit over a six year period were recorded. No other recent study of MI occurrence at BGH has been conducted. It appears from figure 2.3 that the occurrence of MI since 1975 has indeed increased,

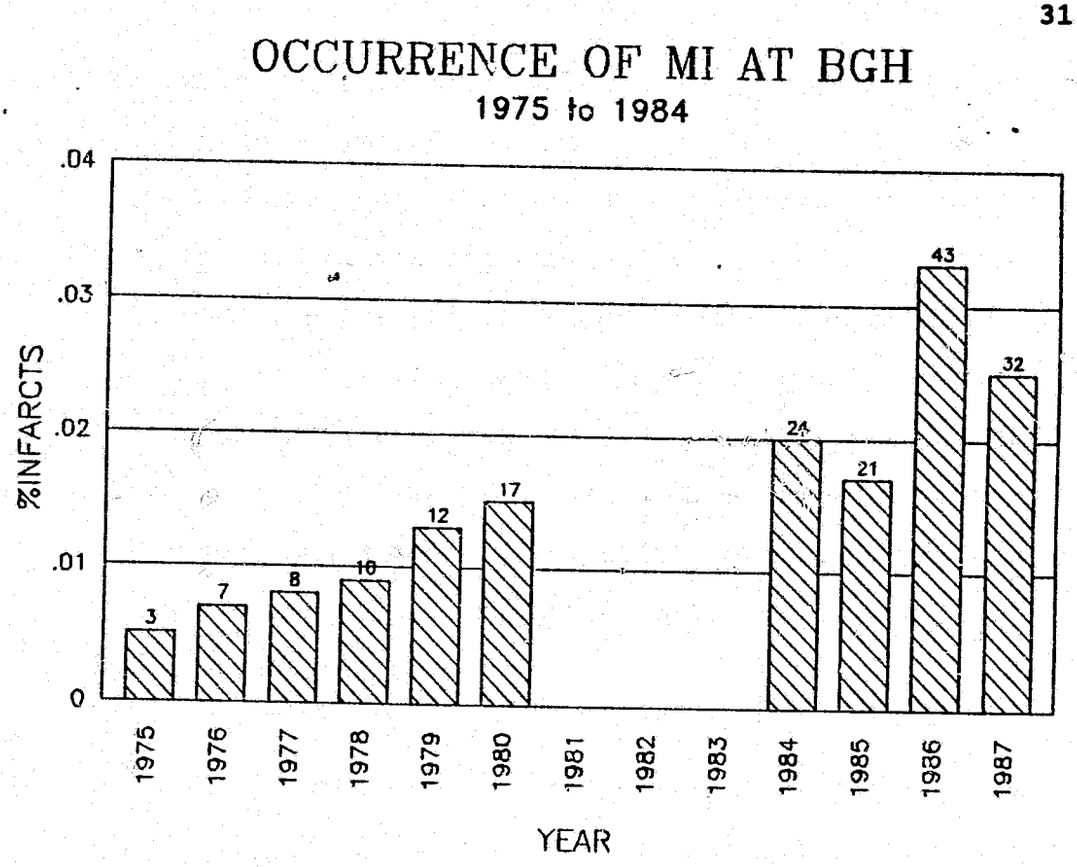



**Figure 2.3. Thirteen Year Occurrance of Myocardial Infarction At Baragwanath Hospital. Levels are given as percent Infarctions in Total Hospital Admissions. Data from 1976 to 1980 Reported by DiBisceglie et al., 1982.**

however these findings may be misleading in that the numbers have not been properly adjusted, and the methodology in detecting individuals with MI in the two studies is quite different.

Given the severe limitations of this study, it appears that the detected occurrence of MI at BGH is very much lower than that of the white hospitals in South Africa. Currently, about 40-50 cases of MI per year are diagnosed at Baragwanath. This hospital serves a black

community with an estimated population of 1-1.5 million, giving an estimated incidence of 5 per 100,000 per year. In the same general area, the incidence of MI in the White Johannesburg population is 1000 per 100,000 per year (Chetty et al., 1988). Differences in diagnostic capability and manpower resources between these institutions, and how this might effect detected levels will be discussed; as will ethnic differences in attitude toward seeking out modern medical care, and the possible effects of childhood mortality due to infectious disease.

Since 1970, the majority of publications dealing with CAD in Black Africans consist of repetition of earlier work (Loock and vanStaden 1983), informative editorials (Walker 1980) and surveys of government publications of vital statistics (Wyndham 1979). In the current study, it was demonstrated that the detected occurrence of MI in the Black South African population is about one-tenth that of the white population. Some feel that this low incidence can be explained almost entirely on the basis of exposure to currently defined risk factors (Seftel 1978), while others suggest the existence of some yet undefined racial factor that imparts a considerable degree of immunity (Walker 1980). While both of these arguments are well presented and convincing, there seem to be at least two aspects that have been ignored.

First, it is assumed that the incidence of CAD in the black population has been effectively demonstrated. Second, the enormous difference in economic availability between the two populations and the effect that this may have on the difference in disease patterns seems to have been overlooked.

One aspect that would tend to mask the true incidence of CAD is the level of medical care afforded this segment of South African society. The adequacy of this service comes under question when published statistics (Wyndham 1979) indicate that one of the leading causes of death in the black population is categorized as "ill defined disease," and that there are thirteen times more blacks in this division than whites. Under the broad classification "disease of the circulatory system," whites had twice the mortality of blacks however, within the subclassification "other forms of heart disease" blacks out numbered whites by six to one. This subclassification contains a number of unrelated heart diseases and also two categories where a definite diagnosis was not made. This black to white disproportion in regards to loosely defined "catch all" categories reflects more a disparity in medical and diagnostic services rather than some biochemical or genetic influence.

A further indication that medical services for the black population are less than adequate is offered by a recent letter published in the South African Medical Journal and signed by seventy medical doctors employed at Baragwanath Hospital. In describing the conditions at Baragwanath they write: "The state of affairs is inhumane. Facilities are completely inadequate. Many patients have no beds and sleep on the floor at night and sit on chairs during the day. The overcrowding is horrendous. Nurses are allocated according to the number of beds and not the number of patients. Ablution facilities are far short of accepted health requirements and ethical standards are undoubtedly compromised." (Abkiewicz, et al., 1987). Conditions such as these must have a considerable bearing on any hospitals' ability to effectively interpret and record the existing medical specifics of the community to which it serves.

One other aspect that bears consideration is the willingness of the black community to present their medical problems to established medical care facilities and how this might effect the level of undetected CAD. Schwab noted in his 1931 clinical study of CAD in Black Americans that black patients did not tend to report to the Texas charity wards until they were in dire need of hospitalization. That the condition of these wards during the depression years in America were far worse

than those currently found at Baragwanath Hospital is quite likely. In South Africa however, the Black African culture offers an alternative to modern medical care that probably was not available to the American Black. In the black urban townships and rural villages a large portion of the population still take their medical problems to local indigenous healers (Buhrmenn 1985). According to one text: "In most rural and many urban areas, traditional herbalists and witch doctors still have considerable influence over their fellow tribesmen. Ill health or the need to obtain suitable medicines to bring fortune or ward off evil leads significant numbers of conservative blacks to visit tribal healers long before seeking help at a hospital or clinic" (Campbell et al., 1982).

In the Framingham Study, twenty-five percent of myocardial infarctions which occurred in the population were not detected in a clinical setting (Kannel and Abbott 1984). This was demonstrated in a society that has perhaps the highest per capita availability of medical care in the world. This availability is further augmented by frequent media campaigns which cajole all individuals over the age of thirty-five to have annual medical examinations. In South Africa, considering the influence of the indigenous healer coupled with ignorance of symptomatology and fear of modern medical services,

the level of undetected CAD in the Black African population could be much higher than that reported for other populations.

As noted previously, the detected occurrence of MI in the white population of Johannesburg is very much greater than that observed for the black population. While availability of medical services and ethnic differences in seeking out modern medical care surely contribute to this difference in recorded disease incidence, other explanations are required in order to account for such an enormous disparity.

The aspect which may have the greatest bearing on the occurrence of CAD in any population is that which may most profoundly effect the constituency of the population at risk. Recent data published by the South African Medical Research Council (1984) indicate that out of all deaths in the black population, approximately 45% occur under the age of 18 years. This is compared to a figure of 12% in the white population. Black infant mortality rate is 94 to 124 per 1000 with 12.3 per 1000 reported for whites. These differences are almost entirely accounted for by differences in parasitic and infectious diseases. Given this enormous level of early life mortality in the black population, it is not difficult to imagine why the occurrence of a later life degenerative

disease such as CAD is considerably less than that of the white population.

It has been suggested that current childhood mortality rates in blacks residing in highly urbanized areas such as Soweto are similar to those of the white population residing in Johannesburg. Current levels however, are not of concern in this context. It is of greater relevance to consider what the levels were 30, 40 or 50 years ago and how this has effected the population which now constitutes the age group at risk for the disease.

Another variable in this complicated equation is the question of the effect on the surviving population from exposure to infectious diseases. Are blacks which survive to middle age generally a healthier group of individuals? Are the ones which die at a young age the same ones who would otherwise succumb to CAD at an older age? Is exposure to a certain pathogen at a young age resulting in immunity to CAD at an older age? There certainly seems to be a strong worldwide inverse association between the incidence of CAD and levels of poverty, malnutrition, overcrowding and inadequate housing. Also, in white populations it is known that the rapid rise in mortality from CAD closely paralleled the sharp decline in mortality from infectious diseases. How these factors may influence the actual incidence of CAD

is complex however, they cannot be ignored when discussing differences in the disease pattern between first and third world populations.

# PART II.

## Biochemical Characterization of Black Africans with Myocardial Infarction.

# CHAPTER 3

# 3. Fatty Acids and Coronary Artery Disease. A Review of the Literature.

## 3.1. Introduction

Since the early years of this century, it has been known that fatty acids are an important source of oxidative energy. It was another thirty years before Burr and Burr (1929) established the essential physiological need for certain long chain polyunsaturated fatty acids in the diet. During the early part of the 1950s developments in gas liquid chromatography made rapid, accurate fatty acid analysis possible on small quantities of material and soon after, the various structures of the long chain fatty acids were established. Later, Bergstrom et al., (1964) isolated and described the structures of the classical prostaglandins, and the relationship between these substances and fatty acids became apparent. Since the 1950s an enormous amount of research has been conducted on the pharmacology, biochemistry, and physical chemistry of fatty acids and their cyclic derivatives. This chapter will attempt to give a brief description of the physical properties and biochemistry of fatty acids, followed by the physiological effects of fatty acids and their precursors as they relate to CAD.

## 3.2. Nomenclature Of Fatty Acids

As with any rapidly changing field, fatty acid

terminology has developed in a random and haphazard fashion. It is not uncommon to see the same fatty acid referred to by three or four different systems of nomenclature in the same journal article. There are three accepted systems of fatty acid identification: the trivial system; the systematic system; and the structural system (Gurr and James 1976). In this thesis, as in the literature, all three systems will be used, consequently, an orientation to all three systems will be offered.

Trivial names for the fatty acids were most often taken from the tissues from which they were first isolated. An example of these are the polyunsaturated eighteen carbon fatty acids which all begin with the prefix lino-, since they occur in large amounts in linseed oil. This system offers nothing in the way of structural information and confusion between similar named species is easy. The systematic method is based on numerical prefixes indicating the number of carbon atoms followed by the number (if any) of the double bonds. At the beginning of the name are the numerical positions of the double bonds relative to their distance from the carboxyl group. With this system oleic acid becomes 9-octadecenoic, indicating an eighteen carbon molecule with one double bond located between carbons nine and ten counting from the carbonyl carbon.

Although this system leaves no doubt as to structure, names tend to be long and cumbersome. Also, naming from the carboxyl group makes the recognition of related compounds with different chain lengths and degree of unsaturation difficult. The abbreviated, or structural system identifies the fatty acid simply by giving the number of carbon atoms followed by the number of double bonds. Position of the double bonds are indicated by the carbon number of the first double bond counting from the methyl terminal (or omega) carbon. Thus, cis-5,8,11,14,17-eicosapentaenoic acid becomes C-20:5 w-3 (Davidson and Cantrill 1985). Figure 3.1 gives the common, systematic, and abbreviated names of the more frequently encountered fatty acids.

## 3.3. Physical Properties Of Fatty Acids.

Fatty acids consist of carbon, hydrogen, and oxygen atoms. They exist mostly as straight aliphatic chains. Most naturally occurring fatty acids are even numbered due to the mode of the acetyl CoA/malonyl-CoA synthase system but odd numbered species occur in small amounts. Many of these fatty acids are polyenoic containing double bonds which are almost exclusively in the cis configuration. The double bonds always occur consecutively and are separated by single methyl groups, an arrangement that has been termed polyallylic. This arrangement is also the result of the specific mechanism of de novo synthesis (Gurr and James 1976).

| COMMON | SYSTEMATIC | ABBREVIATED |
|---|---|---|
| | **Saturated Acids** | |
| Lauric | Dodecanoic | 12:0 |
| Myristic | Tetradecanoic | 14:0 |
| Palmitic | Hexadecanoic | 16:0 |
| Stearic | Octadecanoic | 18:0 |
| Arachidic | Eicosanoic | 20:0 |
| Behenic | Docosanoic | 22:0 |
| Lignoceric | Tetracosanoic | 24:0 |
| | **Monoenoic Acids** | |
| Palmitoleic | cis-9-Hexadecenoic | 16:1 w-7 |
| Palmitvaccenic | cis-11-Hexadecenoic | 16:1 w-5 |
| Oleic | cis-9-Octadecenoic | 18:1 w-9 |
| Vaccenic | cis-11-Octadecenoic | 18-1 w-7 |
| Gadoleic | cis-11-Eicosenoic | 20:1 w-9 |
| Erucic | cis-13-Docosenoic | 22:1 w-9 |
| Cetoleic | cis-11-Docosenoic | 22:1 w-11 |
| | **Dienoic Acids** | |
| Linoleic | cis-9-12-Octadecadienoic | 18:2 w-6 |
| No Common Name | cis-11-14-Ecosadienoic | 20:2 w-6 |
| | **Trienoic Acids** | |
| alpha-Linolenic | cis-9-12-15-Octadecatrienoic | 18:3 w-3 |
| gamma-Linolenic | cis-6-9-12-Octadecatrienoic | 18:3 w-6 |
| dihomo-gamma-Linolenic | cis-8-11-14-Eicosatrienoic | 20:3 w-6 |
| | **Tetra-, Penta-, and Hexaenoic Acids** | |
| Arachidonic | cis-5-8-11-14-Eicosatetraenoic | 20:4 w-6 |
| Timnodonic | cis-5-8-11-14-17-Eicosapentaenoic | 20:5 w-3 |
| Clupanodonic | cis-4-7-10-13-16-19-Docosahexaenoic | 22:6 w-3 |

Figure 3.1. The Common, Systematic, and Abbreviated Names of Fatty Acids.

In 1972 Singer and Nicolsen proposed a unifying theory of membrane structure called the Fluid-Mosaic Model. They suggested that cell membranes were basically a polar lipid bilayer which was a non-rigid structure because the hydrophobic tales of polar lipids consist of an appropriate mixture of saturated and unsaturated fatty acids that are fluid at the functional temperature

of the cell. Proteins, part hydrophobic (immersed within the bilayer), part hydrophilic (exposed to the outer portions of the membrane), which include enzymes and various transport systems are suspended within the bilayer. Due to the absence of covalent bonding between these proteins and the lipid molecules, they are able to move laterally within the membrane structure. This movement of proteins is regulated partly by the fluidity of the membrane and partly by the presence of other proteins .

The physical properties of the individual fatty acids effect the physical properties of acyl lipids which make up the lipid bilayer. The most readily demonstrable effect being the phase transition, or the temperature at which the membrane changes from a liquid state to a rigid crystalline state (Stubbs and Smith 1984). The phase transition decreases with the degree of unsaturation due to kinks produced in the chain by cis double bonds which disrupt the close packing that is possible with the saturated chains. The presence of even a single double bond is sufficient to exert a profound influence on the physical properties of the acyl lipid. The position of the double bond system within the chain is also important. As can be seen from the melting points of the thirteen possible cis isomers of octadecadienoic acid, the nearer the double bond to the

center of the chain, the lower the melting point. The position of the double bond may be a more important influence on melting point in some cases than the actual number of double bonds. The melting point of 18:3 w-6 is not markedly different from that of 18:2 w-6 but is some 28 degrees C lower than that of 18:3 w-3 (Stubbs and Smith 1984). It can be understood then, that the fatty acid makeup of the lipid bilayer between warm blooded vertebrates, cold blooded vertebrates, and plants must be considerably different, given the different temperate environments in which these organisms exist.

3.4. Physiological Effects of Fatty Acids.

Apart from supplying the structural integrity of the lipid bilayer, fatty acids also impart a considerable pharmacological influence. The twenty carbon essential fatty acids, 20:3 w-6, 20:4 w-6, and 20:5 w-3 become oxygenated in the presence of various enzymes to form what have been termed the one-series, two-series, and three-series prostaglandins respectively. The prostaglandins are biologically active substances that are only produced in stimulated cells and tissues. They are not stored, but must be newly synthesized in response to stimulation. They have been implicated in the involvement of regulation of vascular tone, contraction and relaxation of muscle, stimulation and

inhibition of platelet function, activation of leukocytes, regulation of renal blood flow, and possible control of growth and spread of malignant cells (Needleman et al., 1986). The next several sections will deal with the formation and actions of the prostaglandins.

### 3.4.1. Endoperoxides

Early studies on the mechanism behind the transformation of polyunsaturated fatty acids (PUFA) into prostaglandins indicated that endoperoxide structures were involved. When prostaglandin E1 ($PGE_1$) was biosynthesized from 20:3 w-6 in an environment of $[^6O_2]/[^8O_2]$ and enzymes from ram seminal vesicules, it was found that the oxygens of the keto and hydroxyl groups at C-9 and C-11 of $PGE_1$ originated from either $[^6O_2]$ or $[^8O_2]$. This indicated the likelihood of formation of a cyclic peroxide intermediate (Samuelsson 1972). Later it was determined that arachidonic acid may be transformed into the prostaglandin endoperoxide substances ($PGG_2$ and $PGH_2$) by arachidonic acid induced platelet aggregates (Hamberg and Samuelsson 1974). This was determined by: aggregating platelets with labeled arachidonic acid; time dependent extraction of formed products into acidified methanol; and isolating the resulting methyl ester substances by TLC and identifying these substances

by mass spectrometry.

The formation of $PGH_2$ and $PGG_2$ were found to be facilitated by an endoperoxide synthase in a two step reaction. Arachidonic acid was first oxygenated to form $PGG_2$, and $PGG_2$ was reduced to form $PGH_2$. These cyclo-oxygenase and peroxidase activities reside in a single protein which has been termed prostaglandin endoperoxide synthase (PES) (Samuelsson et al., 1975).

Pagels et al., (1983), using antibodies prepared against PES determined that in ram seminal vesicle microsomes, PES is a membrane associated protein with a molecular weight of 72,000. He further determined that each subunit requires a molecule of heme for maximal catabolic activity. Non-steroidal anti-inflammatory drugs (aspirin, indomethacin, flurbiprofen, flufenamic acid, mefemamic acid, sulindac sulfide and clidanac) selectively inhibit the cyclo-oxygenase activity of this enzyme. The hydroperoxide activity however, is scarcely effected by any of these drugs (Mizuno et al., 1982). It appears that aspirin acetylates the cyclo-oxygenase portion of the enzyme, and this causes irreversible inhibition. Interestingly, it has been shown that treatment by aspirin or indomethacin, actually protects the hydroperoxidase activity of the enzyme from inactivation at higher temperatures or alkaline pH

(Mizuno et al., 1982).

The prostaglandin products of the endoperoxides, $PGE_1$ (from 20:3 w-6) and $PGE_2$ (from 20:4 w-6) differ radically in their actions. It was shown that $PGE_1$ is a very active inhibitor of platelet aggregation whereas $PGE_2$ stimulated ADP induced aggregation of rat, rabbit and human platelets (Samuelsson et al., 1975). When comparing the physiological effects of the active 2-series prostaglandins to those of the endoperoxides it was shown by Samuelsson (1975) that the two groups of substances had a similar ability to contract gastrointestinal smooth muscle. On the other hand, the ability of $PGG_2$ and $PGH_2$ to stimulate vascular and airway smooth muscle of rabbits and guinea pigs were considerably greater than those of $PGE_2$ or $PGF_2$-alpha. Intravenous administration of $PGG_2$ and $PGH_2$ to guinea pigs produced an increase in blood pressure which was greater than that found for $PGE_2$ or $PGF_2$-alpha.

### 3.4.2. Thromboxanes

When indomethacin treated washed human platelets were incubated with [1-$^{14}$C]-arachidonic acid for five minutes and the reaction terminated by addition of acidified methanol, chromatographic analysis of the resulting methylated structures revealed the formation

of the previously described substances thromboxane B2 ($TxB_2$), HHT, and HETE. Termination of the same reaction after thirty seconds revealed the formation of an additional product. Consequent mass spectrum analysis and oxygen labeling found the substance to be a highly unstable epimer of $TxB_2$. This instability was due to the presence of an oxetane ring which bridged carbons nine and eleven of the pententane ring. The oxetane ring of the unstable substance spontaneously hydrolyzes ($t_{1/2}$=30 sec) to the hemiacetal $TxB_2$. The unstable substance was termed thromboxane $A_2$ ($TxA_2$) and was postulated to be the same as the rabbit aorta contraction substance (RCS) which had been described by Piper and Vane (1969) several years earlier (Hamberg et al., 1975). $TxA_2$ not only contracts smooth muscle but also induces platelet aggregation and serotonin release at nanomolar concentrations. $TxB_2$ is virtually inactive. In the platelet, $TxA_2$ is the principal metabolite of $PGH_2$ (Needleman et al., 1986). Recently, $TxA_2$ has been synthesized, and the original structure proposed by Hamberg et al., (1975) was proved correct (Bhagwat et al., 1985).

The exact mechanism by which $TxA_2$ induces platelet aggregation and vascular contraction is not known however, it appears to be involved in a host of biological reactions which result in both normal

maintenance of vascular hemostasis, and cardiovascular disease (Fitzgerald et al., 1987). Evidence suggests that some cases of myocardial ischemia which occur while at rest result from constriction of the larger coronary arteries (Ogletree 1987). An acute reduction in flow resulting from coronary constriction could also conceivably trigger myocardial infarction or cardiac arrhythmias (Coker et al., 1981). Several studies have in fact shown that $TxA_2$ does increase significantly during both animal induced and human acute myocardial infarction (Smith et al., 1982; Coker et al., 1981; Brezinski et al., 1985).

### 3.4.3. Prostacyclin

Following the discovery of the thromboxane pathway investigations were conducted to determine if this substance was produced in tissues other than platelets. Moncada et al., (1976) described a labile substance found in microsomal fractions of blood vessels which was enzymatically metabolized from the endoperoxide $PGH_2$. Rather than displaying activity similar to a thromboxane however, this substance relaxed strips of rabbit mesenteric and coeliac arteries and inhibited platelet aggregation to arachidonic acid. This substance was later termed prostacyclin ($PGI_2$) and was determined to be an enol-ether which was the primary metabolite of arachidonic acid in vascular endothelial cells (Johnson

et al., 1976). It had an in vitro half-life of ten minutes at pH 7.4 and 23 degrees C, an in vivo half-life of three minutes, and spontaneously hydrolyzed to 6-keto-$PGF_1$-alpha (Moncada and Vane 1978). In later work, $PGI_2$ was shown to relax coronary as well as splanchnic vascular strips in vitro, dilate vascular beds in vivo, and had strong antithrombotic activity in vivo (Dusting et al., 1982). Weksler et al., (1978) demonstrated that cultured human endothelial cells were induced to secrete $PGI_2$ when stimulated with sodium arachidonate, thrombin, the ionophore A231867 or trypsin. Production of $PGI_2$ activity was abolished by treatment of the cells with indomethacin, tranylcypromine, or 15-hydroperoxy arachidonic acid.

$PGI_2$ inhibits platelet aggregation by stimulating adenylate cyclase which in turn leads to an increase in intracellular cyclic adenosine 3' 5'-monophosphate (cAMP). cAMP inhibits platelet activation by a number of mechanisms. It inhibits phospholipases through activation of a protein kinase, and this in turn inhibits the liberation of arachidonate from membrane phospholipids. It also inhibits the conversion of arachidonic acid to endoperoxides by inhibiting PES. Furthermore, it causes intracellular membrane sequestration of free calcium ions which impairs phospholipase activity and the platelet contractile

process (Gorman et al., 1977). Finally, cAMP appears to have an inhibitory effect on the action of $TxA_2$ since it blocks aggregation induced by $PGG_2$ and $PGI_2$ but does not interfere with the formation of $TxA_2$ from the PG endoperoxides (Fitzpatrick and Gorman 1979).

While the exact mechanism behind $TxA_2$ induced platelet aggregation is not known, it is believed that human platelet involved haemostasis is to an extent controlled by the reciprocal regulation of cyclic AMP levels by $PGI_2$ and $TxA_2$ (Tateson et al., 1977). Agents that elevate cyclic AMP such as $PGI_2$ (and $PGE_1$) block both PES dependent and PES independent aggregation. In fact, $PGI_2$ inhibits platelet aggregation regardless of the aggregating stimulus (Gorman and Sparks 1982). In contrast to $PGI_2$, $TxA_2$ inhibits platelet adenylate cyclase and promotes haemostasis by acting as a vasoconstrictor and inducer of platelet aggregation. The dynamic balance of production of vascular $PGI_2$ and platelet $TxA_2$ is an important determinant of the state of platelet-vessel wall interactions and primary haemostasis.

A correlation between circulating levels of $PGI_2$ and myocardial infarction has been demonstrated in a number of studies. Coker et al., (1981) found that both $TxA_2$ and $PGI_2$ are released from the acutely ischemic

myocardium in dogs, and that the balance between $TxA_2$ and $PGI_2$ release in the period immediately following coronary artery ligation is related to the occurrence of arrhythmias. The animals which demonstrated the most ventricular ectopic activity also showed the largest increases in local coronary venous $TxB_2$ concentration. In humans, a prolonged increase in the in vivo synthesis of $TxA_2$ and $PGI_2$ during the acute phase of myocardial infarction has been demonstrated (Henriksson et al., 1986). Also, increased levels of $PGI_2$ sensitive platelet aggregates have been demonstrated in whole venous blood of patients with acute myocardial infarction (Splawinska et al., 1987). Finally, $PGI_2$ has been shown to have a considerably decreased half-life in individuals who have recently experienced a myocardial infarction. The half-life returned to normal fifteen days after the occurrence of the infarct and in fifteen angina patients, no change in $PGI_2$ half-life was detected (Sinzinger et al., 1987). Jaschonek et al., (1986) however, demonstrated an increased stability of $PGI_2$ when bound to serum albumin. Perhaps the decreased stability could be related to a fall in serum albumin following an MI.

### 3.5. OMEGA-3 FATTY ACIDS.

Since the discovery that the thromboxane/prostacyclin ratio to a large extent regulates platelet to

vessel wall interactions, attempts to alter this ratio to favor a non-thrombotic situation have been intensively studied. Various thromboxane synthase inhibitors and $TxA_2$ agonists have proved most useful. An alternate strategy that may have considerable potential would be to employ dietary supplementation with certain fatty acids. The fatty acids which could replace arachidonic acid could alter platelet metabolism and thereby reduce aggregation and thrombotic events. This next section will review the various effects of, and postulated mechanisms behind, the anti-thrombotic nature of the omega-3 fatty acids.

Following elucidation of the actions and mechanisms of the 2-series prostaglandins, Needleman et al., (1976) demonstrated that 5,8,11,14,17-eicosapentaenoic acid (EPA) was enzymatically converted by sheep seminal vesicle PES into the PG endoperoxide $PGH_3$. The purified $PGH_3$ was enzymatically converted by human platelets into thromboxane $A_3$ ($TxA_3$), or by blood vessel microsomes into delta-17-prostacyclin ($PGI_3$). It was subsequently found that $PGH_3$ or $TxA_3$ did not cause platelet aggregation, while $PGI_2$ and $PGI_3$ exerted similar effects both on vascular smooth muscle and on platelets (Needleman et al., 1979).

The findings of Needleman were given physiological

significance by the findings of Dyerberg et al., (1978). Through epidemiological studies in North Greenland they determined that Eskimos have a very low incidence of acute myocardial infarction. They live on a traditional marine diet which is rich in long chain omega-3 polyunsaturated fatty acids. In subsequent studies it was found that in certain plasma and platelet lipids, arachidonic acid was partly replaced by EPA and in certain in vitro systems, tissue formation of the three-series prostaglandins occurred. Finally it was shown that with EPA feeding in man, $PGI_3$ dinor products could be detected in the urine, indicating in vivo metabolism of EPA (Fisher and Weber 1984).

Following these early findings, studies have been conducted on the effects of a fish fatty acid diet on virtually every variable which has been positively correlated with the occurrence of CAD. Many investigators have determined that EPA feeding decreases platelet aggregation (Thorngren and Gustafson 1981; Goodnight et al.). A decrease in $T_XB_2$ formation has been observed (1981; Siess et al., 1980; Lorenz et al.), as have decreases in platelet count (Hay et al., 1982), platelet retention to glass beads (Goodnight et al., 1981), blood pressure (Singer et al., 1984) and blood viscosity (Kobayashi et al., 1981). Many studies report a fall in serum triglycerides and very low density

lipoprotein cholesterol (Mortensin et al., 1983; Terano et al., 1983; Bradlow et al., 1983; Sanders et al., 1983; Fehily et al., 1983; Rogers et al., 1984). Fewer report decreases in total serum cholesterol (Goodnight et al., 1981; Saynor et al., 1984). Interestingly, one report indicated a decrease in total serum cholesterol which correlated with an increase in platelet membrane cholesterol and platelet membrane cholesterol/protein ratio (Goodnight et al., 1981). EPA feeding has been shown to increase heparin thrombin clotting time (Rogers et al., 1987), decrease plasma levels of platelet factor four ($PF_4$) and decrease plasma beta-thromboglobulin (Hay et al., 1982).

While it is obvious that marine lipids cause profound changes in various homeostatic profiles, the mechanism of action behind these changes remains a clouded issue. Most theories concerning the effect of fish oils revolve around the observation that increased levels of EPA result in a shift from the oxygenated products of arachidonic acid ($TxA_2$ and $PGI_2$) to those of EPA ($TxA_3$ and $PGI_3$). Dyerberg et al., (1978) proposed that this shift is created by increased intra-cellular EPA/arachidonate ratio, while Needleman et al., (1980) proposed that the shift was caused by a reduced conversion of released arachidonic acid by competitive inhibition of EPA for the PES enzyme. Finally Siess et

al., (1980) argues that the shift is created by a reduction in the amount of arachidonate liberated from phospholipids in stimulated cells. More recent studies have shown that while EPA does change the balance of intra-cellular prostaglandin content, this may not be the whole story behind the effect of fish fatty acids on cells in vivo.

Unstimulated platelets have an active deacylation/ reacylation pathway which allows the fatty acid in the 2-position of the phospholipid to be replaced continually (Lands and Samuelsson 1968). By this mechanism, dietary fatty acids may be incorporated rapidly into platelet phospholipids. This incorporation is selective, with specific fatty acids being esterified to specific phospholipids (Shattil and Cooper 1979). On the inner leaflet of the platelet membrane, arachidonic acid is esterified mostly to phosphatidylethanolamine (PE) and is fairly evenly distributed among phosphatidylcholine (PC), phosphatidylserine (PS) and phosphatidylinositol (PI) (Chap et al., 1984).

The turnover of arachidonate is most active in PI (Stubbs and Smith 1984). While EPA appears to be taken up by PI in platelets in vitro, the degree of incorporation differs drastically depending on whether the cells are incubated with EPA in buffer, in buffer

plus albumin, or in platelet rich plasma (Weaver and Holub 1985; Ahmed et al., 1983). With fish oil feeding in normal volunteers, EPA is incorporated into PC and PE at the expense mostly of arachidonate however, EPA is not incorporated into PI or PS and the arachidonate content of these two latter phospholipids is not effected by the presence of EPA (Galloway et al., 1985). If this is truly the case in vivo, then the reported beneficial effects of EPA on hemostasis cannot be explained exclusively on the basis of its turnover in the platelet membrane pool in place of arachidonate.

Needleman et al., (1979) determined that in order to achieve a 50% reduction in arachidonic acid cyclo-oxygenation in vitro a 1:1 ratio of arachidonate to EPA was required. It was shown by vonSchaky et al., (1985) that a 50% reduction of TxB2 synthesis in vivo could be achieved with a 20% increase in total omega-3 fatty acids in platelets. This tends to support the notion that a change in the arachidonate to EPA ratio in phospholipids cannot be the only factor effecting the production of cyclo-oxygenation products in vivo. Further proof of this was given by Thorngren and Gustafson (1981) who determined that aspirin given before a long term fish fatty acid diet prolonged bleeding time by as much as did the diet alone. Aspirin given during the diet prolonged bleeding time by

somewhat more than the sum of the increases caused by aspirin and by the EPA diet separately. Also, Chetty et al., (1988) demonstrated that aggregation and serotonin release by washed platelets in vitro were similarly inhibited by EPA and indomethacin. Inhibition by EPA plus indomethacin to aggregation and serotonin release by U46619 stimulus, was considerably greater than inhibition with EPA and indomethacin alone. Finally, when washed human platelets are incubated with EPA or docosahexaenoic acid (DHA) or EPA plus DHA, the strongest inhibition is observed with the longer chain fish fatty acid. Also DHA is poorly liberated from phospholipid compared to EPA when platelets are stimulated with thrombin or the calcium ionophore. DHA is also a much poorer substrate for PES than is EPA (Croset and Lagarde 1986). This final bit of evidence may indicate that EPA exerts a considerable part of its effect through modification of the platelet membrane. This could lead to certain physio-chemical changes such as membrane receptor availability or affinity, lateral movement of membrane bound proteins or enzymes, or changes in membrane dependent intra-cellular signaling devices.

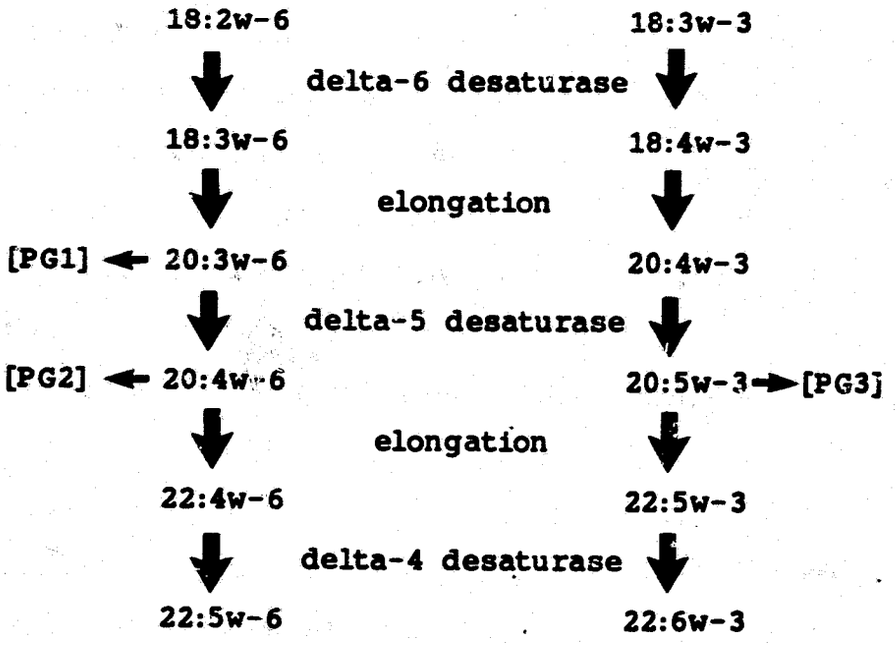
### 3.6. Endogenous Fatty Acid Synthesis.

De novo synthesis of sixteen and eighteen carbon

saturated fatty acids occurs by means of an acetyl CoA/malonyl CoA synthase system, and this mechanism is identical in plants and animals. Also both plant and animal enzymes are able to introduce a double bond to the omega-9 position of oleic acid. Animals, however are only able to introduce additional double bonds between the existing double bond and the carboxyl group. Plants on the other hand only introduce the new double bond between the existing double bond and the terminal methyl group. When indicating position of the double bond in relation to the methyl terminal carbon only three separate families of fatty acids can by achieved. These are termed the omega-9, omega-6 and omega-3 series of fatty acids, and the first members of each family are oleic acid (18:1w-9), linoleic acid (18:2w-6) and alpha-linolenic acid (18:3w-3) respectively (Gurr and James 1976).

Linoleic acid and its metabolites are necessary to maintain animals in a healthy condition. The isomer 18:2w-9, is inadequate in this respect. The inability of animals to desaturate oleic acid towards the methyl end of the chain therefore means that linoleic acid must be supplied in the diet from plant sources. In this respect, it is known as an essential fatty acid (Holman 1978). Animals can, however convert dietary linoleic acid by a series of alternate desaturations and elongations into other members of the omega-6 series.

The theoretical sequence of animal synthesis of longer chain fatty acids from the plant omega-6 and omega-3 precursors are as follows:




Two of the metabolites of linoleic acid, alpha-linoleic and arachidonic acids, are the precursors of the one-series and two-series prostaglandins respectively.

It has been known for some time that patients with CAD tend to have a low linoleic acid content in plasma cholesterol ester (CE) (Lewis 1958). More recently, low levels of linoleic acid in platelets, red blood cells and adipose tissue have been correlated with the occurrence of CAD (Simpson et al., 1982; Wood et al., 1987). Also, several cross-sectional population surveys

between localities demonstrating varying rates of CAD have found decreased levels of linoleic acid in the populations demonstrating higher levels of CAD (Nikkari et al., 1983; Salo et al., 1985). These low levels of linoleic acid may reflect dietary differences, however other explanations have been considered. These include differences in patterns of absorption, metabolism and storage in individuals prone to CAD.

The mechanism by which high levels of linoleic acid protect against the occurrence of CAD are unknown however, some suggestions have been offered.

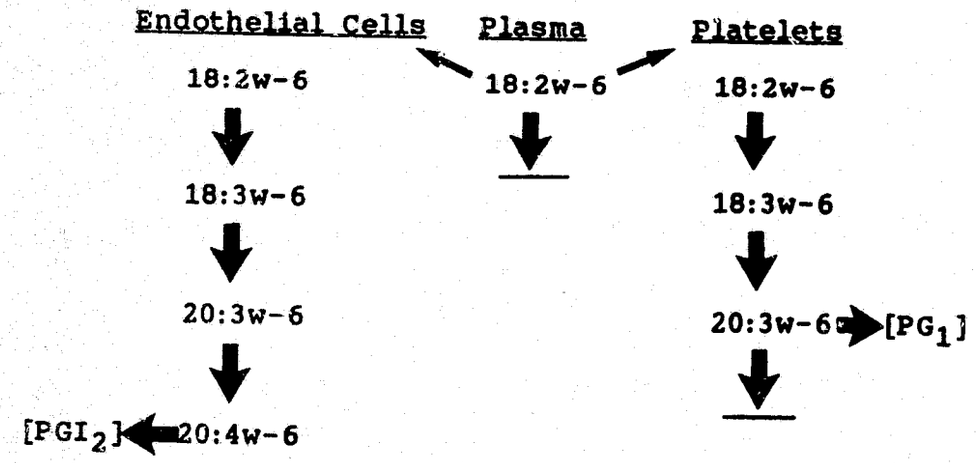
Nordoy (1979) incubated various fatty acids bound to albumin with platelet rich plasma (PRP) plus endothelial cell monolayers (ECM). The four fatty acids of the linoleic acid metabolic sequence, 18:2w-6, 18:3w-6, 20:3w-6 and 20:4w-6, had an inhibitory effect on ADP and collagen induced platelet aggregation when both platelets and endothelial cells were present. Only 20:3w-6 had such an effect when the pre-incubation was performed without the presence of ECM however, this effect was not as pronounced as when ECM was included. The saturated and monounsaturated fatty acids 18:0 and 18:1 had no effect under any circumstances. These results indicate that while platelets probably do not to any great extent convert less saturated fatty acids to

arachidonic acid, endothelial cells (at least in vitro) may readily accomplish this with subsequent production of $PGI_2$.

In a recent study by Boberg et al., (1985) correlation analysis among platelet phospholipid fatty acids and fatty acids in plasma lipid esters was carried out. This group determined that in plasma CE there is a negative correlation between linoleic acid and its metabolites 18:3w-6, 20:3w-6 and 20:4w-6. This suggests that the relationship between linoleic acid and its metabolites in the plasma is primarily a dietary one and that there exists a limited capacity of desaturation between linoleic acid and 18:3w-6. This observation was confirmed by Singer et al., (1986) who examined serum lipids following linoleic acid feeding in humans. Boberg also demonstrated that in the platelet, there is a positive correlation between linoleic acid and 20:3w-6, and no correlation between linoleic acid and 20:4w-6. This suggests a limitation in desaturation between 20:3w-6 and 20:4w-6. Given this data, Boberg suggested that high dietary intake of linoleic acid would not lead to high levels of 20:4w-6 in plasma or platelets, however it could lead to elevated levels of 20:3w-6 in the platelets. As mentioned, 20:3w-6 is the precursor of the one-series prostaglandins which have been shown to lead to inactive or non-thrombotic

substances in platelets.

Given the data from Nordoy (1979) and Boberg et al., (1985) the sequence of events which may lead to a non-thrombotic state with high levels of linoleic acid could be as follows:




It can be seen from these reaction sequences that high levels of serum linoleic acid would lead to increased levels of PGI2 in the endothelium, and one-series prostaglandins in the platelet, both of which could lead to a non-thrombotic state. Furthermore, several studies have shown negative correlations between linoleic acid and 20:4w-6 in various serum lipid fractions. This would tend to decrease 20:4w-6 availability to the platelet (Boberg et al., 1985; Valles et al., 1984).

# CHAPTER 4

# 4. Lipids and Coronary Artery Disease. A Review of the Literature.

## 4.1. Introduction.

Lipids can be divided into two general groups, the acyl lipids, and sterols. Acyl lipids are a large heterogeneous group of compounds which are to a greater or lesser extent soluble in organic solvents. They are primarily responsible for the storage and transport of metabolic energy and serve as structural and functional components of biological membranes. Acyl lipids are basically divided into the neutral lipids and the polar lipids. The neutral lipids are fatty acid esters of glycerol and there may be one to three fatty acyl groups esterified to the glycerol moiety. The fatty esters may be the same or different, and it is the nature of the fatty esters that impart the characteristic physical traits such as melting point and solubility. The polar lipids are those which contain an electrically charged functional group. The largest class of polar lipids are the glycerophosphatides or phospholipids. These consist of a polar head group (an amino alcohol or inositol) esterified to diacylglycerol by a phosphate group at the number three carbon of the glycerol backbone. Finally there are the steroids. These are water insoluble substances which are derived from a four membered ring compound termed the cyclopentanoperhydropenanthrene

ring system. Sterols are those members of the steroid class which contain a hydroxyl group (usually at carbon number 3 of the ring system) capable of forming an ester linkage. The sterol, cholesterol is an important member of this class as it is the precursor of a number of essential metabolites, namely the bile acids, adrenocortical hormones and the male and female sex hormones (Lehninger 1982). Also a strong association has been established between the incidence of CAD and increased levels of total serum cholesterol (Gordon and Kannel 1971; Keys 1975).

This review will focus primarily on the biosynthesis, absorption, physical chemistry, laboratory analysis and protein association during transport of the triglycerides and cholesterol. Also, it will included an overview of what is currently known concerning these substances and their involvement in CAD.

## 4.2. *Nomenclature of Lipids*.

The lipid terminology used in this text will follow that suggested by the IUPAC-IUB commission on biochemical nomenclature (1977). The neutral fats, which are mono, di, or tri esters of glycerol, will be termed monoacylglycerol, diacylglycerol, or triacylglycerol as appropriate. In order to designate the configuration of glycerol derivatives, the carbon atoms of glycerol are

numbered stereospecifically. The carbon atom that appears on top in that Fischer projection that shows a vertical carbon chain with the hydroxyl group at carbon 2 to the left is designated as C-1. To indicate that this particular numbering system is in use, the prefix "sn" (for stereospecifically numbered) is used.

## 4.3. Biosynthesis of Triacylglycerols.

The two common precursors of Triacylglycerols (TG) are fatty acyl groups in their activated CoA thiolester form (fatty acyl-CoA) and sn-glycerol 3-phosphate. Fatty acyl-CoAs can be supplied either from dietary ingestion of acyl lipids or from de novo synthesis from acetyl-CoA in the presence of the acyl carrier protein (ACP). Glycerol phosphate can be formed either from dihydroxyacetone phosphate (DHAP) which is generated during glycolysis or from glycerol plus ATP by the action of a glycerol kinase. The sn-1 and sn-2 free hydroxyl groups of glycerol phosphate are acylated by two molecules of fatty acyl-CoA to yield diacylglycerol-sn-3-phosphate which is more commonly termed phosphatidic acid. Phosphatidic acid is then hydrolyzed by phosphatidate phosphatase to form sn-1,2-diacylglycerol. At this point sn-1,2-diacylglycerol can become esterified by another molecule of fatty acyl-CoA to form the triacylglycerol or it can react with various pyrimidine nucleotides to

form the phospholipids. This mechanism of TG synthesis is referred to as the glycerol phosphate pathway and it has been shown to occur in the endoplasmic reticulum of liver cells, mammary gland and adipose tissue (Packter 1973). Another pathway, which occurs mainly in the endoplasmic reticulum of the small intestinal mucosal cells of many species including man, involves the sequential acylation of a monoacylglycerol and is usually referred to as the monoglycerol pathway. In this pathway fatty acyl-CoAs are consecutively acylated to a monoacylglycerol by monoglycerol and diglycerol acyltransferases (Gurr and James 1976). The synthesis of triacylglycerol involves a sequence of enzymes which are capable of handling water-insoluble intermediates. In higher animals, the cell has developed a multienzyme complex similar to the fatty acid acyl carrier protein termed diacylglycerol acyltransferase (DGAT) which may be regulated by cAMP (Maziere et al., 1986).

4.3.1. Ether Linkages.

As mentioned previously, the three carbon glycerol backbone of TG may arise from the dehydrogenation of DHAP. In the presence of fatty acyl-CoA, ATP, $Mg^{2+}$, and coenzyme A a fatty acid can be esterified directly to the sn-1 position of DHAP. In a subsequent reaction, the fatty acyl group of acyl-DHAP can exchange with a long chain fatty alcohol creating an ether linkage at

the sn-1 carbon rather than an ester linkage, forming alkyl-DHAP. Alkyl-DHAP may then form either TGs or phospholipids by the previously discussed mechanisms (Natarajun et al., 1983). Plasmalogens are phospholipids with a vinyl ether linkage at the sn-1 position of the glycerol backbone and they are formed from 1-alkyl-2-acyl-sn-glycerol (Gurr and James 1976). The substance 1-alkyl-2-acetyl-sn-glycerol-3-phosphocholine (commonly termed platelet activating factor or PAF) is a potent stimulator of the aggregation and degranulation of both platelets and neutrophils. PAF represents the first bona fide example of a biologically active phospholipid (Hanahan 1986).

### 4.3.2. Regulation of Triaclyglycerol Oxidation and Synthesis.

Biosynthesis and oxidation of TG occur in a steady state and are regulated to a certain extent by metabolic needs and caloric intake. If carbohydrate, fat or protein are consumed in excess, the excess calories are stored in adipose tissue in the form of TG. Both carbohydrates and the carbon chains of amino acids can give rise to acetyl-CoA resulting in increased biosynthesis of fatty acids and TGs. The rate of TG biosynthesis/oxidation is regulated by the action of several hormones which are in turn influenced by the cellular requirement for high

energy nucleotides. Insulin for example promotes the conversion of carbohydrate into TG. TG metabolism is also influenced by the secretion of pituitary growth hormone, adrenal cortical hormones and by glucagon (Martin 1987).

4.4. Biosynthesis of Cholesterol.

Since the discovery of cholesterol in gallstones during the early 1800s, contributions toward the understanding of cholesterol synthesis have resulted from a series of world wide investigations. Beginning in the 1940s various epidemiological studies demonstrated a correlation between elevated serum cholesterol levels and CAD. Soon after, with the development of radio isotope techniques knowledge of the de novo synthesis of cholesterol developed rapidly. This work was pioneered by Rittenberg and Block (1945) who monitored the metabolism of [$^{14}$C]-acetate in mice. The elucidation of the complete biosynthetic pathway (one of the most complex known) was the result of numerous investigations led mainly by Cornforth et al., (1960).

The de novo synthesis of cholesterol is a complex multi-step sequence of events that involves over ten different enzymes. Also several nonsteroid products which are essential to cell survival are produced from the metabolites of cholesterol. Given this lengthy

metabolic process, cholesterol synthesis can be regulated by a large number of enzymes. The key regulatory enzyme has been found to be 3-hydroxy-3-methyl-sn-glutryl Co-enzyne A (HMG CoA) reductase (Brown and Goldstein 1980). Many of the other enzymes in the sequence have also been shown to exhibit regulatory control however the amount of inhibition is less and the response time is slower (Clark et al., 1987).

All of the regulatory enzymes of cholesterol synthesis may possibly be controlled by a single substance or regulatory factor. This common controlling factor appears to be activated by the presence of low density lipoprotein cholesterol (LDL-C) (Brown and Goldstein 1980).

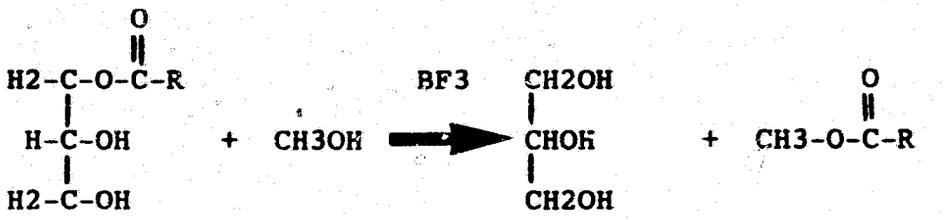
4.5. Lipid Analysis.

Lipids are most easily extracted by procedures which operate on the principal that organic and inorganic solvents, when mixed, form two phases. If biological material is present, the polar constituents migrate to the inorganic solvent while the nonpolar ones migrate to the organic solvent (Gurr and James 1976). No extraction system based on differential solubility provides 100% separation of all classes however, various techniques offered by Folch et al., (1957) and Bligh and

Dyer (1959) give total lipid recoveries of 90% to 95% with most animal tissues (Zweig and Sherma 1984). Separation of the various components of the crude lipid extract are most often carried out by thin layer chromatography using bonded silica as the stationary phase. Direct identification of the structural components of the crude lipid extract may be accomplished by acid hydrolysis of the constituent lipids, methylation of the liberated fatty acids, and identification of the resulting fatty acid methyl esters by gas liquid or capillary gas chromatography.

Fatty acid hydrolysis can be accomplished by a number of methods. Those using a strong Lewis acid in methanol are most convenient. Boron trifluoride-methanol has served as a reliable reagent for the rapid esterification of lipids and demonstrates a minimum of undesirable side reactions (Morrison and Smith 1964). A level of 14% boron trifluoride in methanol does not cause double bond hydrolysis of unsaturated fatty acids or polymer formation when used at the recommended reaction temperature (85-95 degrees C). The reaction of lipid and methanol producing glycerol (or a glycerol like compound) and fatty acid methyl ester (FAME) in the presence of boron trifluoride can be likened to a simple

acid/base reaction as given by:




Due to the extreme electropolarity of boron trifluoride, methanol acts as a strong acid and at 100 degrees C, the reaction proceeds 97% to 99% to completion. Boron trifluroide functions as a true catalyst in the reaction medium as can be seen by the reaction mechanism given in figure 4.1. (Solomons 1980).

During the methylation procedure dimethylacetals (DMA) are formed from plasmalogens. As discussed, most plasmalogens are composed of long chain hydrocarbons bound to the sn-1 position of glycerol by a vinyl ether linkage rather than an ester bond. The vinyl ether linkage is probably cleaved by boron trifluoride with the formation of an oxonium salt intermediate resulting in the ultimate formation of a long chain aldehyde. An unstable hemiacetal is formed via the nucleophilic addition of methanol to the carbonyl group of the aldehyde. With boron trifluoride acting as a catalyst, the hemiacetal reacts with another molecule of methanol forming a DMA which is stable in aqueous base (Solomons

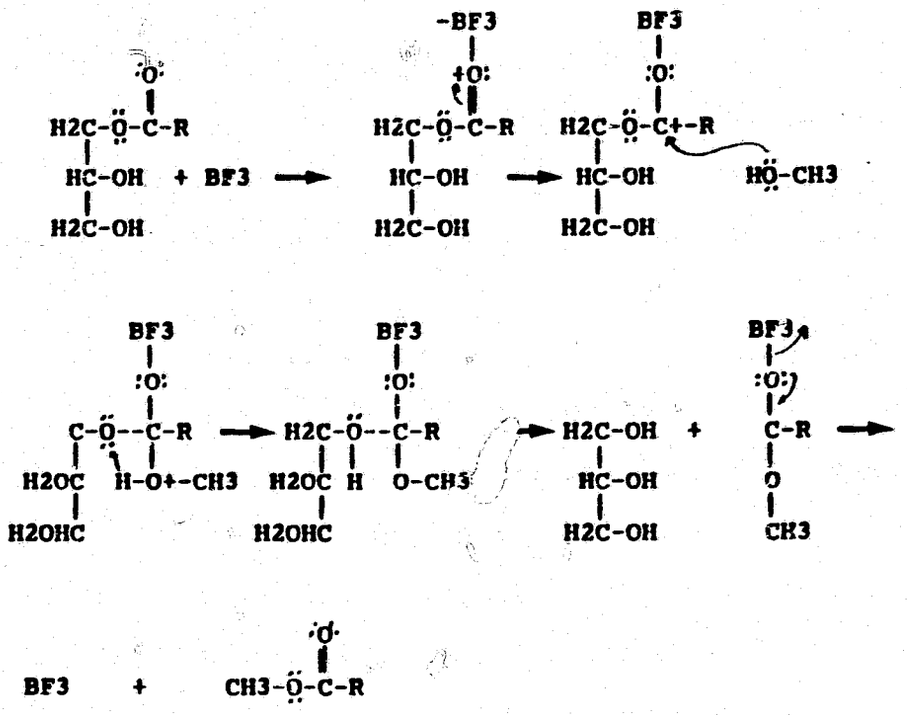


Figure 4.1. Reaction mechanism of the transmethylation of a monoacyl lipid in boron trifluoride methanol.

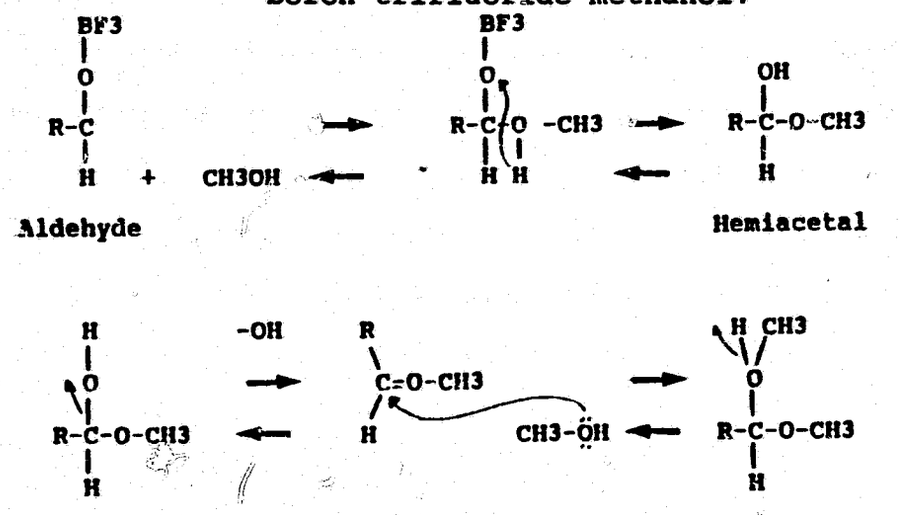


Figure 4.2. Reaction mechanism of DMA formation from a long chain aldehyde in boron trifluoride methanol.

1980). If extracted into an acidic or neutral medium the DMA will revert back to an aldehyde and is lost in the final extraction procedure. The mechanism of DMA formation is given in figure 4.2 (Solomons 1980).

Crude extractions of whole lipid contain cholesterol. In the presence of boron trifluoride fatty acid residues esterified to the 3-beta position of cholesterol are hydrolyzed and transformed to fatty acid methyl esters via the same mechanism as transesterification of lipids, leaving cholesterol in its native 3-beta sterol form. Cholesterol at this point acts as an alcohol, and in the strong acid medium dehydrates in an equilibrium reaction with the formation of 2,5-cholestadiene, 3,5-cholestadiene and $H_2O$. 3,5-cholestadiene is the major product of the two cholestadienes due to the inherent stability of the conjugated unsaturated system. At a concentration of 14% boron trifluoride in methanol the ratio of cholesterol to cholestadiene formation will be 1:1 (Solomons 1980).

## 4.6. Lipoproteins.

### 4.6.1. Physical Properties Of Lipoproteins.

All lipid molecules are amphiphatic to a certain degree. The oxygen of hydroxyl, carbonyl or ether groups can take part in hydrogen bond formation while the hydrocarbon chains of fatty acyl residues and the

ring system of sterols bind together by Van der Waals forces. Although triacylglycerol contain ester groups which are permanent dipoles, the hydrocarbon chains of the fatty acyl groups override this polar tendency and render the molecule almost completely nonpolar. The same is true for cholesteryl ester. These substances tend to associate with each other in an aqueous environment and form spherical droplets whose surface area is minimal (Gurr and James 1976).

Phospholipids in water behave quite differently. Due to their highly polar head groups they tend to form characteristic patterns in which their charged groups associate with the water molecules while their fatty acyl tails associate with each other. These arrays are termed micelles and they can take on lamellar, hexagonal or spherical forms. If phospholipids or cholesterol are introduced into a nonpolar lipid/water system, they will tend to arrange themselves at the lipid-water interface with their nonpolar groups buried in the lipid droplet and their polar groups at the surface. This interaction among lipids of increasing polarity results in a more stable dispersion of the fat termed an emulsion.

The forces which stabilize large protein molecules and maintain their secondary and tertiary structures are similar to those which cause lipids to form emulsions and

micelles. Proteins with appropriately polarized domains will bind to the outer layer of the lipid globule and render further stabilization. These lipid-protein structures are termed lipoproteins and the surface protein units are called apolipoproteins. Lipoproteins represent the functional unit of transport for water insoluble lipids in the blood. Classes of lipoproteins contain different ratios of lipid types and different types of proteins. This heterogeneity causes variations in electrophoretic mobility, density and water solubility. Lipoproteins are currently classified according to their densities, however it must be born in mind that lipoproteins *in vivo* are not static substances. Due to the rather loose arrangement of lipids and proteins these components readily exchange with other substances in the blood such as endothelial cells, red blood cells, platelets and other lipoproteins. Furthermore, lipoproteins are continuously being altered by an array of enzymes and transfer proteins. The five major groups of lipoproteins, in their order of increasing density are chylomicrons, very low density lipoproteins (VLDL), low density lipoproteins (LDL), high density lipoproteins (HDL) and albumin-fatty acid complexes (Assman 1982).

### 4.6.2. Apolipoproteins.

Apolipoprotein A1 (apo A1) is the major protein

constituent of HDL. HDL levels have been inversely correlated with susceptibility to CAD (Miller and Miller 1975) and recently the same association has been demonstrated for serum apo A1 (Brunzell et al., 1984). Apo A1 is thought to participate by two mechanisms in the reverse transport of cholesterol from tissues to the liver for excretion: (1) apo A1 can promote cholesterol efflux from tissues; and (2) apo A1 displays co-factor activity for the lecithin cholesterol acyltransferase (LCAT) enzyme which is responsible for almost all plasma cholesterol esterification (Miller and Small 1987). This later reaction is thought to play a role in transforming nacent HDL to mature HDL particles.

Apolipoprotein AII (apo AII) is the second most abundant protein in HDL comprising approximately 20% of its polar surface structure. Apo AII is able to displace apo A1 from HDL particles (Lagocki and Scanu 1980). Also it is an activator of hepatic lipase.

Apolipoprotein B (apo B) is the major protein constituent of LDL and is also found in chylomicrons and VLDL. Human apo B is a glycoprotein which occurs in two forms, designated B-100 and B-48 (Deeb et al., 1985). Apo B-100 is the predominant structural apoprotein in LDL and VLDL and is synthesized continuously in the liver. Apo B-48 is produced by the mucosal cells of the

gut and is the major structural protein of chylomicrons (Packard et al., 1984; Shepherd and Packard 1987). The clearance of LDL and VLDL from plasma is mediated by a receptor which is specific for apo B-100 and Apo E. The production rate of Apo B-100 is instrumental in regulating plasma LDL levels. Obese patients and some patients with premature CAD have high VLDL and LDL apo B levels (Kesaniemi et al., 1985). Monoclonal antibodies which bind to apo B-100 inhibit cellular uptake of LDL however, anti-apo B-48 monoclonal antibodies do not block uptake of chylomicron remnants suggesting that apo B-100 but not apo B-48 contains the domain responsible for the cellular recognition of lipoproteins (Schonfeld et al., 1987).

Apo E is a single polypeptide chain containing 299 amino acids. Abnormalities in Apo E result in defective receptor binding of VLDL, LDL and chylomicrons.

Apolipoprotein CII (apo CII) comprises about 10% of the total protein of VLDL as well as 1-2% of the total protein of HDL. It is an important activator of lipoprotein lipase, which catalyzes the hydrolysis of triacylglycerol in chylomicrons and VLDL. The physiological importance of apo CII in activating lipoprotein lipase has been established by the finding of patients with inherited apo CII deficiency who are

severely hypertriglyceridemic and have functional lipoprotein lipase deficiency (Havel et al., 1979).

Apolipoprotein CIII (apo CIII) is a constituent of VLDL and HDL and comprises about 50% of VLDL protein and 2% of HDL protein. In vitro, Apo CIII has been shown to inhibit the activities of both lipoprotein lipase and hepatic lipase.

4.6.3. Chylomicrons.

Nacent chylomicrons are formed exclusively in the intracellular endoplasmic reticulum compartments of intestinal enterocytes. They are composed of 90% TG, 5% phospholipid, 3% esterified and nonesterified cholesterol and 2% protein. The fatty acid content of chylomicron TG and phospholipid closely resemble that of exogenous (dietary) fat. The principal apolipoprotein components are A1, AII, and B-48. (Assman 1982).

After entering the general circulation via the thoracic duct, chylomicrons undergo several major compositional changes. Both apoproteins and lipids are exchanged between the chylomicron and plasma elements such as red blood cells and other lipoproteins. Cholesterol is transferred from blood components to chylomicrons independently of protein carriers probably via spontaneous movement along a concentration gradient

(Pyorala 1987). Cholesteryl ester and phospholipids are delivered to HDL by the actions of cholesteryl ester transfer protein (CETP) and phospholipid transfer protein (PTP) respectively (Tall et al., 1987). Also apo A1 transfers from chylomicrons to HDL when nacent lipoproteins are incubated with plasma and it is likely that a large portion of phospholipid is carried over in this exchange.

Apo CII and E peptides transfer from HDL to chylomicron. When chylomicrons become activated by apo CII in the peripheral circulation, they attach to the capillary walls and undergo degradation by lipoprotein lipase which is bound to cell surface glycosaminoglycans and can be released from its binding site by heparin (Nestel et al., 1987a). As a consequence of this lipase action, a large percentage (>75%) of the chylomicron TG is degraded to fatty acids and monoacylglycerols which eventually enter muscle or fat cells. A small portion of the chylomicron phospholipid is also cleaved to fatty acid and lysophospholipid by lipoprotein lipase. Following degradation of the nacent chylomicron to the core remnant, apo C peptide is transferred back to HDL thus halting delipidation. The chylomicron remnant is now taken up by the liver and the recognition by the hepatic receptor of the chylomicron remnant is mediated by apo E. The chylomicron receptor is distinct from

receptors that bind other lipoproteins and apo B-48 does not appear to be involved (Schonfeld et al., 1987). The chylomicron receptor may however be involved in hepatic uptake of large VLDL remnants as will be discussed in the next section.

4.6.4. Very Low Density Lipoproteins.

VLDL particles are the soluble carriers of endogenous (liver synthesized) lipids. The density of these lipoproteins range from 0.95 to 1.006 g/ml (Sf 20-400) indicating a large diversity of chemical content. On average, VLDL contain 60-70% TG, 10-15% phospholipid, 8-10% cholesterol ester, 3-5% cholesterol and about 7-10% protein. The protein content consists of 30-35% apo B-100, 50% C apoproteins (C1, CII, CIII), and about 10% apo E (Assman 1982). Intermediate density lipoproteins (IDL) result from the delipidation of VLDL and are the precursors of LDL. IDL span the density range of Sf 12-20 (Miller and Small 1987).

The intracellular steps in VLDL assembly include synthesis of the individual lipid and protein components to form the nascent lipoprotein. TG synthesis occurs via the glycerol phosphate pathway and the requisite fatty acids may originate either from plasma albumin bound fatty acids or from hepatic de novo synthesis. Ultimately, the synthesis of VLDL is dependent on the

synthesis of the apoproteins. This was demonstrated by Marcel et al., (1981) who effected a complete block of VLDL synthesis in rats following administration of cycloheximide, an inhibitor of protein synthesis. Also, individuals with abetalipoproteinemia who are unable to produce apo B have no chylomicrons, VLDL or LDL particles resulting in extremely low plasma TG levels (Malloy et al., 1981).

Diet also has a profound effect on VLDL synthesis. As noted in a previous section, studies on the effects of dietary fish oils have found that VLDL levels are considerably reduced following long term fish diets. Nestel et al., (1984) determined that this VLDL lowering effect was the result of decreased apo B synthesis and while this occurs to a certain extent with omega-6 PUFA feeding, the results are not nearly as dramatic. Also, as will be discussed below, carbohydrate feeding increases the overall size of VLDL while cholesterol feeding tends to decrease the size. This may be a strong determining factor in the metabolic fate of VLDL remnants in the plasma.

The intravascular catabolism of VLDL is regulated, at least initially in a manner similar to that of chylomicrons. There is early transfer of apo CII and apo E from HDL and this controls the rate at which

delipidation and remnant removal takes place. Remodeling of VLDL remnants is also required and this involves the transfer of unwanted surface lipid and protein to HDL. It was originally believed that nascent VLDL were relatively homogenous in size and chemical composition and that the wide range of densities seen in VLDL were exclusively the result of catabolism. It was demonstrated by Havel et al., (1979) however, that hepatocytes in culture synthesize and secrete nacent particles which cover a broad size range. More recent studies have determired that these heterogenecus nacent particles follow quite different metabolic channels.

It has been known for some time that the basic scheme of VLDL catabolism involves delipidation of VLDL to form IDL and remodeling of IDL to form LDL. Recent studies however, have added several modifications to this initial model. Packard et al., (1984) followed the transit of radio labeled apo B-100 (a non-exchangable protein) from nacent VLDL to LDL in man and found that little of the radioactivity reached LDL however, the labeled protein was cleared from the plasma in a normal fashion. Following separation of several classes of particles within the VLDL density spectrum it was found that 90% of all the $^{125}I$-labeled Sf 100-400 VLDL appeared in the Sf 12-100 density range. There after, it failed to progress into the Sf 0-12 LDL density

range, but was cleared from the circulation. Furthermore, when arginine residues on the B protein were modified, it did not effect the rate of large particle delipidation but it did retard the clearance of the large particle remnants (Packard et al., 1985). From this it was concluded that metabolic channeling within the VLDL flotation interval most likely occurs and that LDL cannot originate from large VLDL but is possibly derived from smaller particles in the Sf 20-100 fraction. Furthermore, it was shown that apo B was directly involved in clearance of the large VLDL derived remnants. These findings may explain the observation that carbohydrate feeding which leads to large VLDL results in a decrease in LDL, whereas dietary cholesterol loading decreases the size of VLDL and at the same time raises the plasma LDL concentration (Shepherd and Packard 1987).

### 4.6.5. Low Density Lipoproteins.

Low density lipoproteins are the primary transport units of cholesterol in man. The particle size and composition of LDL are less heterogeneous than that of VLDL and the density range is considerably narrower (1.019 to 1.063 g/ml) (Sf 0-12) (Assman 1982). As discussed, LDL originate from the catabolism of mostly small VLDL. This involves loss of TG from the core of VLDL and IDL with the subsequent transfer of surface

lipid (phospholipid and cholesterol) and apoproteins E and C to HDL. This leaves a particle that is composed primarily of cholesteryl ester and apo B-100. In the absence of metabolic disease in man all of the LDL arises from the catabolism of VLDL however, in states of abnormal lipoprotein metabolism some LDL may possibly be secreted directly by the liver (Nestel 1987a). The conversion of small VLDL and IDL to LDL occurs mainly within the hepatic circulation and this involves the activity of hepatic TG lipase (Rubinstein et al., 1985). This was demonstrated in vivo in monkeys where the blocking of hepatic lipase delayed the conversion of IDL to LDL. Also, patients with hepatic TG lipase deficiency demonstrate an accumulation of IDL. It is not known to what extent hepatic receptors are involved in the conversion of IDL to LDL however, it is known that IDL particles bind with high affinity to hepatic LDL receptors (Brown and Goldstein 1983a).
Cellular uptake of cholesterol occurs mainly via endocytosis of LDL and this is mediated by the LDL receptor. The LDL receptor in the liver is often termed the B/E receptor however, this protein in all tissues has been shown to be the same molecule (Brown and Goldstein 1983a).

Plasma LDL are catabolized primarily in the liver. This was dramatically demonstrated when a liver transplant

from a normal donor into a FH homozygote child resulted in a lowering of plasma LDL cholesterol levels from 988 to 184 mg/dl (Bilheimer et al., 1984). A biochemical basis of receptor mediated clearance of LDL and its subsequent regulation of cholesterol metabolism was characterized by Brown and Goldstein who received the Nobel Prize for Medicine and Physiology in 1985 for this achievement (Brown 1985). The following is a brief review of their findings.

The LDL receptor is expressed on virtually every cell which has been tested for its presence however, it occurs in varying levels depending of the cells cholesterol requirement. When the cell requires cholesterol, synthesis of the LDL receptor protein is increased and it is inserted into the plasma membrane. Most of these receptor proteins associate spontaneously with coated pits and those that do not are induced to migrate to coated pits by the binding of LDL. Coated pits are regions of the cell membrane which contain large amounts of the protein clathrin. When these regions become saturated with LDL molecules, they invaginate to form coated vesicles which become the LDL/LDL receptor intracellular transport vehicles. The coated vesicles rapidly lose their clathrin coat and fuse with other vesicles to form larger vesicles called endosomes. Upon fusing with an endosome, the LDL

dissociates from the receptor. The receptor recycles to the surface and the uncoated vesicles fuse with lysosomes where both apoprotein and lipid are degraded by lysosomal enzymes.

The LDL cholesterol released upon lysosomal hydrolysis by an acid cholesteryl ester hydrolase suppresses further synthesis of the receptor which limits continued uptake of LDL. The released cholesterol also down-regulates HMG-CoA reductase. Excess cholesterol not needed for membrane or steroid hormone synthesis is re-esterified by acyl CoA:cholesterol acyltransferase (ACAT) which is activated by LDL uptake and stored primarily as cholesteryl oleate in the cytoplasm. The efficient down regulation of the LDL receptor, especially in extra-hepatic tissues, followed by increased LDL in circulation, with increased hepatic uptake and consequent down regulation of hepatic HMG-CoA reductase serves to feedback regulate the cholesterol synthetic pathway in the liver according to the demand for peripheral cholesterol supply.

LDL receptors can by activated by thyroxine and by pharmacologic doses of estrogen. Hepatic LDL receptors decline when rabbits are fed a diet composed mostly of casein and in dogs, hepatic receptors decline with ageing. All of these changes in LDL receptor activity are reflected by plasma LDL levels. Whenever hepatic

LDL receptors are depressed plasma LDL levels rise. Conversely, when the receptors are stimulated, plasma LDL levels fall.

LDL which has been chemically modified *in vitro* with acetic anhydride or malondialdehyde (MDA) is no longer recognized by the LDL receptor but is bound in a high affinity by the scavenger receptor (Brown and Goldstein 1983b). The scavenger receptor, which was first discovered on macrophages, appears to be present on only a few cell types. Among these are endothelial, Kupffer and parenchymal cells. It was demonstrated by Nëgelkerke *et al*., (1983) that endothelial cells take up acetylated LDL five times more readily than Kupffer cells and 31 times more readily than hepatocytes.

Macrophages in culture can be converted into foam cells by the uptake of acetylated or MDA modified LDL (Brown and Goldstein 1983b). Foam cells are cells which are laden with cholesteryl esters and which are found to be a predominant component of atherosclerotic plaques *in vivo*. The scavenger receptor leads to an accumulation of cholesterol in the macrophage because it is not subject to the same feedback regulation as is the LDL receptor.

It has been postulated that *in vivo* modification by MDA,

which is released as a consequence of $TxA_2$ formation by the platelet, may give rise to a modified LDL that would be recognized by the scavenger receptor (Fogelman et al., 1980). Also, incubation of LDL with endothelial cells generated an altered LDL which is a substrate for the scavenger receptor (Soutar and Knight 1982). The modification of LDL by endothelial cells is accomplished by extensive hydrolysis of phosphatidylcholine to lysophosphatidylcholine and this can be completely inhibited by the presence of antioxidants (Steinbrecher et al., 1984). Furthermore, endothelial cell modification of LDL was accompanied by the generation of thiobarbituric acid-reactive material indicating that lipid peroxidizing agents, possibly arising from endothelial cell fatty acid oxidation-reduction, may be involved.

Serum LDL levels appear to vary considerably among the various populations. This phenomenon was addressed in a recent study in which 35 to 49 year old men were recruited at random in the United Kingdom, Finland, Italy, Spain and South Africa (Lewis et al., 1986). In each population LDL cholesterol (LDL-C), LDL apo B turnover and LDL-C fractional clearance rates (FCR) were determined. The highest LDL-C levels were observed in the United Kingdom and Finland, and the lowest were seen in the black population of Cape Town. In each

population, the higher the LDL-C level, the greater was the LDL apo B synthesis. Also, in each population there was an inverse correlation between FCR values and LDL-C levels. The variations between LDL-C levels among the European populations were best explained by variations in the LDL FCR rather than by the LDL apo B production rate. In contrast, the South African Blacks were able to maintain very low LDL levels solely by very low LDL apo B production rates.

In all the countries except Finland, these differences could be explained solely on the basis of diet. After further investigation in Finland, it was found that the elevated LDL-C levels correlated positively with increased levels of cholesterol absorption. This data suggests that, at least within the Finnish population, effective absorption of cholesterol may overload the liver cells with cholesterol resulting in a shut down of the hepatic LDL receptors with a subsequent decrease in the LDL FCR and an increase in the plasma LDL-C.

4.6.6. <u>High Density Lipoproteins.</u>

The basic structure of HDL resembles that of the other lipoproteins. The mature particles contain a neutral lipid core surrounded by a surface monolayer of phospholipid, cholesterol and apoproteins. A property which distinguishes HDL from the other lipoproteins is

that its particle mass consist of about 70% surface components (Assman 1982). The two major apolipoproteins in HDL are apo A-1 and apo A-II with smaller amounts of the apo C proteins. Apo A-1 is the major protein component and constitutes about 60-70% of the surface mass. While both apo A-1 and apo A-II are capable of activating LCAT, apo A-1 is the most potent (Lagocki and Scanu 1980).

HDL particles vary in respect of size, composition and density. The HDL density fraction ranges from 1.063 to 1.210 g/ml, and with moving boundary ultra-centrifugation, two major fractions can be isolated. These are termed $HDL_2$ and $HDL_3$. $HDL_2$ is the larger and less dense of these subfractions (Groot and Scheek 1984).

Mature or spherical HDL particles are derived from precursor HDL particles. The precursor particles are formed in one of three ways. The liver and intestine are capable of synthesizing nacent HDL and these are secreted into the circulation. Nacent HDL may also arise from the components of triglyceride rich lipoproteins during lipolysis. Finally, HDL can be formed by spontaneous interaction between free apolipoproteins and phospholipid in the circulation. All of these precursor HDL are disk shaped bilayers

composed of phospholipid, cholesterol and apolipoproteins and are consequently termed discoidal HDL (Patch and Gotto 1987).

There are several theories as to why HDL levels negatively correlate with the occurrence of CAD. One theory considers HDL to have a primary influence in protecting against the disease by scavenging excess cholesterol from the peripheral circulation (Glomset 1968). Another suggests that HDL levels may simply be a measure of an effective lipid transport system and that high levels of HDL are the result of the phenomenon which protects against atherosclerosis rather than the cause of it (Patch and Gotto 1987). A brief review of HDL metabolism and enzyme involvement in HDL maturation and subspecies formation and how these may relate to the above theories of HDL involvement in CAD will be reviewed.

The transformation of discodial HDL to spherical HDL is primarily due to the activity of LCAT. At the disk surface, LCAT transesterifies a fatty acid from the sn-2 position of phosphatidylcholine to the 3-beta hydroxyl group of cholesterol. Due to its increased hydophobicity, cholesteryl ester migrates to the particle core thus splitting the bilayer disc until a spherical particle is formed (Fielding et al., 1983).

The findings in patients with Tangier disease (absence of apo A-1) and LCAT deficiency suggest that both apo A-1 and LCAT are absolute requirements for the formation of spherical HDL (Patch and Gotto 1987). As a result of the action of LCAT, there is little reciprocal transfer of cholesterol back from HDL to cells. This forms the basis for the idea that the primary function of HDL is to transfer cellular and dietary excess cholesterol for removal.

In most clinical laboratories HDL levels are estimated by measuring cholesterol levels of fasting serum after precipitation of apo-B containing lipoproteins and subtracting this from the total serum cholesterol measurement. The mechanisms controlling plasma distribution of HDL subfractions therefore may be of considerable importance in that measurements of HDL-cholesterol (HDL-C) correlate strongly with $HDL_2$ levels since it is this fraction which carries the bulk of cholesterol (Hamsten et al., 1986). The ratio of $HDL_2$ to $HDL_3$ may be primarily the result of the actions of lipoprotein lipase and hepatic lipase with involvement of LCAT and lipid transfer proteins. The clearance of triglyceride rich lipoproteins are largely due to the activity of triglyceride lipase and hepatic lipase. Patch and Gotto (1987) proposed a mechanism whereby lipoprotein lipase may cause the

conversion of $HDL_3$ to $HDL_2$. According to this hypothesis, surface components liberated from the triglyceride rich lipoproteins undergoing lipolysis are transferred to $HDL_3$ which then become enriched in lipid with a consequent decrease in density to that of $HDL_2$. LCAT is most certainly involved in this process by altering the neutral lipid core of $HDL_3$ (Saku et al., 1985).

Given normal lipoprotein metabolic activity, chylomicrons are efficiently cleared from the circulation resulting in low postprandial lipemia. Under these conditions, chylomicron derived phospholipids are transferred to $HDL_2$. Hepatic lipase serves to remove the excess phospholipid from $HDL_2$ allowing it to be recycled to participate in further chylomicron metabolism. Given an active hepatic lipase and low postprandial lipemia, $HDL_2$ levels remain at a steady state (Nestel 1987b). In conditions of high postprandial lipemia (for whatever reason) chylomicrons accumulate in the blood. This results in a lipid transfer protein mediated exchange of triglycerides and cholesteryl ester between chylomicrons and $HDL_2$ resulting in increased levels of cholesteryl ester being deposited in cells from chylomicrons. Also, in this instance, hepatic lipase not only removes phospholipid from the surface of $HDL_2$ but also triglycerides from

the core resulting in transformation of the particle to $HDL_3$ (Fielding 1987).

Given these latter two theories of HDL metabolism it can be seen that low postprandial lipemia coupled with an active lipoprotein lipase and hepatic lipase would result in elevated levels of $HDL_2$ with consequent increases in HDL-C measurements. Conversely, either high postprandial lipemia or decreased activity of lipoprotein lipase would result in lower levels of HDL-C.

# CHAPTER 5

## 5. Materials and Methods.

### 5.1. Patient Selection.

Patient volunteers for the study were obtained from the Baragwanath Outpatient Cardiology Clinic in Soweto under the supervision of Dr. P. Serali and under the approval of the University of the Witwatersrand Ethics committee. These patients presented to the clinic most often on a basis of need, frequently to obtain renewal of medication. There was no scheduling of patient visits by appointment and the cardiology clinic did not maintain documentation of cardiology patients. The patient charts were filed in a central repository by name with no cross reference to diagnosis. No reasonable method existed for contacting patients once they left the hospital. Over a 12 month period 24 male patients who had experienced an MI were found by reviewing the outpatient charts of all cardiology patients as they presented to the clinic. All patients had been instructed to fast before visits to the cardiology clinic because of routine blood coagulation tests. Diagnosis of MI was based on typical history, recorded cardiac enzyme elevation (CK > 195U/L or CKMB > 12% of total) and positive ECG. Diagnosis had been confirmed in 12 of the patients by angiography. All patients between six and eighteen months post MI, were not on anticoagulant therapy, and were in a good general

state of health as determined by the cardiology registrar. Seven patients were taking no medication. Most of the other 17 were taking dipyridamol (25mg Tid), a furosemide diuretic (40mg Bid) and nitroglycerin PRN for chest pain. No lipid lowering drugs were used, and no dietary advice had been given to the patients. All patients that had previously smoked had been advised to quit, although compliance was seen to be very low. The details of the study were fully explained to each volunteer and signed consent forms were obtained.

5.1.2. Normal Control Selection.

These patients were compared to 26 apparently healthy male subjects who were employed by the South African Institute for Medical Research as cleaners, laboratory assistants and security guards. Volunteers had been instructed to fast from midnight the day before. They admitted to no medication and none were under the care of a physician. On all individuals, informed consent was obtained.

5.1.3. Phlebotomy.

The puncture site was cleaned with ethanol swabs and blood was drawn with a nineteen gauge butterfly needle. Fasting blood was withdrawn into two 50 ml plastic syringes and aliquoted into various containers within two minutes. Bloods were drawn on patients and

| TEST | NUMBER OF CONTAINERS x AMOUNT/ CONTAINER | TYPE OF CONTAINER | FINAL CONCENTRATION OF ANTICOAGULANT IN BLOOD (mg/ml BLOOD) |
|---|---|---|---|
| PLATELET, PLASMA & RBC FATTY ACID ANALYSIS | 3 x 10ml | 15 ml plastic with screw top (Falcon) | E.D.T.A. 1.5 mg/ml (Zucker and Borrelli 1958) |
| PLATELET CHOLESTEROL & PROTEIN ASSAY | 3 x 10ml | 15 ml plastic with screw top (Falcon) | E.D.T.A. 1.5 mg/ml (Zucker and Borrelli 1958) |
| LIPOGRAM, URIC ACID & APOLIPOPROTEINS | 2 x8ml | siliconized glass vacutainer | None |
| CLOTTING FACTORS & INHIBITORS | 2 x 9ml | 15 ml plastic with screw top (Falcon) | CITRATE 3.8 mg/ml Dacy & Lewis (1984) |
| FULL BLOOD COUNT | 1 x 3ml | siliconized glass vacutainer | E.D.T.A. 1.5 mg/ml (Zucker and Borrelli 1958) |

**Figure 5.1.** Disposition of Bloods and Anticoagulants Used.

controls between eight and ten in the morning. The amount of blood per tube, anticoagulants used and disposition of bloods are listed in figure 5.1.

## 5.2. Fatty Acid Analysis.

### 5.2.1. Centrifugation.

All centrifugations were done at 4 degrees C. using a refrigerated Mistral 6L preparative scale centrifuge (model RJ450). The radius of the rotor head to the center of the angled centrifuge tubes was 20.0 cm. Calculations of revolution per minute (RPM) to g force

were determined by: g force = $1.18 \times 10^{-5} \times r \times (RPM)^2$ where r = radius of the rotor head (Dacie and Lewis, 1984). All tubes were capped or covered with aluminum foil during centrifugation.

5.2.2. Sample Preparation.

EDTA blood in 15ml graduated plastic tubes (Falcon) was centrifuged at 135 x g for 10 minutes and the upper platelet rich plasma was transferred into a glass 30ml centrifuge tube. The remaining plasma and buffy coat in the Falcon tubes were removed by suction, and these plus the platelet rich plasma containing tube were centrifuged at 950 x g for 15 minutes. A 5.0 ml aliquot of the platelet poor plasma from the glass centrifuge tube was placed in an acid washed ground glass top tube with 10.0 ml 2:1 (v/v) chloroform-methanol and stored at -20 degrees C for fatty acid analysis. The remaining plasma supernate was discarded and the platelet button re-suspended in 10.0 ml cold normal saline and gently agitated with a Pasteur pipette. The upper plasma fraction and a portion of the red blood cell fraction from the Falcon tubes was once again removed by suction and 3.0 ml of the concentrated red blood cell sediment was placed into a 30 ml glass centrifuge tube. The red cells were re-suspended in 10.0 ml cold normal saline and gently agitated with a Pasture pipette. The red cells and platelets were washed twice again in 10.0 ml

cold normal saline and spun at 950 x g for 15 minutes. Prior to the third centrifugation a platelet count was obtained from a 1:3000 dilution of platelets in Isoton-I with a Coulter Thrombocounter.

Following centrifugation the platelet button was re-suspended in sufficient saline to give a platelet count of 1 x $10^6$/ul. 1.0 ml of the concentrated platelet suspension was placed in a 3.0 ml glass screw top vial and the platelet count was checked again in a 1:6000 dilution of platelets in Isoton-I. This platelet count, multiplied by a factor of 2, was used in the final protein and cholesterol calculation. The remaining platelet and red blood cell sediments were stored at -20 degrees C in 10.0 ml 2:1 (v/v) chloroform-methanol for fatty acid analysis.

5.2.3. Solvent and Glassware Preparation.

All organic solvents used in the lipid extraction, methylation, CG analysis and cholesterol determination were fractionally re-distilled using a 45 cm jacketed coil condenser with ground glass connectors. De-ionized water used in reagent preparation was also twice distilled using a similar apparatus. Boron trifluoride in methanol was aliquoted into 10 ml glass screw top vials and stored at -20 degrees C in a desiccator with de-humidifing granules. All glassware that came into

contact with organic solvents was soaked in a dichromic acid solution (20% dichromate in 2M HCl) for 48 hours, rinsed in de-ionized water, oven dried and stored in either heat sealable plastic bags,or air tight plastic containers. After cleaning, rubber gloves were used at all times when handling the glassware.

5.2.4. Lipid Extraction.

Lipids were extracted by the method of Folch et al., (1957). Platelets and red blood cells were freeze-thawed to -20 degrees C. three times in chloroform-methanol to effect cell rupture. To the platelet, red blood cell and plasma samples in chloroform-methanol was added 2.0, 2.5 and 3.0 ml (.2 x the volume) of 0.73% NaCl respectively. This was vortexed for 15 to 20 seconds and centrifuged at 950 x g for 15 minutes. The lower lipid containing phase was removed with a volumetric pipette and filtered through chloroform washed No.1 Watman filter paper into 50 ml graduated glass centrifuge tubes. The remaining upper phase was re-extracted by adding 0.66 it's volume of pure lower phase, (PLP) (chloroform-methanol-water; 86:14:1 v/v) mixing, and centrifuging. The resulting lower phase was removed and filtered as was the first lipid extract. Inorganic contaminants were removed from the combined filtrates by washing three times with 1.5 volumes of pure upper phase (PUP) (chloroform-

methanol-.58% NaCl; 3:48:47 v/v). After each centrifugation, the upper phase was removed as completely as possible. Following the third washing, remaining upper phase was reconstituted to lower phase by addition of 0.5 ml of methanol. The resulting complex lipid extract was stored under nitrogen in a Teflon lined screw cap vial at -20 degrees C.

5.2.5. Lipid Transmethylation.

Lipids were transmethylated by the method of Morrison and Smith (1964). A 1.0 ml aliquot of the complex lipid mixture was blown to dryness with nitrogen in a 32 mm x 12 mm glass reaction vial. Boron trifluoride-methanol 14% (w/v) (Supelco) was added under nitrogen in an amount to ensure at least 1.0 ml reagent per 10.0 mg of lipid, and the vial was crimp-sealed with a Teflon coated septum cap. The vial was heated in a 95 degree C glycerol bath for 90 minutes. The vial was removed, cooled to room temperature with tap water and the contents placed into a 10 ml ground glass test tube with 2.0 ml hexane. After one minute of gentle agitation with a Pasteur pipette, 2.0 ml of 5N NaOH was added slowly and the tube was shaken for one minute, centrifuged at 4 degrees C while stoppered, and the upper hexane layer was removed and placed into a tare weighted 2.0 ml serum type reaction vial. The vial was crimp sealed with a Teflon coated septum cap and stored

at -20 degrees C for later analysis. For CG analysis, the cap was gently removed from the tare weighted vial, the hexane blown to dryness with nitrogen and the weight of the contents determined with a Sartorius model 2442 analytical balance to the nearest 0.1mg. The contents were re-suspended in an amount of hexane to give a concentration of 10.0 ug/ul. 1.0 ul of the sample was injected for CG analysis with a Hamilton 10 ul syringe which had been pre-rinsed with hexane, and the remaining sample stored at -20 degrees C.

### 5.2.6. Gas Chromatography of Fatty Acid Methyl Esters.

#### 5.2.6.1. Chromatography Procedure.

All chromatographic separations were performed with a Hewlett Packard model 5890A gas chromatograph equipped with a hydrogen flame ionization detector. The injection port was maintained at 220 degrees C and the outlet manifold at 220 degrees C. The carrier gas was helium with a flow rate of 0.6 ml/min. Column head pressure was 30 psi. The detector was operated at 17 psi hydrogen and 31 psi compressed air. Gas pressures were maintained at the gas tanks with two stage stainless-steel diaphragm pressure regulators supplied by Afrox. Helium was passed through a model 6063 gas filter located distill to the pressure regulator and was also supplied by Afrox. The chromatograph was fitted

with a 100 m glass capillary column of 0.25mm I.D. coated with the cyanopropylsiolxane SP2340 (Supelco). The oven temperature program was as follows: 50 minutes at 175 degrees C then increase to 200 degrees C at 1 degree/min and hold for 35 minutes, then increase to 220 degrees C at 1 degree/min and hold until end of run. Split vent ratio was 1:50 and total time of run was 160 minutes with cholesterol and its breakdown products eluting last.

5.2.6.2. Calculations.

Peak retention time and area were determined with an SP 4270 computing integrator (Spectro Physics). Relative peak percentage was calculated from peak integrals on a D Base III computer program. The amount of DMA was calculated as a percentage of the integrated total fatty acids plus the two contributing DMA peaks. The relative percent of each individual fatty acid was calculated independently of both the DMA peaks and cholesterol. The fatty acid to cholesterol ratio was the sum of the integration of the total fatty acids over the sum of the cholesterol peak and the two cholestadiene peaks.

5.2.6.3. Chromatogram Interpretation.

Fatty acid peaks were identified by comparing retention times to known standards, plotting the log of the adjusted retention time (t'R) against the carbon number

and comparison of reported values in the literature.

5.2.6.3.1. Standards.

The major fatty acid peaks were identified by comparing retention times with those of Nu Check Prep (Elysian NM, USA) reference standard GLC 68A, which contained the following FAMEs: 14:0, 14:1, 16:0, 16:1, 18:0, 18:1, 18:2, 18:3, 20:0, 20:1, 20:2, 20:3, 20:4, 20:5, 22:0, 22:1, 22:6, 24:0, 24:1. The omega-3 esters were identified with a methylated sample of Maxepa (Efamed), a pharmaceutical fish oil preparation. The amounts of each fatty acid present in Maxepa were reported by Sanders and Younger (1981) and we demonstrated good agreement with these values in our lab.

5.2.6.3.2. Log Plot.

As illustrated by figure 5.2, the elution times of a homologous series of FAMEs separated on a capillary column do not yield a linear plot when the log t'R is plotted against the number of carbon atoms in the fatty acid chain, but demonstrate a quadratic relationship (Nelson 1974). Nelson offers an equation which utilizes the retention times of three members of a series to calculate the retention time of consecutive members however, such standards are not readily available. Such a graph does however, offer a device whereby unknown peaks can logically be identified based on the elution

LOG ADJUSTED RETENTION TIME Vs. CARBON NUMBER

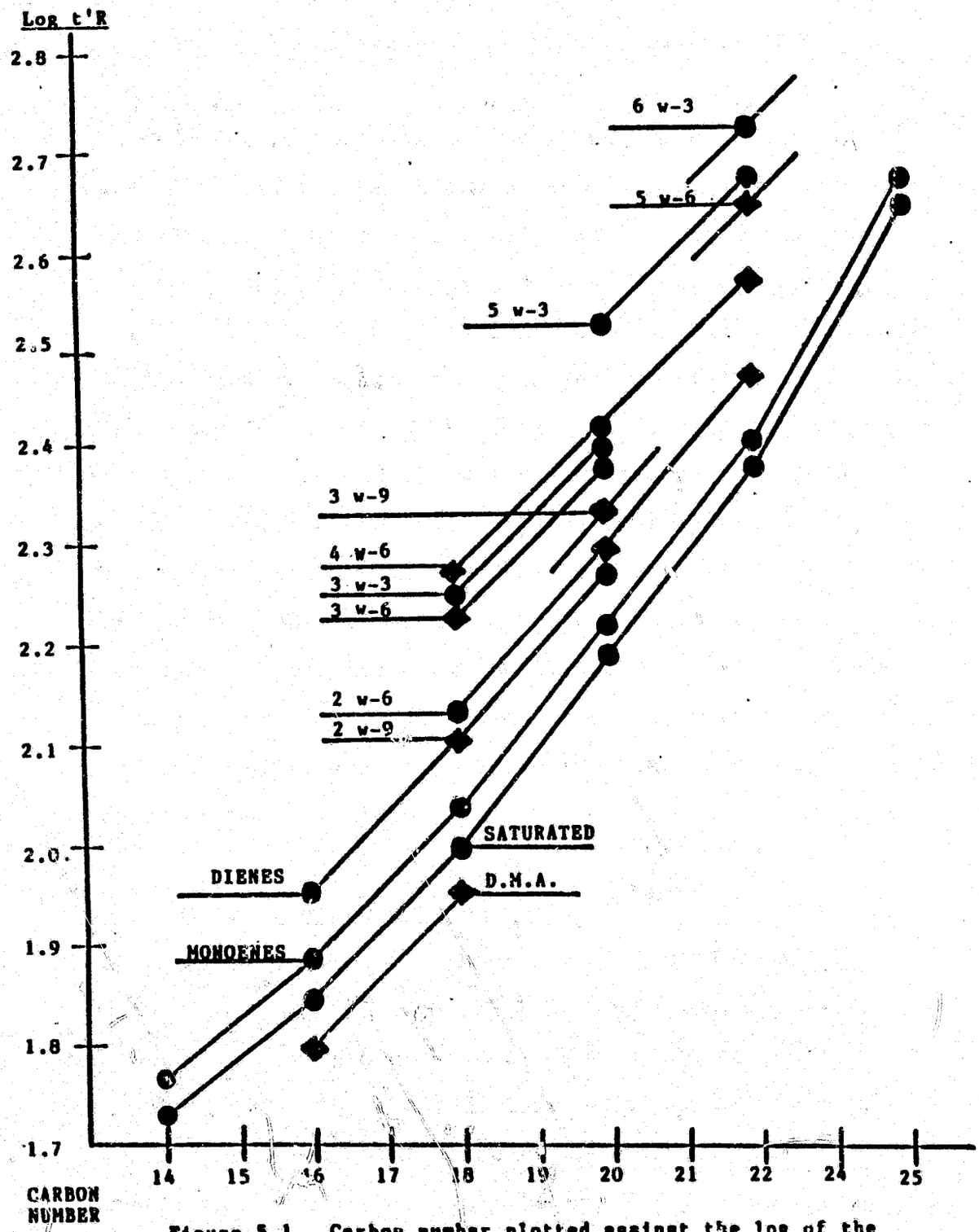



Figure 5.1. Carbon number plotted against the log of the retention time which has been adjusted to the retention time of 18:0. FAMEs identified by known standard (●) and FAMEs identified by retention relationships to known standards (◆) are indicated.

pattern of known compounds and likewise the retention times of unknown compounds can readily be estimated

The major peaks were identified in numerous isothermal runs of platelets, red blood cells and plasma by comparison to the retention times in the standards. The retention times for these peaks were adjusted according to the retention time of 18:0 in each individual run and the adjusted retention times combined to give peak identification numbers that coincided to all known peaks in all runs. The log t'R was calculated for all peaks by: Log [(R.T. peak/R.T.18:0)100] and this was plotted against the carbon number of the saturated FAMEs. Homologous FAMEs were lined up according to the base line curve demonstrated by the saturated species. FAMEs identified by known standard and those by structure retention relationships to known standards are characterized in figure 5.1.

5.2.6.3.3. Literature Values.

The order of elution of FAMEs on a 100m SP2340 wall coated capillary column with an isothermal oven temperature of 180 degrees C was determined by Slover and Lanza (1979). Retention time for a given series of FAME is increased with degree of unsaturation and decreased as the location of the double bonds approach the carbonyl carbon. Thus for the 18 carbon series, the

order of elution would be 18:0, 18:1, 18:2, 18:3 and among the double bond positional isomers of 18:3 the elution order would be 18:3w-9, 18:3w-6, 18:3w-3. Fatty acids in platelets, red blood cells and plasma were identified based on levels reported by Boberg et al., (1985), Heckers et al., (1977) and Holman et al., (1979) respectively, who used similar GC apparatus. The fatty acid composition of Maxepa was obtained from Sanders and Younger (1981).

#### 5.2.6.4. Quality Control Data for Fatty Acid Analysis.

Lipids were extracted from ten 5.0 ml portions of PPP prepared from human bank blood. Lipids were transmethylated, and FAMEs were stored in hexane at -20 degrees C. These ten preparations were analyzed on ten consecutive GC runs over a three day period. Extractions, transmethylations and GC conditions were exactly as described for patient and control platelets, RBCs and PPP.

## 5.3. Platelet Protein and Cholesterol Determination.

### 5.3.1. Protein Assay.

The Folin-Ciocalteu photometric assay (Lowry et al., 1951) was used to determine platelet protein concentration. This procedure employs two color forming reactions. The first involves the reaction of alkaline

copper ions with the peptide bonds to form the characteristic biuret color. The second involves reduction of the phosphomolybate-phosphotungstinate salts in the Folin reagent by tryptophan and tyrosine residues giving a blue-green color (Clark and Switzer 1977). Rather than give a quantitative determination of total platelet protein, the Lowry assay provided a consistent color reaction which was dependent primarily on platelet concentration and in this study, served as a constant in the expression of total platelet cholesterol content.

The procedure for this assay was identical to that given by Lowry et al., (1951) with the exception of protein denaturation in 2% deoxycholate and initial volumetric suspension of 40.0 ul of the $10^6$/ml platelet solution in calcium free, phosphate free tyrodes (pH 6.4). All assay reagents were mixed thoroughly for 10-15 seconds when added to the platelet suspension. The amount of protein in the unknown was always 100 to 200 ug and the standard range was 20 to 200 ug using serial dilutions of BSA (1 mg/ml calcium free phosphate free tyrodes). At 45 minutes following the addition of the Folin reagent, the optical density was determined at 750 nm on a Pye Unicam SP-6-350 dual channel spectrophotometer using matched glass curvets. The amount of protein in $10^9$ platelets was calculated by: (ug protein in

sample/40)/platelet count.

5.3.2. Cholesterol Assay.

The quantitative cholesterol content of whole platelets was determined by the method of Zlatkis et al., (1953) in which cholesterol reacts with ferric ions in acetic acid followed by the addition of concentrated sulfuric acid. A 100.0 ul aliquot of the platelet suspension was taken to 0.5 ml with re-distilled isopropanol in a 5 ml glass test tube. 1.0 ml of $FeCl_3$ in glacial acetic acid (1 mg/ml) was added and the contents mixed well. 1.0 ml of concentrated $H_2SO_4$ was slowly layered under the acetic acid-isopropanol mixture and the contents were mixed immediately and thoroughly. Color development was determined after 10 minutes at 540 nm on a Pye Unicam SP-6-350 dual channel spectrophotometer using matched glass curvets. Standards were run with each individual assay and the range was 0 to 32 ug cholesterin (Boehringer Mannheim) in isopropanol. The amount of cholesterol in $10^9$ platelets was calculated by: (ug cholesterol in sample/.1)/platelet count.

5.3.3. Quality Control Data For Platelet Total Cholesterol and Protein Content.

Platelets were prepared from 15.0 ml fresh citrated blood taken from a normal fasting volunteer. From the citrated blood 1.5 ml of PRP (platelet count adjusted to 1 x $10^6$/ml with PPP) was prepared. The PRP was

divided into 10 100.0 ul, and 10 40.0 ul portions. Total cholesterol and total protein content were determined on ten each of the platelet preparations (cholesterol on 100.0 ul portions and protein on 40.0 ul portions) over a four hour period. PRP preparations and cholesterol and protein analysis were conducted exactly as described for patients and controls.

## 5.4. Lipogram, Apolipoprotein, Uric Acid, and Blood Glucose assays.

Routine laboratory enzymatic colorimetric methods were used to determine serum lipids and uric acid. Blood glucose levels were determined on an IL 50B (Instrumentation Laboratory) chemical analyzer, which measures the optical density gradient generated by a glucose oxidase/peroxidase reaction. These were performed by the staff of the SAIMR Biochemistry Department at Baragwanath Hospital. Kits for serum lipids (with the exception of triglycerides) and uric acid were supplied by Boehringer Mannheim. The kit for triglyceride determination was supplied by Bio Merieux. HDL-cholesterol was determined by precipitation of apo B containing lipoproteins with phosphotungstic acid and magnesium followed by enzymatic determination of cholesterol. LDL and VLDL-cholesterol were estimated by calculations using HDL-cholesterol, total cholesterol, and total triglycerides after the method of Friedewald et al., (1972). Apolipoprotein A1 and B immuno-chemical

assays were done by the SAIMR Central Laboratories using kits supplied by Orion Diagnostica on a Multistat III analyzer.

5.5. Coagulation Factors and Inhibitors.

Fibrinogen and factor VII assays were performed by conventional technique. Plasminogen and protein C were performed by the staff at the Johannesburg General Hospital. Methods used are described by Dacie and Lewis (1984).

5.6. Statistical Evaluation.

A data base was created using the D Base III. program by Dr I.A.F. Dukes, and statistical analysis was performed on a Sperry PC using the epistat programme. For normally distributed data, the Student-t test was used. For non-parametric data, the Wilcoxon rank test was used. Differences were considered significant at a p value of less than 0.05 (Siegel 1956).

# CHAPTER 6

# 6. Results

## 6.1. Characteristics of Cases and Controls.

Characteristics of controls and patients with previous history of myocardial infarction can be seen in Table 6.1. There was an eleven year difference in the mean age of the two groups. Two patients were over the age of seventy and no matching normal volunteers could be found. Consequently, the age distribution was nonparametric, and with the Wilcoxon rank test, the ages did not show to be statistically different. Patients and controls differed little in their body mass index (BMI), blood pressure, and smoking habits. Socioeconomic background, as judged by the weekly take-home salary of those currently employed, was also similar. None of the test subjects maintained knowledge of parental or sibling history of CAD however, of those whose parents were deceased, many did not know exactly how they had died.

Table 6.1. Characteristics of Cases and Controls. Mean ± SD.

| | CONTROLS | PATIENTS |
|---|---|---|
| Number Of Subjects | 26 | 24 |
| Age (mean) | 45 ± 10 | 56 ± 11 |
| Age Range | 32 - 65 | 28 - 72 |
| BMI (kg/m2) | 26.0 ± 6.1 | 25.9 ± 8.6 |
| Blood Pressure (mmHg) | | |
| Systolic | 138 ± 20 | 141 ± 24 |
| Diastolic | 90 ± 10 | 89 ± 14 |
| Weekly Take Home Salary | R190 ± 25 | R165 ± 22 |
| Smoking > 10 Cigarettes/Day | 88% (22) | 92% (22) |
| Family History of CAD | None | None |

## 6.2. Gas Chromatogram Analysis of Platelets, Platelet Poor Plasma and Red Blood Cells.

In table 6.2, the saturated fatty acids myristic (14:0) and stearic (18:0) were significantly elevated in the case platelets ($p<.05$). Also in the case platelets, the total saturated fatty acids were significantly higher

Table 6.2. Relative Percent of Individual Fatty Acid Methyl Esters (FAME), Combined Dimethyl Acetyls (DMA) and Cholesterol to Total Fatty Acid Ratio of Case and Control Platelets. Mean ± SD.

| FAME | | CONTROLS n=25 | PATIENTS n=23 |
|---|---|---|---|
| 14:0 | * | 1.08 ± 0.87 | 1.53 ± 0.53 |
| 16:0 | | 18.26 ± 2.5 | 19.07 ± 2.4 |
| 16:1 w-7 | | 0.91 ± 0.45 | 0.87 ± 0.30 |
| 18:0 | * | 17.96 ± 2.1 | 19.44 ± 2.0 |
| 18:1 | | 17.68 ± 2.5 | 16.72 ± 2.3 |
| 18:2 w-6 | | 8.24 ± 3.1 | 6.83 ± 2.8 |
| 18:3 w-3 | | 1.80 ± 0.28 | 1.89 ± 0.28 |
| 20:0 | | 1.50 ± 0.24 | 1.05 ± 0.21 |
| 20:1 | | 0.45 ± 0.12 | 0.43 ± 0.11 |
| 20:2 w-9 | | 0.14 ± 0.40 | 0.15 ± 0.10 |
| 20:2 w-6 | | 0.46 ± 0.08 | 0.57 ± 0.32 |
| 20:3 w-9 | | 0.07 ± 0.04 | 0.10 ± 0.09 |
| 20:3 w-6 | | 1.04 ± 0.24 | 1.05 ± 0.23 |
| 20:4 w-6 | | 19.38 ± 2.8 | 20.06 ± 2.7 |
| 20:5 w-3 | ** | 0.44 ± 0.17 | 0.24 ± 0.09 |
| 22:0 | | 2.87 ± 0.40 | 2.95 ± 0.62 |
| 22:4 w-6 | | 1.73 ± 0.37 | 1.91 ± 0.52 |
| 22:5 w-6 | | 0.21 ± 0.04 | 0.29 ± 0.28 |
| 22:5 w-3 | | 1.32 ± 0.28 | 1.16 ± 0.28 |
| 22:6 w-3 | | 1.78 ± 0.36 | 1.72 ± 0.43 |
| 24:0 | | 1.48 ± 0.24 | 1.39 ± 0.47 |
| 24:1 | | 1.20 ± 0.20 | 1.20 ± 0.33 |
| Total DMA | | 5.52 ± 1.7 | 4.72 ± 2.3 |
| Cholesterol/FAME | | 0.17 ± 0.08 | 0.14 ± 0.04 |
| Total Saturated | * | 42.65 ± 2.9 | 45.87 ± 3.2 |
| Total Monoenoic | | 20.24 ± 2.9 | 19.48 ± 2.9 |
| Total PUFA | * | 37.11 ± 2.8 | 34.52 ± 2.3 |

* $p<.05$ ** $p<.01$ *** $p<.001$

than the controls while the total polyunsaturated fatty acids (PUFA) were significantly decreased ($p<.05$). This trend was also seen in plasma (Table 6.3), although it was not statistically significant. There was little difference in the total saturated and total

TABLE 6.3. Relative Percent of Individual Fatty Acid Methyl Esters (FAME), Combined Dimethyl Acetyls (DMA) and Cholesterol to Total Fatty Acid Ratio Of Control and Case Platelet Poor Plasma. Mean ± SD.

| FAME | | CONTROLS n=25 | PATIENTS n=23 |
|---|---|---|---|
| 14:0 | | 1.27 ± 0.80 | 1.13 ± 0.62 |
| 16:0 | | 24.79 ± 3.2 | 25.20 ± 2.7 |
| 16:1 w-7 | | 3.37 ± 1.8 | 3.06 ± 1.4 |
| 18:0 | | 8.15 ± 0.85 | 8.73 ± 1.5 |
| 18:1 | | 21.04 ± 4.3 | 21.60 ± 3.9 |
| 18:2 w-6 | | 24.27 ± 7.2 | 22.44 ± 5.5 |
| 18:3 w-3 | | 0.35 ± 0.15 | 0.33 ± 0.14 |
| 20:0 | | 1.18 ± 0.50 | 1.25 ± 0.43 |
| 20:1 | | 0.13 ± 0.04 | 0.13 ± 0.037 |
| 20:2 w-9 | | 0.45 ± 0.15 | 0.52 ± 0.38 |
| 20:2 w-6 | | 0.22 ± 0.15 | 0.19 ± 0.09 |
| 20:3 w-9 | | 0.22 ± 0.10 | 0.18 ± 0.09 |
| 20:3 w-6 | | 1.62 ± 0.45 | 1.72 ± 0.33 |
| 20:4 w-6 | | 6.61 ± 1.3 | 7.11 ± 1.8 |
| 20:5 w-3 | ** | 0.85 ± 0.27 | 0.47 ± 0.19 |
| 22:0 | | 0.68 ± 0.30 | 0.87 ± 0.33 |
| 22:4 w-6 | | 0.26 ± 0.05 | 0.32 ± 0.09 |
| 22:5 w-6 | | 0.21 ± 0.20 | 0.21 ± 0.19 |
| 22:5 w-3 | | 0.57 ± 0.15 | 0.56 ± 0.14 |
| 22:6 w-3 | | 2.41 ± 0.70 | 2.40 ± 0.81 |
| 24:0 | | 0.58 ± 0.25 | 0.71 ± 0.28 |
| 24:1 | | 0.77 ± 0.25 | 0.87 ± 0.38 |
| Total DMA | | 0.97 ± 0.40 | 0.82 ± 0.19 |
| Cholesterol/FAME | | 0.17 ± 0.15 | 0.23 ± 0.09 |
| Total Saturated | | 36.65 ± 2.9 | 37.89 ± 3.8 |
| Total Monoenoic | | 25.31 ± 2.4 | 25.66 ± 3.1 |
| Total PUFA | | 38.04 ± 3.1 | 36.45 ± 3.5 |

* $p<.05$ ** $p<.01$ *** $p<.001$

polyunsaturated fatty acids in RBCs which are shown in table 6.4. Linoleic acid was decreased in the patient platelets and plasma but it was nonsignificant. Linoleic acid levels were very similar in the RBCs of cases and controls. Only eicosapentaenoic acid (20:5 w-3) demonstrated a statistical difference in all

Table 6.4. Relative Percent of Individual Fatty Acid Methyl Esters (FAME), Combined Dimethyl Acetyls (DMA) and Cholesterol to Total Fatty Acid Ratio Of Control and Case Red Blood Cells. Mean ± SD.

| FAME | CONTROLS n=25 | PATIENTS n=23 |
|---|---|---|
| 14:0 | 0.37 ± 0.13 | 0.32 ± 0.14 |
| 16:0 | 25.76 ± 1.6 | 24.38 ± 2.2 |
| 16:1 w-7 | 0.68 ± 0.30 | 0.64 ± 0.28 |
| 18:0 | 11.47 ± 0.83 | 12.98 ± 1.5 |
| 18:1 | 14.36 ± 1.8 | 13.97 ± 1.3 |
| 18:2 w-6 | 10.97 ± 1.6 | 10.07 ± 2.2 |
| 18:3 w-3 | 0.[illegible] ± 0.20 | 0.65 ± 0.21 |
| 20:0 | 1.03 ± 0.20 | 0.94 ± 0.45 |
| 20:1 | 0.17 ± 0.05 | 0.15 ± 0.04 |
| 20:2 w-9 | 0.38 ± 0.08 | 0.37 ± 0.11 |
| 20:2 w-6 | 0.12 ± 0.08 | 0.10 ± 0.05 |
| 20:3 w-9 | 0.15 ± 0.23 | 0.13 ± 0.14 |
| 20:3 w-6 | 1.33 ± 0.36 | 1.42 ± 0.21 |
| 20:4 w-6 | 11.73 ± 1.9 | 12.12 ± 1.4 |
| 20:5 w-3 *** | 0.85 ± 0.32 | 0.52 ± 0.25 |
| 22:0 | 1.71 ± 0.46 | 1.93 ± 0.47 |
| 22:4 w-6 | 2.16 ± 0.48 | 2.42 ± 0.52 |
| 22:5 w-6 | 0.89 ± 0.37 | 0.98 ± 0.61 |
| 22:5 w-3 ** | 1.91 ± 0.28 | 1.70 ± 0.33 |
| 22:6 w-3 | 4.87 ± 0.86 | 4.82 ± 1.3 |
| 24:0 | 4.87 ± 0.82 | 5.70 ± 1.7 |
| 24:1 | 3.51 ± 0.39 | 3.33 ± 0.57 |
| Total DMA | 6.91 ± 1.5 | 5.01 ± 2.3 |
| Cholesterol/FAME * | 0.56 ± 0.09 | 0.46 ± 0.08 |
| Total Saturated | 45.90 ± 2.3 | 46.25 ± 3.4 |
| Total Monoenoic | 18.72 ± 2.1 | 18.09 ± 2.2 |
| Total PUFA | 35.38 ± 3.1 | 35.66 ± 3.0 |

* p<.05 ** p<.01 *** p<.001

three tissues studied and it was found to be decreased in the patients ($p<.05$ in platelets and plasma; $p<.001$ in RBCs). There was a tendency for the cholesterol to FAME ratio to be decreased in the platelets of the patients and increased in the plasma of the patients but it was not significant. The cholesterol to FAME ratio was significantly decreased in the patient RBCs.

Table 6.5. Quality Control Data on the Determination of Relative Percent FAME, combined Dimethyl Acetyls (DMA) and Cholesterol to Total Fatty Acid Ratio from Bank Blood Platelet Poor Plasma.

| COMPOUND | MEAN ± S.D. (Rel %) n=10 | C.V. (%) | C.V. (%)* |
|---|---|---|---|
| 14:0 | 0.18 ± 0.07 | 38.0 | - |
| 16:0 | 18.59 ± 1.20 | 6.4 | 3.5 |
| 16:1 w-7 | 1.72 ± 0.18 | 10.5 | - |
| 18:0 | 11.78 ± 0.31 | 2.6 | 4.8 |
| 18:1 | 28.45 ± 0.29 | 1.0 | 3.0 |
| 18:2 w-6 | 19.00 ± 0.13 | 0.7 | 1.9 |
| 18:3 w-3 | 0.37 ± 0.12 | 32.4 | - |
| 20:0 | 0.26 ± 0.01 | 3.8 | 7.8 |
| 20:1 | 0.21 ± 0.02 | 9.5 | - |
| 20:2 w-9 | 0.13 ± 0.04 | 7.6 | - |
| 20:2 w-6 | 0.31 ± 0.05 | 16.1 | 10.2 |
| 20:3 w-9 | 0.19 ± 0.03 | 15.8 | - |
| 20:3 w-6 | 1.66 ± 0.08 | 4.8 | 6.5 |
| 20:4 w-6 | 9.73 ± 0.38 | 3.9 | 3.1 |
| 20:5 w-3 | 0.55 ± 0.02 | 3.6 | - |
| 22:0 | 0.14 ± 0.02 | 14.3 | 4.5 |
| 22:4 w-6 | 0.03 ± 0.006 | 20.0 | - |
| 22:5 w-6 | 0.39 ± 0.02 | 5.1 | - |
| 22:5 w-3 | 1.14 ± 0.20 | 17.5 | 4.6 |
| 22:6 w-3 | 4.37 ± 0.29 | 6.6 | 7.0 |
| 24:0 | 0.05 ± 0.009 | 18.0 | 4.0 |
| 24:1 | 0.74 ± 0.14 | 18.9 | 2.9 |
| Total DMA | 0.36 ± 0.19 | 52.7 | - |
| Cholesterol/FAME | 0.17 ± 0.13 | 76.5 | - |
| Total Saturated | 31.00 ± 1.01 | 3.2 | - |
| Total Monoenoic | 30.01 ± 3.08 | 10.3 | - |
| Total PUFA | 37.98 ± 0.98 | 3.2 | - |

* Data obtained from M[illegible]skiet et al., 1983.

GC quality control data for within-run precision are given in Table 6.5. The CV for the major FAMEs demonstrated good within-run precision and agreed well with quality control data generated by other laboratories (Muskiet et al., 1983). Native cholesterol demonstrated poor reproducibility on the polar SP 2340 column. Also, the DMA to total fatty acid ratio demonstrated unacceptable reproducibility possibly due to the inherent instability of DMAs.

## 6.3. Serum Lipids, Lipoproteins, Apolipoproteins, Uric Acid and Blood Glucose.

Serum lipids, lipoproteins, apolipoproteins and uric acid values for 24 controls and 25 patients are shown in Table 6.6. The two values most strongly associated with CAD among the serum lipids and lipoproteins were total

Table 6.6. Serum Lipids, Lipoproteins, Apolipoproteins and Uric Acid. Mean ± SD. Lipid and Lipoprotein Values given in mMol/L. Apolipoproteins are given in g/L.

| | | CONTROLS | PATIENTS |
|---|---|---|---|
| Number Of Subjects | | 24 | 25 |
| Total Serum Cholesterol | ** | 4.99 ± 0.77 | 5.82 ± 0.85 |
| Serum Triglycerides | | 2.10 ± 1.5 | 2.30 ± 1.4 |
| Serum HDL Cholesterol | | 1.09 ± 0.44 | 0.93 ± 0.35 |
| Serum VLDL Cholesterol | | 1.09 ± 0.68 | 1.06 ± 0.65 |
| Serum LDL Cholesterol | * | 2.92 ± 0.93 | 3.86 ± 0.99 |
| Serum Apolipoprotein A1 | | 1.27 ± 0.39 | 1.35 ± 0.55 |
| Serum Apolipoprotein B | * | 0.86 ± 0.24 | 1.13 ± 0.25 |
| Serum Uric Acid | *** | 0.37 ± 0.05 | 0.47 ± 0.10 |
| Blood Glucose | *** | 4.47 ± 1.12 | 6.05 ± 2.42 |

* p<.05 ** p<.01 *** p<.001

serum cholesterol and serum LDL cholesterol ($p<.01$ and $p<.05$ respectively). In the patients, the total serum cholesterol and LDL cholesterol were elevated. Of the apolipoproteins, only serum apolipoprotein B was significantly different and was found to be increased in the patients ($p<.05$). Serum uric acid and blood glucose were also shown to be elevated in the patients ($p<.001$).

## 6.4 Platelet Cholesterol, Platelet Protein and Cholesterol to Protein Ratio.

As can be seen from table 6.6, there was a significant difference in the cholesterol to protein ratio, with an decreased level found in the patients ($p<.001$). The large variation seen in the total platelet cholesterol is probably the result of problems encountered in acquiring a consistent day to day platelet count of the

Table 6.7. Total Platelet Cholesterol and Protein Content. Cholesterol to Protein Ratio. Mean ± SD. Values are Given as ug/$10^9$ Platelets.

| | | CONTROLS | PATIENTS |
|---|---|---|---|
| Number Of Subjects | | 15 | 18 |
| Total Cholesterol | | 251.6 ± 65.0 | 214.7 ± 65.7 |
| Total Protein | | 3.46 ± 1.1 | 3.36 ± 0.89 |
| Cholesterol/Protein | *** | 74.8 ± 7.4 | 64.0 ± 10.1 |

* $p<.05$ ** $p<.01$ *** $p<.001$

saline washed platelets. As indicated in Table 6.8, the cholesterol to protein ratio demonstrated acceptable within-run precision.

*Table 6.8.* Quality Control Data on the Determination of Platelet Total Cholesterol, Total Protein and Cholesterol to Protein Ratio. Values are given as ug/$10^9$ Platelets.

| COMPOUND | MEAN ± SD n=10 | C.V.(%) |
|---|---|---|
| Total Cholesterol | 182.7 ± 15.87 | 8.7 |
| Total Protein | 3.62 ± 0.27 | 7.5 |
| Cholesterol/Protein | 49.8 ± 1.65 | 3.3 |

## 6.5 *Coagulation Factors and Inhibitors.*

Selected coagulation factors and inhibitors were analyzed and these results are given in table 6.9. Fibrinogen demonstrated a large variation in both groups but appeared to be normally distributed. Fibrinogen was significantly higher in the patients ($p<.001$). Factor VII was also slightly elevated in the patients although this was not statistically significant. Of the inhibitors, only plasminogen demonstrated a significant difference, and was elevated in the patients ($p<.01$). Protein C was similar among the two groups. There was a large variation of the numbers of subjects in each group because some of the samples were lost while in transit to the Johannesburg General Hospital.

Table 6.9. Selected Coagulation Factors and Inhibitors. Mean ± SD.

| | | No. Of Subjects | CONTROLS | No. Of Subjects | PATIENTS |
|---|---|---|---|---|---|
| Fibrinogen(g/L) | *** | 23 | 275 ± 120.4 | 18 | 439 ± 127.2 |
| Factor VII(%) | | 21 | 125 ± 54.0 | 15 | 164 ± 72.0 |
| Plasminogen(%) | ** | 26 | 84 ± 12.8 | 20 | 98 ± 13.5 |
| Protein C(%) | | 26 | 103 ± 32.1 | 16 | 122 ± 32.0 |

* $p<.05$ ** $p<.01$ *** $p<.001$

# CHAPTER 7

# 7. *Discussion and Conclusions.*

## 7.1. *Discussion.*

### 7.1.1 *Fatty Acids.*

The detected occurrence of MI among urban Black Africans of the Witwatersrand area has, over the past decade, increased steadily. Many feel that the emergence of CAD in white populations is largely the result of increased consumption of animal protein and animal fat. (Keys 1975; Renaud et al., 1986). Intrapopulation studies have determined that dietary intake of saturated fat is the environmental factor most closely associated with CAD (Keys 1975). Also, a number of studies conducted in white populations have associated interpopulation variation in the saturated and polyunsaturated fatty acid content of platelets, plasma and RBCs with the occurrence of MI (Renaud et al., 1970; Valles et al., 1979; Lea et al., 1982; Wood et al., 1984; Boberg et al., 1985). In order to determine the extent of dietary influence on the progression of MI in the urban black population, the total fatty acid content of platelets, plasma and RBCs was determined in Black African males who were between six and eighteen months post MI. These values were compared to a normal urban black group of similar age, geographical location and socioeconomic status.

While variations in methodology and availability of

patient and control material make comparisons difficult, the platelet fatty acid profile of post MI Black Africans is similar to that found in white male patients who have experienced an MI. The levels of total saturated fatty acids were increased in the African group and this resulted almost entirely from increased levels of myristic (14:0) and stearic (18:0) acids. The levels of total PUFA were significantly decreased in patient platelets and this was mostly the result of decreased levels of linoleic acid (18:2 w-6).

Elevated levels of 18:0 in platelets of white males with MI have been demonstrated in whole platelet preparations by Renaud et al., (1970) and in the platelet phospholipid fraction by Valles et al., (1979). The mechanism whereby saturated fats influence platelet function is not known however, cause and effect relationships indicate that increased levels of saturated fats in the diet lead to hyperaggragable platelets (Renaud et al., 1986; Hornstra et al., 1973). Also, in vitro, 18:0 is a more potent inducer of platelet aggregation than are the unsaturated fatty acids (Hoak et al., 1967).

Decreased levels of linoleic acid in platelets have been demonstrated in white post MI males (Renaud et al., 1970; Valles et al., 1979; Nikkari et al., 1983; Wood et al., 1987). In the Black African group linoleic acid

was decreased in platelets however it was nonsignificant. It should be mentioned that these studies conducted in white populations had access to large numbers of individuals with the disease and because of this, were able to closely match cases and controls. There was a difficulty in matching cases and controls in the current study due to the low incidence of the disease, an inability to contact patients on an appointment basis and a lack of normal volunteers in the appropriate age group.

From what is known about the metabolism of fatty acids to prostaglandins, arachidonic acid in platelets has the greatest potential to create a thrombotic tendency (Needleman et al., 1986). It has been suggested that differences in thrombotic disorders among populations may partially be due to differences in dietary intake of arachidonic acid (Meade et al., 1985). While it has been shown that the arachidonic acid content in platelets is highest in urban White South Africans, lower in urban Black South Africans and lowest in rural Black South Africans (Chetty et al., 1988), the involvement of arachidonic acid in the development of CAD remains questionable. While it is known that arachidonic acid aggregates platelets in vitro, the release of arachidonic acid in vivo is dependent on phospholipase activity and this has not been shown to be effected by dietary fats. Furthermore, there is a

propensity for the aggregating effects of free arachidonic acid *in vivo* to be counteracted by endothelial production of $PGI_2$ from arachidonic acid. There was no difference in the levels of arachidonic acid in either platelets, plasma or red blood cells, indicating that differing levels of this substance may not contribute to the occurrence of MI in this population.

In the current study, eicosapentaenoic acid (20:5 w-3) was decreased in the patient platelets. Greenland Eskimos who ingest large amounts of omega-3 fatty acids have low incidences of thrombotic disorders (Dyerberg and Bang 1979). This observation was given a biochemical rationale by the finding that 20:5 w-3 is a competitive inhibitor of the cyclo-oxygenation of 20:4 w-6, and is itself metabolized by the platelet to $TxA_3$, a non-stimulating prostaglandin (Dyerberg *et al.*, 1978; Needleman *et al.*, 1980) The extent to which 20:5 w-3 is affecting platelet function in the African group is difficult to surmise given that the levels in both groups were very low. During fish eating trials, changes in platelet function have been associated with considerably increased levels of 20:5 w-3 and 22:6 w-3 together with a significant decrease in the levels of the w-6 fatty acids in platelet phospholipids (Thorngren and Gustafson 1981). In Greenland Eskimos, the 20:5 w-3 to 20:4 w-6 ratio in

platelets is near unity (Dyerberg and Bang 1979). While the therapeutic dose of 20:5 w-3 has not been established, slight but significantly lower levels of 20:5 w-3 in the platelets of white males who have had an MI has been demonstrated (Boberg et al., 1985). Also, a recent study indicates that platelet omega-3 fatty acids have a higher discriminating power between high risk and low risk patients with MI and normal controls than the polyunsaturated 18 carbon fatty acids (Aursnes et al., 1986). The omega-3 fatty acids may effect platelet function by means other than the prostaglandin pathway (Thorngren et al., 1984), possibly by inhibiting formation of the inositol phosphates (Chetty et al., 1988). These recent findings may suggest a mechanism whereby 20:5 w-3 could influence hemostasis even though levels are very much less than that of 20:4 w-6.

The possible effects of dipyridamole (Persantine) on the levels of platelet total fatty acids must be mentioned as 17 out of 24 members of the patient group were receiving 75 mg/day of this antiplatelet medication. While the exact mechanism for the antiplatelet effect of dipyridamole is not known, the hypothesized mode of action involves two separate yet related aspects. First, dipyridamole inhibits the active uptake of adenosine by red blood cells and endothelial cells thus allowing for the increased passive diffusion of this

substance into platelets. Adenosine inhibits platelet function via stimulation of adenylate cyclase. Second, dipyridamole may inhibit the degradation of cAMP via inhibition of phosphodiesterase (Fitzgerald et al., 1987). This is believed to be potentiated mainly in the presence of $PGI_2$ (Moncada et al., 1978). In vivo, dipyridamole is bound to an acid glycoprotein that limits its availability to bind to platelets (Mahoney et al)., 1982). Consequently, high doses of the drug are required to demonstrate reduced platelet function in vitro.

Increased levels of cAMP in platelets may effect the turnover rate of eicosanoid producing fatty acids via inhibition of phospholipases $A_2$ and C (Needleman et al., 1986). However, the possibility of this altering the absolute levels of individual platelet polyunsaturated fatty acids as measured in the current study is doubtful. In well nourished individuals, characteristic fatty acid levels are maintained by a rapid deacylation-reacylation cycle, with precursor being acquired primarily from the dietary fatty acid pool (Lands and Samuelsson 1968; Neufeld et al., 1983). Because of this, measurements of depletion or sparing of specific platelet fatty acids have not proven to be accurate indicators of platelet activation (Prescott and Majerus 1981) or deactivation (Chetty, personal communication 1990). Finally, no study has demonstrated

that treatment with drugs which alter platelet metabolism cause shifts in the ratio of PG precursor fatty acids (e.g., 20:4 n-6/18:2 n-6 or 20:4 n-6/20:6 n-3) in a manner similar to that seen in long term dietary alterations.

In the current study, the platelet mean EPA level of patients receiving dipyridamole (0.21 $\pm$ 0.07, n=17) was compared to the mean of the patients not receiving the drug (0.26 $\pm$ 0.08, n=6). These levels were not significantly different (p=0.246). Also, when the mean EPA level of the individuals not receiving the drug was compared to that of the control group (0.44 $\pm$ 0.17, n=25) the difference remained significant (p=0.007).

Finally, studies which have shown decreased platelet aggregation to ADP and collagen following oral doses of 200 to 400 mg/day of dipyridamole have been unable to demonstrate increases in intracellular levels of cAMP (Gregov et al., 1987; Tsien and Sheppard, 1981; Mehta et al., 1982; Weiss et al., 1981). It seems unlikely that free drug concentrations sufficient to increase platelet cAMP levels would be achieved with a dose of 75 mg/day.

The fatty acid content of platelets in normal subjects may not accurately reflect the dietary intake of polyunsaturated fatty acids (PUFA) (Dougherty et al.,

1987). While plasma lipid esters have been shown to differ between patients with CAD and normal controls (Kirkeby et al., 1972a; Miettinen et al., 1982), plasma may not be a good indicator of fatty acid intake due to fast turnover, hepatic involvement in fatty acid synthesis and variations in plasma lipase activity. Due to a lack of metabolic activity the fatty acid content of red blood cell (RBC) acyl lipids is controlled almost exclusively by dietary fat intake (Pittman and Martin 1966; Farquhar and Ahrens 1963). Moreover, the fatty acid levels are unlikely to be altered by the presence of vascular disease or recent dietary intervention. The evaluation of RBC total fatty acid composition is a valid indicator of dietary fatty acid intake due to the somewhat uniform distribution of fatty acids among the various phospholipid classes (Dougherty et al., 1987; Farquhar and Ahrens 1963).

Many retrospective studies conducted in white populations, by measuring the fatty acid content of fatty acid stable tissues such as RBCs or adipocytes, have related differences in the occurrence of MI to differences in dietary fatty acid intake (Kirkeby et al., 1972b; Lea et al., 1982; Simpson et al., 1982; Wood et al., 1984; Rapley et al., 1987). The fatty acids most often implicated have been 18:2 w-6 (Simpson et al., 1982; Wood et al., 1984; Rapley et al., 1987) and/or 20:5 w-3 (Kirkeby et al., 1972b; Lea et al.,