ETHNIC DIFFERENCES IN ADIPOGENESIS AND THE ROLE OF ALKALINE PHOSPHATASE IN THE CONTROL OF ADIPOGENESIS IN HUMAN PREADIPOCYTES AND 3T3-L1 CELLS

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A thesis submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in fulfilment of the requirement for the degree of Doctor of Philosophy.
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Declaration

This is to certify that the thesis, **Ethnic Differences in Adipogenesis and the Role of Alkaline Phosphatase in the Control of Adipogenesis in Human Preadipocytes and 3T3-L1 Cells** , presented for the Degree of Doctor of Philosophy , at the University of the Witwatersrand, Johannesburg, is my own work and has not been presented for a Degree at any other University

A.T. ALI
M Sc
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Abstract

Alkaline phosphatase (ALP) is a ubiquitously expressed enzyme, that has been shown to play a role in cell differentiation and organogenesis. One study has also demonstrated ALP activity in rat adipocytes. The purpose of the present study was therefore to determine whether ALP is expressed in preadipocytes and what role it may have in adipogenesis. ALP activity was detected in the murine preadipocyte cell line, 3T3-L1, and in human preadipocytes isolated from mammary tissue, and from subcutaneous abdominal fat depots. In all the cell types studied ALP activity increased in parallel with adipogenesis. In the 3T3 –L1 cell line the tissue- non -specific ALP inhibitors, levamisole and histidine inhibited ALP activity, and adipogenesis, whereas the tissue specific ALP inhibitor Phe-Gly-Gly did not inhibit ALP or adipogenesis.

In human preadipocytes, histidine inhibited adipogenesis and ALP activity, whereas levamisole inhibited adipogenesis, but did not block ALP activity in intact cells. However, levamisole did inhibit ALP activity by 50% in cell extracts. Levamisole was able to inhibit adipogenesis in human preadipocytes. The tissue specific ALP inhibitor, Phe Gly Gly, did not inhibit ALP activity or adipogenesis in human preadipocytes.

ALP activity and adipogenesis, were compared in preadipocytes isolated from mammary tissue taken from black (13) and white (15) female subjects. Both ALP activity and adipogenesis, were lower in white compared to black female subjects.

Immunocytochemical, analysis of the 3T3-L1 cell line and human preadipocytes demonstrated that ALP activity was restricted to the lipid droplets of these cells.

ALP activity was also measured in serum samples obtained from 100 African subjects (74 females and 26 males) of varying BMI. ALP activity was found to be higher in obese than lean subjects, whereas, the other liver enzymes or products measured in serum were not. In fact these variables correlated to varying degrees with waist-hip ratio, whereas ALP levels did not. This suggest that liver function is predominantly influenced by abdominal obesity whereas serum ALP levels are more influenced by overall body adiposity.

In conclusion, ALP, may be involved in the control of adipogenesis, in the 3T3-L1 preadipocyte cell line and in human preadipocytes isolated from mammary adipose tissue and subcutaneous abdominal adipose tisssue. The presence of ALP activity in lipid droplets in 3T3-L1 cells and human preadipocytes, and the ability of ALP inhibitors to block adipogenesis strongly suggest that ALP plays a role in the control of adipogenesis.

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DEDICATION

I dedicate this work to my father Tariq Ali Dawood.

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CHAPTER ONE

INTRODUCTION

Introduction

Obesity is defined as an excess accumulation of body fat associated with increased fat cell size and number. Obesity is a common and serious medical problem all over the world especially in industrial countries. Although obesity is associated with stigma in western countries, the public opinion of obesity and overweight in the Middle East is different being associated with health (i.e. a person is eating "well" and is also not HIV positive, which is associated with weight loss) and wealth. This is also the case in some African societies.

Obesity occurs when energy intake is greater than energy expenditure. The surplus energy will be stored as fat in the adipose tissue. In the last decade, there has been a plethora of data relating to the fact that adipose cells are not just a storage depot for excess calories, but rather a metabolically active tissue Leptin and more recently, a number of additional cytokines have been reported to be secreted by adipocytes and to have paracrine as well as endocrine effects on a variety of target tissues .Relative to the issue of cytokines and insulin resistance ,tumour necrosis factor α (TNFα) has been reported to modify the insulin signalling pathway via serine phosphorylation of insulin receptor substrate-1,a primary substrate of the insulin receptor (Gregoire et al., 1998;Frühbeck et al., 2001).

Adipogenesis is the transformation of fibroblast like preadipocytes into mature adipocytes as a response to insulin and other metabolic processes. Although many of the molecular details about adipogenesis are still unknown,

studies found many factors involved in this processes. Some stimulators include, peroxisome proliferator-activated receptor γ (PPAR γ), insulin-like growth factor I (IGF-I), Macrophage colony stimulating factor, fatty acids, prostaglandins and glucocorticoids. Inhibitors include glycoproteins, transforming growth factor-β(TGF-β), inflammatory cytokines and growth hormone (MacDougald and Mandrup, 2002). Beside those factors, there are others like age, gender, life style that may affect this process in one way or another (Frühbeck et al., 2001).

Obesity is associated with stress, incontinence , complications of pregnancy , menstrual irregularities , excess facial hair , increased surgical risk, and psychological disorders such as depression (Jung, 1997; Kopelman, 2000; Stevens , 2000), type 2 diabetes (Maggio and Pi-Sunyer, 2003; Must et al., 1999), cardiovascular disease (Kannel et al., 1996; Lovejoy et al., 2001), sexual dysfunction (Hertz et al., 1979; Franks, 1998), and cancer (Flegal et al.,1998). Weight gain in adulthood is associated with significant increased mortality. Epidemiological evidence shows that the BMI associated with the lowest mortality falls within the range of 18.5-24.9, and that thinner people live longer than over weight and obese people (Stevens, 2000).

Adipogenesis could lead to central obesity if it occurs in the abdominal fat depot and peripheral obesity if it occurs in subcutaneous fat tissue .While the total number of adipocytes is increased with increasing fat mass, the increased number and percentage of large adipocytes may partially account for the inability

of adipose tissue to function properly and this may contribute to some of the health problems associated with obesity (Rosen et al., 2000).

Many enzymes and hormones play major roles in adipogenesis and obesity. However the enzyme alkaline phosphatase (ALP) has not been studied for its role in adipogenesis and obesity. ALP is found in a number of different tissues, but its precise function in many of these tissues is not known. However it is thought that, ALP may play a role in cellullar differentiation and bone mineralization. Therefore, the present study was performed to determine whether ALP is expressed in human preadipocytes or the murine 3T3-L1 preadipocyte cell line and if so, whether it is involved in adipogenesis. Furthermore, because ethnic differences exist in the prevalence of obesity within different South African population group's investigations were performed to determine whether ethnic differences are present in the level of adipogenesis.

CHAPTER TWO

LITERATURE REVIEW

Prevalence of obesity

Obesity is defined medically as a state of increased body weight, more specifically of increased fat tissue mass. It is associated with increased fat cells size and number, and usually is followed by serious health consequences if not controlled. The word obesity is derived from the Latin word *obesus*, which means fattened by eating. Body weight and composition, and the storage of energy as triglyceride in adipose tissue, are determined by the interaction between genetic, metabolic, environmental, and psychological factors. These influences ultimately act by changing the balance, between energy intake and expenditure (Spiegelman and Flier, 2001).

Any disorder in the energy balance can lead to obesity .The term `obese` denotes excessive body weight relative to height. The most common medical assessment of obesity is body mass index (BMI) which is calculated based on body weight (measured in kilograms) divided by height (measured in meters),squared . People are considered overweight if they have a BMI 25-29.9, and they are considered obese if their BMI is more than 30 (Flegal et al., 1998). Some researchers divide obesity into three divisions according to the degree of obesity. When BMI is 30-35 the subject is considered to be obese, from 35-40 very obese and the state of the BMI over 40 as severe obesity. See table 2.1.

Table 2.1 WHO standard classification of obesity.

WHO Classification	Popular Description	BMI(kg/m2)	Risk of co-morbidities
Underweight	Thin	<18.5	Low
Normal range	Normal	18.5-24.9	Average
Pre-obese	Overweight	25-29.9	Increased
Obese Class I	Obese	30-34.9	Moderate
Obese Class II	Obese	35-39.9	Severe
Obese Class III	Morbidly Obese	>40.0	Very severe

The World Health Organization (WHO) reported in 2002 that the majority of adults in the United States are over weight , with increasing numbers being clinically obese, and the trend is increasing .There are at least 300 million people worldwide who are clinically obese. Among these about half a million people die as result of obesity –related diseases every year in the Untied States and Canada(220,000 deaths) and in 20 countries of Western Europe (320,000 deaths)(WHO , 2002).In the United States of America 65% of Americans were clinically overweight (Loos and Bouchard , 2003).

The World Institute reported in 2000 that, the number of overweight and obese people in the world was 1.1 billion (cited in Eckersley, 2001) .This year the WHO International Task Force (ITF)revealed that out of the world population of six billion there are 1.7 billion who are overweight or obese. Five of the top 10 global diseases identified by WHO are caused by obesity. More than 20% of European

children between the age of five and seventeen are now considered to be overweight or obese. North Africa, the Middle East and Asia are seeing a marked rise in children`s obesity, while the USA headed the list with more than 37%of children overweight or obese (cited in Abdel-Aziz, 2003).

Current data shows that the range of obesity prevalence in European countries is from 10% to 20% for men and 10% to 25% for women. Prevalence of obesity has increased by between 10-40% in the majority of European counties in the past 10 years .The most dramatic increase has been in the UK where it has more than doubled between 1980 and 1995; from 6% to 15% among men and from 8% to 17% among women, and has increased to 21% and 23.5% for men and women respectively in 2001 (National Audit Office, 2001). In USA the majority of adults are overweight with an increasing number being medically classified as obese. In Australia 67% of adult men and over 52% of the adult women are overweight or obese .Those figures in 1980 were 48% for adult men and 27% for adult women. The proportion of adult Australians who are obese has more than doubled over the past 20 years to 19% for men and 22% for women. Almost a quarter of Australian children and adolescent are over weight or obese (Cited in Eckersley, 2001).

The impact of obesity has social and economic relevance. The economic cost of obesity in the USA is estimated to be $117 billion, about 52.3% of those costs via direct health care of obesity and 47.7% indirect costs which represent by lost wages because people are unable to work because of morbidity. Overweight and

obesity is associated with higher risks of morbidity and premature death. There are more than 30 medical conditions associated with obesity (Colditz, 1999). Industrialisation and urbanisation have an important effect on the nutritional status of a population. It has lead to changes in lifestyle and diet. While these changes mean improvement in the standard of living, they also imply negative consequences such as unhealthy dietary patterns, diminishing physical activity and in parallel a growth in diet-related diseases.

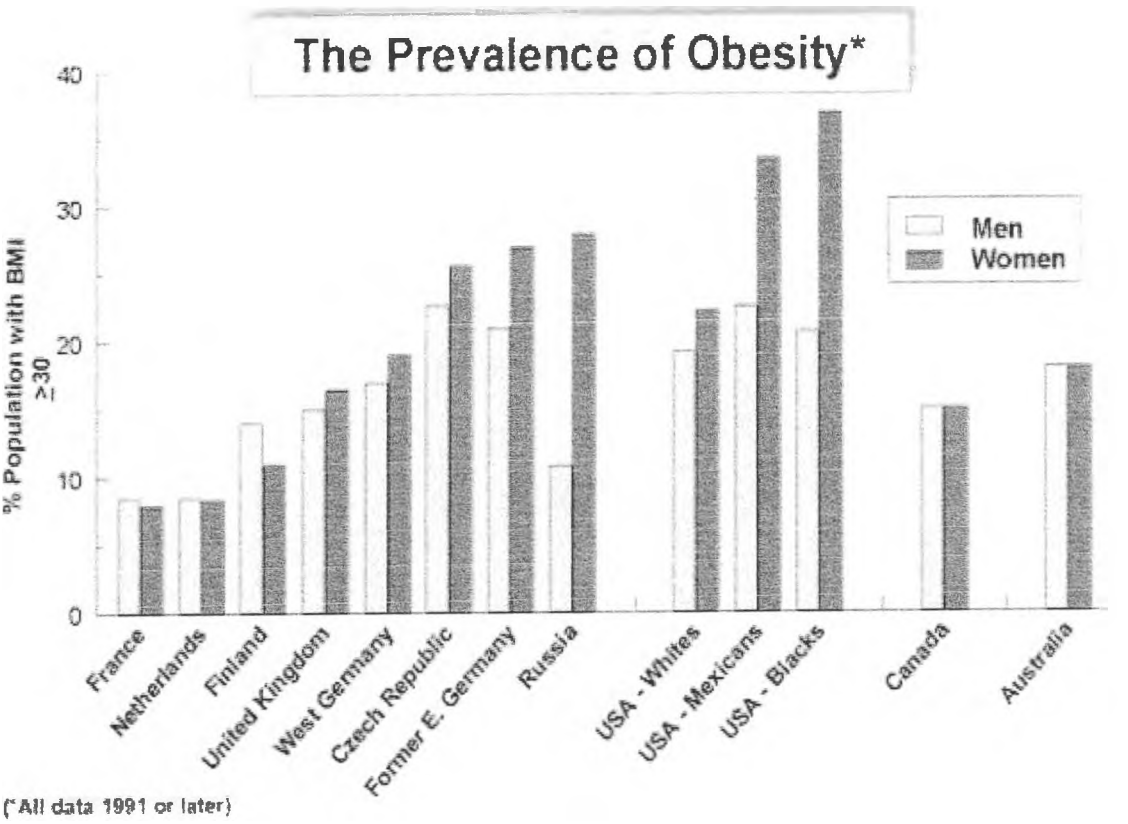


Figure 2.1 shows the prevalence of obesity in some countries, adapted from the The Global Challenge of Obesity and the International Obesity task force site page: www.iuns.org/features/obesity/tabfig.htm.

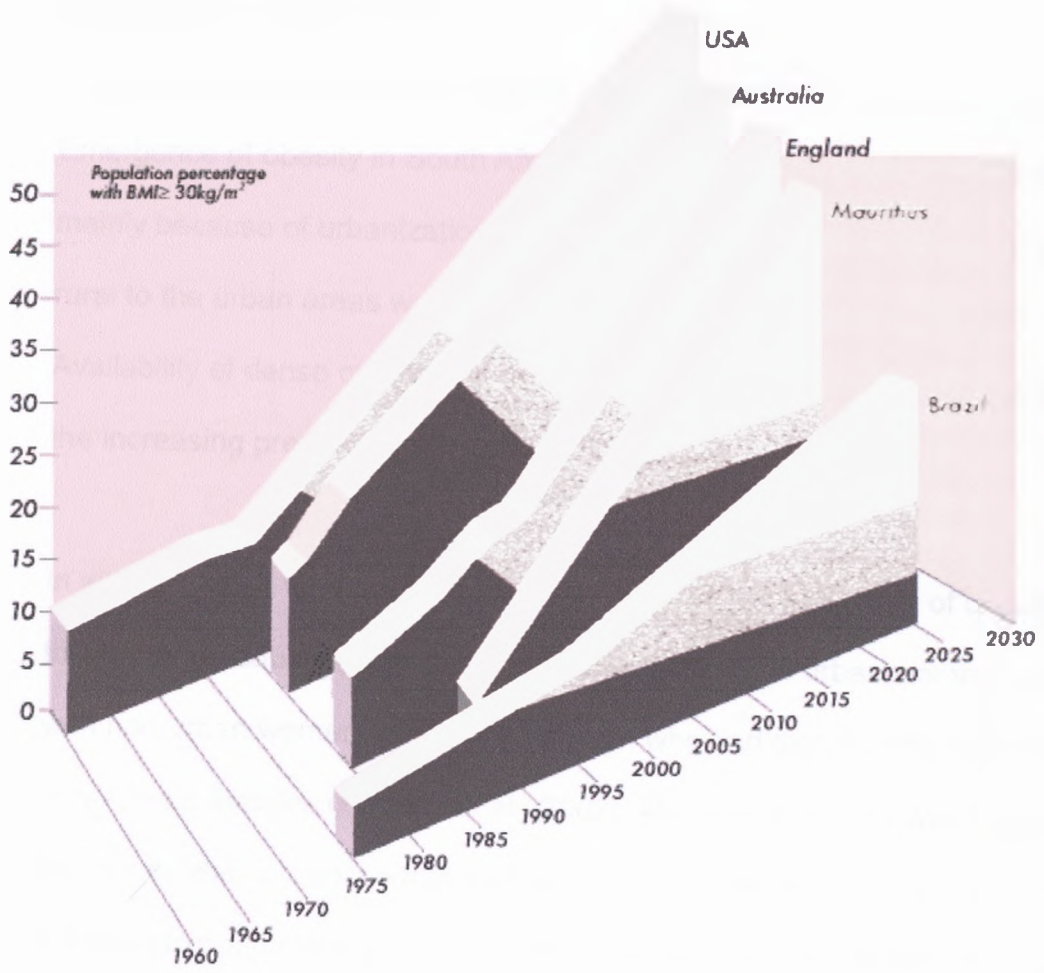


Figure 2 shows the increasing prevalence of obesity to 2025. Adapted from Kopelman, 2000.

Obesity in South Africa

South Africa is one of the developing countries that have been hit by obesity. Emergence of obesity in South Africa began (at least in black society) after 1994, mainly because of urbanization. Moving of the population in this country from rural to the urban areas was associated with huge changes in life style. Availability of dense caloric and fatty food is also another important factor behind the increasing prevalence of obesity in this country.

In women, those who are older have a significantly higher level of obesity than their younger counterparts. The same effect is seen in urban women compared with non-urban women and African women who had significantly higher BMI than non-African females (Puoane et al., 2002). Abdominal obesity was found to be highest in white urban women and non urban African women .This type of obesity is known to have more adverse health consequences than peripheral obesity (Donahue et al., 1987).

Obesity in African societies is viewed positively as it is popular opinion that it reflects affluence and happiness. These opinions are obviously not born out by the many studies showing the increased morbidity and mortality associated with obesity. Obesity and overweight are also viewed by African populations as reflecting healthy people, as the high prevalence of HIV-AIDS in South Africa has lead to the common misbelief that weight loss and leanness are signifiers of HIV

status and that being obese means you are not HIV positive. In some societies in South Africa obese and overweight women reflect the affluence of the men (Puoane et al., 2002).Researchers found that fat intake in African people increased from16% of the total calories in 1940 to 26% in 1990 to 30% in 2002 (Fox, 1940 cited by Puoane et al., 2002).These reasons make the management and controlling of obesity in South Africa very difficult.

According to the Heart Foundation in South Africa, there are about 56.6% of women and 29.2% of men over weight or obese. Abdominal obesity is linked to hypertension and diabetes in studies done in South Africa (Steyn et al., 1996; Levitt et al., 1993). Obesity in South Africa emerges women in early years of life. Thus a 10% of women were obese at age of 15 to 24 years (Puoane et al., 2002). In this a same study education was found to correlate positively with obesity, this was explained as the women with low education tend to do more manual work than their better educated counterparts. Table 2.2 provides a comparison of the rate of obesity from some African countries.

Country	Year	Ages (years)	Prevalence of obesity %	Prevalence of obesity %
			Men	Women
Ghana	1987	>20		0.9
Mali	1991	>20		0.8
Mauritius	1992	25 to 74	5	15
USA	1988-94	20 to 74	19.9	24.9
Tanzania	1986/89	35 to 64	0.6	3.6
South Africa				
Cape Peninsula, Africans	1990	25 to 64	13.9	48.6
Mangaung,Africans	1990	25 to 64	12.9	43.9
QwaQwa, Africans	1990	25 to 64	12.7	40.2
Cape Peninsula,Mixed	1982	25 to 64	7.2	31.4
South Western Cape,whites	1988	25 to 64	17.6	20.4
Durban, Indians	1990	25 to 69	3.5	17.6
Demographic&Health Survey ,African	1998	15	7.7	30.5
Demographic&Health Survey ,Mixed	1998	15	9.1	28.3
Demographic&Health Survey,Asian	1998	15	8.7	20.2
Demographic&Health Survey,Whites	1998	15	19.8	24.3

Table 2.2 Obesity Prevalence in South Africa and selected countries. Adapted from Puoane et al., 2002

Causes of Obesity

Overeating

Overeating is an important factor in the pathophysiology of obesity. Any change in food intake over time can markedly alter the level of energy storage. Eating a diet with a high glycemic index has been suggested to increase total energy intake and to promote obesity (Ludwig, 2000) .Indeed for most obese individuals, overeating must be counted as the primary cause of obesity. Overeating causes hyperinsulinaemia, which promotes more weight gain.

Thus a vicious circle leads to increased weight gain and increased hyperinsulinaemia (Cosford, 1999). There are many factors affecting food intake, such as the price of food, the size of portions , the protein content , taste of food, the sweetness of food ,fast foods, variety ,and accessibility of foods .Beside all these factors ,some societies eat food according to special principles like halal and kosher food. From epidemiological studies however, evidence for a high-fat diet promoting a positive energy balance and development of obesity is not definitive (Seidell, 1998).

Environmental Factors

The global epidemic of obesity is driven by an environment that encourages overeating and discourages physical activity, creating a consistent bias toward a positive energy balance. Environmental factors related to life style and cultural or socio-economic conditions are strong influences of obesity by influencing eating behaviour and physical activity .A marked change in BMI is frequently witnessed in migrant studies, where a population with common genetic heritage live under new environmental circumstances. Pima Indians, for example, living in the United States are on average 25 kg heavier than Pima Indians living in Mexico (Ravussin, 1995). A similar trend is seen for Africans living in the United States. In Nigeria the mean of BMI for men is 21.7 and for women 22.6 while in the United States the BMI for the Nigerian men and women is 27.1 and 30.8 respectively (cited in Kopelman 2000) .The same effect is observed in Aboriginals in Australia when they moved to urban areas (Thompson et al., 2003).

Genetic Factors

Obesity tends to run in families, suggesting a genetic link. Studies on animals and humans suggest that genetic factors affect energy balance and thus may act as determinants of BMI. The genetics of human obesity has been evaluated mostly from twin studies, which led to the conclusion that there is a strong genetic factor. In one study the BMIs of adopted Danes were evaluated many years after adoption and compared with the BMIs of their biologic and adoptive

parents. A correlation was observed between BMI at all levels of leanness and fatness between the biological parents and their offspring, who had been given up for adoption. This relationship was much weaker between children and their adoptive parents. These studies demonstrate that genetic factors are more important than environmental factors in determining body weight. Studies have confirmed the role of genetic predisposition in the aetiology of life-long obesity (Koeppen-Schomerus et al., 2001; Poulsen et al., 2001; Kunesova et al., 2002). If one of two parents is obese, the chances of their offspring developing obesity are 40%; if both parents were overweight all their lives, the chances increase to 70% that the offspring will also be overweight (Adams and Murphy, 2000). More than 300 genes, markers, and chromosomal regions have been defined to be associated with human obesity phenotypes (Chagnon et al., 2003). The first monogenic human obesity syndrome was reported in 1997, and was congenital leptin deficiency. A study of two severely obese cousins was found to have undetectable levels of serum leptin (Montague et al., 1997a).

Psychological Factors

Psychological factors also influence eating habits. Many people eat in response to negative emotions such as boredom, sadness, or anger, and the development and perpetuation of some of the human obesities could have a largely psychological basis. Depression and some neurological problems can lead to overeating and obesity (Doll et al.2000; Rippe et al. 1998).

Some other factors related to overweight and obesity

1. Age

As a general rule as people grow older their metabolic rate slows down and they do not require as many calories to maintain their weight. If caloric intake remains constant or increases they will therefore gain weight (Ajlouni et al., 2000; Flagel et al., 1998).

2. Ethnic influence

Studies have shown differences in prevalence of obesity among different ethnic groups (Björntorp ,1993a;Flagel et al., 1998; Puoane et al., 2002;Swinburn et al.,1996).The interpretation of these differences have been attributed partly to the effect of obesity related genes, which becomes clear especially when individuals life style improved (WHO, 2000).The Asian Indians have high percentage of body fat in relation to body mass index and muscle mass, this accompanied with a proportionate increase in visceral fat (Banerji et al.,1999). Obesity and abdominal obesity are twice as prevalent in African American women compared with white women (Ferdinand, 2004). In a South African study it was found that old white men have the highest rate of obesity in men in South Africa (Puoane et al., 2002), whilst middle aged African females have the highest prevalence of obesity.

3. Educational level

There is a strong inverse association between obesity and educational status especially in women .This has been reported in many populations (Ajlouni et al., 2001; Monteiro et al., 2001, Lipowicz, 2003). Ajlouni et al. (2001) found that the obesity is highly related to illiteracy in Jordan. Another study performed in Brazil found that in the less developed regions female obesity was negatively associated with education, and in a more developed regions obesity was also found to be associated negatively with education. In men it was found to be negatively associated with education, but to lesser degree than in women (Monteiro et al., 2001). Another study done by Lipowicz (2003) in the USA found that the level of education of the husband correlated negatively with the prevalence of obesity in the wives. This may be due to a higher level of education and economic status determining the dietary intake of the family, and an increased peer pressure to remain thin. In a study in South Africa (Puoane et al., 2002) women with no education had lower BMIs than those with schooling .These women tend to do more manual labour than their better educated counterparts. The same study found that women with tertiary education had a lower BMI than those with some schooling.

4. Economic status

An Australian study showed that compared with middle and high socio-economic status, even after controlling for socio-economic differences in age and height, BMI was significantly higher among low socio-economic than middle and high

socio-economic participants for the total group (O`Dxx, 2003).In urban areas of Brazil, over the period 1989-1997, increases in the prevalence of obesity have been relatively higher for the poorest population strata (Monterio et al., 2001).

5. Gender

Males have a higher resting metabolic rate than females, so males require more calories to maintain their body weight .Studies have confirmed that women have more body fat than men (Sjöström et al., 1972; Arner et al., 1991). Additionally when women become post-menopausal, their metabolic rate decreases significantly. That is part of the reason why many women start gaining weight after menopause. Studies found that women have a higher BMI than men, especially after the age of 50 year (Björntop, 1993a; Flagel et al., 1998). Body composition differences also play an important role between genders. Men have more skeletal muscle than women, and these differences have been found to be very clear in the upper body (Janssen et al., 2000). Men also have more visceral fat than women (Albright and Stern, 1998).

Health risks associated with obesity

Obesity is a prevalent health hazard, especially in developed countries, and is associated with a number of pathological disorders, including hypertension, type 2 diabetes mellitus, cardiovascular diseases, cancer, gallstones, respiratory system problems, and sleep apnoea. The degree of health impairment is determined by three factors: first is the amount of fat, second is the distribution of fat and third is the presence of other risk factors. The fat deposition may be generalized, or may occur preferentially in different adipose tissue compartments. Two major variants of obesity are distinguished for their different impact on morbidity of cardio-vascular diseases (CVD): truncal and peripheral obesity (Bray, 2003).

Predominant accumulation of fat in the truncal area, which includes subcutaneous and visceral fat divisions, is the main feature of android obesity. This form of obesity is more found in men and his associated with high risk for CVD, independent of generalized adiposity. While accumulation of fat in the gluteofemoral area is the main feature of gynoid obesity .This form of obesity is more frequently found in premenopausal women and apparently is not associated with increased risk for CVD (Bray, 2003).

Cardiovascular	Hypertension, Coronary heart disease, Cerebrovascular disease Varicose veins, deep venous thrombosis, Hypertension
Respiratory	Breathless, Sleep apnea, Hypoventilation syndrome
Gastrointestinal	Hiatus hernia , Gallstones and cirrhosis, Hemorrhoids, Hernia, Cancer colorectal
Metabolic	Hyperlipidaemia, Insulin resistance ,Diabetes mellitus, Polycystic ovarian Syndrome
Neurology	Nerve entrapment
Renal	Proteinuria
Breast	Breast cancer, Male gynaecomastia.
Uterus	Endometrial cancer , Cervical cancer
Urological	Prostate cancer, Stress incontinence
Skin	Sweat rashes , fungal infection, Lymphodaema, Cellulitis, Acanthosis nigricans
Orthopaedic	Osteoarthritis, Gout
Endocrine	Growth hormone and IGF1 reduced , reduced prolactin response, Hyperdynamic ACTH response to CRH, Increased urinary free cortisol, Altered sex hormones
Pregnancy	Obstetric complications, Caesarean operation, Large babies, Neural tube defects.

Table 2.3 Morbidity in obesity (adapted from Jung, 1997)

2.2.1. Hypertension

The most prominent condition associated with obesity is hypertension. According to the WHO up to 20% of the population in developed countries may suffer from this condition (Cited in Baynes and Dominiczak, 2003).Obesity alone possibly accounts for 78% and 65% of essential hypertension in men and women respectively (Garrison et al,1987). Another study shows that 5-10% of obese patients have severe hypertension (Alexander, 1964-cited in Adams and Murphy, 2000). Some of the major characteristics associated with obesity associated hypertension in humans are the activation of the renin–angiotensin system (Hall et al., 2001; Hall and Louis, 1994; Domfeld et al., 1987), high levels of circulating leptin (Hall et al., 2002; Hirose, 1998), reduced growth hormone concentration and an activation of the sympathetic nervous system (Hall et al., 2001; Hall et al., 2002). Figure 2.3 summarises the effect of obesity on blood pressure and renal and cardiovascular function.

Studies have shown that blood pressure is positively related to weight (Hirose, 1998). The prevalence of high blood pressure in obese adults is 38.4 % for men and 32.2 % for women compared with 18.2 % for men and 16.5 % for women with BMI <25. Obesity, in some ethnic groups is not enough to cause hypertension, suggesting that genetics may play a role in this process. Thus, obese African American women and Pima Indians living in the USA display milder elevations in blood pressure (El-Atat et al., 2003).

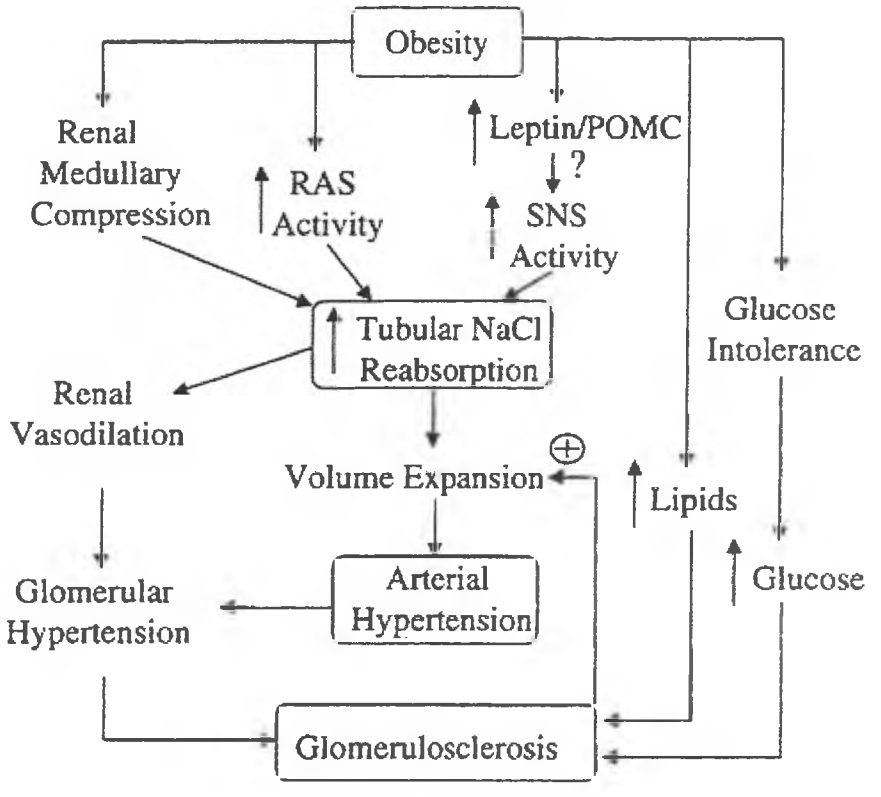


Figure 2.3 Summary of mechanisms by which obesity increases renal tubular sodium reabsorption, impairs pressure natriuresis, and causes hypertension as well as progressive glomerular injury. Adapted from Hall, 2003.

2.2.2. Diabetes Mellitus

The association between obesity and type 2 diabetes mellitus is well recognized (Björntorp, 1993b) .The majority of patients with type 2 diabetes are obese (Boden, 1996) approximately 85-95% in most population studies (Maggio and Pi-Sunyer, 2003). However, only 10% of obese patients are diabetic (cited in Nadler et al. 2000). Unfortunately, the increase in adolescent obesity is associated with an increase in adolescents with type 2 diabetes. Approximately 85% of children diagnosed with type 2 diabetes mellitus in the USA are obese (Mokdad et al., 2000). Additionally, in a recent study, impaired glucose tolerance / insulin resistance was diagnosed in 25% of obese children, 4-10 years old (Sinha et al., 2002). Serious health consequences may follow diabetes, such as renal failure, blindness, and lower-limb amputation, heart disease and stroke.

Obesity not only increases the risk of developing type 2 diabetes mellitus but also complicates its management. The presence of obesity exacerbates the metabolic abnormalities of type 2 diabetes, including hyperglycaemia, hyperinsulinaemia, and dyslipidaemia .Obesity also increases insulin resistance and glucose intolerance. Obesity may contribute to excessive morbidity in type 2 diabetes. The risk of developing hypertension and cardiovascular disease is increased with coexistence of obesity and type 2 diabetes (Maggio and Pi-Sunyer, 2003).

The risk of developing diabetes increases by approximately 25 % for each additional unit of BMI over 22 kg/m^2. Thus, when BMI is 35 kg/m^2 the risk of

developing diabetes will be 38 fold higher than if BMI were <22 (Colditz et al., 1995). In the USA, the prevalence of type 2 diabetes is more common in African Americans than in whites (Ferdinand, 2004). It has been found that weight gain is responsible for 27% of new cases of type 2 diabetes in the USA (Ford et al., 1997). Both cross sectional (Despers et al.,1989; Haffner et al. 1991; Sparrow et al., 1986) and longitudinal studies (Chan et al.,1994; Lundgren et al. 1989) indicated that central obesity is the main risk factor for type 2 diabetes. Epidemiological studies confirm that although obesity is a very important risk factor, it may not be enough to produce type 2 diabetes. A genetic susceptibility to diabetes may be necessary for obesity to induce overt disease.

2.2.3. Coronary Heart Disease (CHD)

The negative effects of obesity on health and cardiovascular disease are well documented (Wikstrand, et al.1993; Lovejoy et al., 1996; Jung, 1997; Lovejoy et al., 2001). Body weight, independent of several traditional risks factors, is directly related to the development of CHD: a study by Willett et al. (1995, cited in Jung, 1997) showed that overweight and obese women have an increased risk of CHD of 2 and 3.6 fold respectively. Obesity, especially with high abdominal fat, is associated with increased morbidity and mortality from CHD (Fujioka et al.,1987) .The effect of increased body fatness on cardiovascular function is predictable (as shown in figure 2.4). Visceral adipose tissue is the strongest predictor of cardiovascular disease in white and African American women (Lovejoy et al., 2001).The nature of the postulated association between insulin resistance and

coronary heart disease remains obscure. It has been suggested that tissue insulin resistance is the primary initiating defect which leads to compensatory hyperinsulinaemia and increased levels of other atherogenic risk factors (Laws and Reaven, 1993).

It is well known that obesity is a leading risk factor for chronic arterial hypertension and coronary heart disease. Observational studies have shown that overweight, obesity and excess abdominal fat are directly related to cardiovascular risk factors, including high levels of total cholesterol, LDL-cholesterol, triglycerides, blood pressure, fibrinogen and insulin (Haffner et al., 1991) and low levels of HDL-cholesterol.

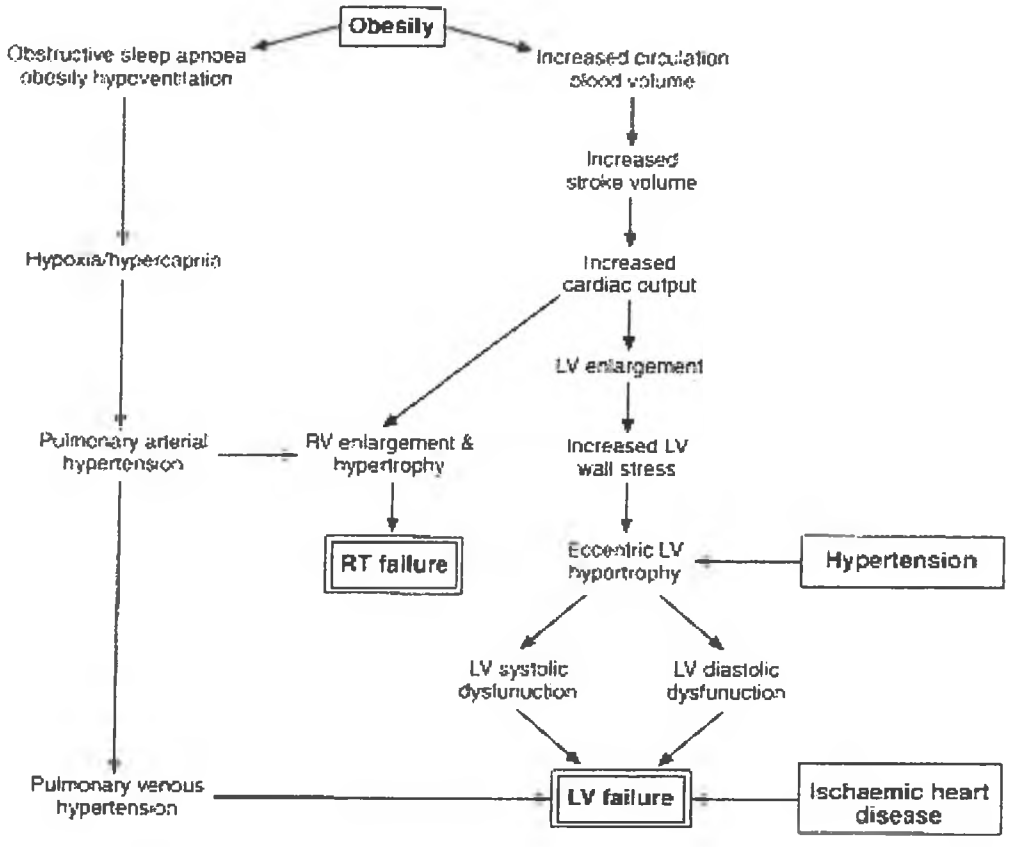


Figure 2.4 The aetiology of obesity cardiomyopathy, and its association with right-sided heart failure, systemic hypertension and ischaemic heart disease.(adapted from Adams and Murphy, 2000).

2.2.5. Cancer

2.2.5.1. Breast Cancer

Breast cancer is the commonest cancer in women in westernised countries, and accounts for about 20% of all female cancers, and it occurs mainly (80%) in postmenopausal women (Pharoah et al., 1998). Studies have confirmed that obesity has a direct relationship to mortality from breast cancer mainly in postmenopausal women (Lew and Garfinkel, 1979; Morimoto et al., 2002; Huang et al., 1997). More than 100 epidemiological studies have examined the relation of breast cancer with BMI, fat distribution, and weight gain at different ages (Morimoto et al, 2002; Huang et al, 1997; Sellers et al., 1992; Friedenreich, 2001).Taken together those studies found that women who were overweight or obese had a 30-50% greater risk for postmenopausal breast cancer development than leaner women . The International Agency for Research on Cancer estimates that 25% of breast cancer cases world wide are due to obesity and a sedentary lifestyle (McTiernan, 2003).

2.2.5.2. Colon Cancer

Obese men and women have much higher risk of developing colon cancer. A study performed by the Centre for Disease Control and Prevention showed when BMI is more than 24 the chances of developing colon cancer doubles and triples when the BMI passes 30.

2.2.5.3. Other kinds of Cancer and Obesity

A study performed in Sweden by Hill and Austin (1996) detected a relationship between obesity and cancer risk. The researchers compared the incidence of cancer in obese patients with the incidence in the general Swedish population. They found 33% more cases of cancer among the obese people than in the general population (25% more among men and 37% more among women). The obese patients had an increased risk of Hodgkin's disease among man and cancers of the endometrium, kidney, gallbladder, colon, pancreas, bladder, cervix, ovary and brain. Figure 2.5 shows the contribution of overweight and obesity to mortality from cancer in the USA.

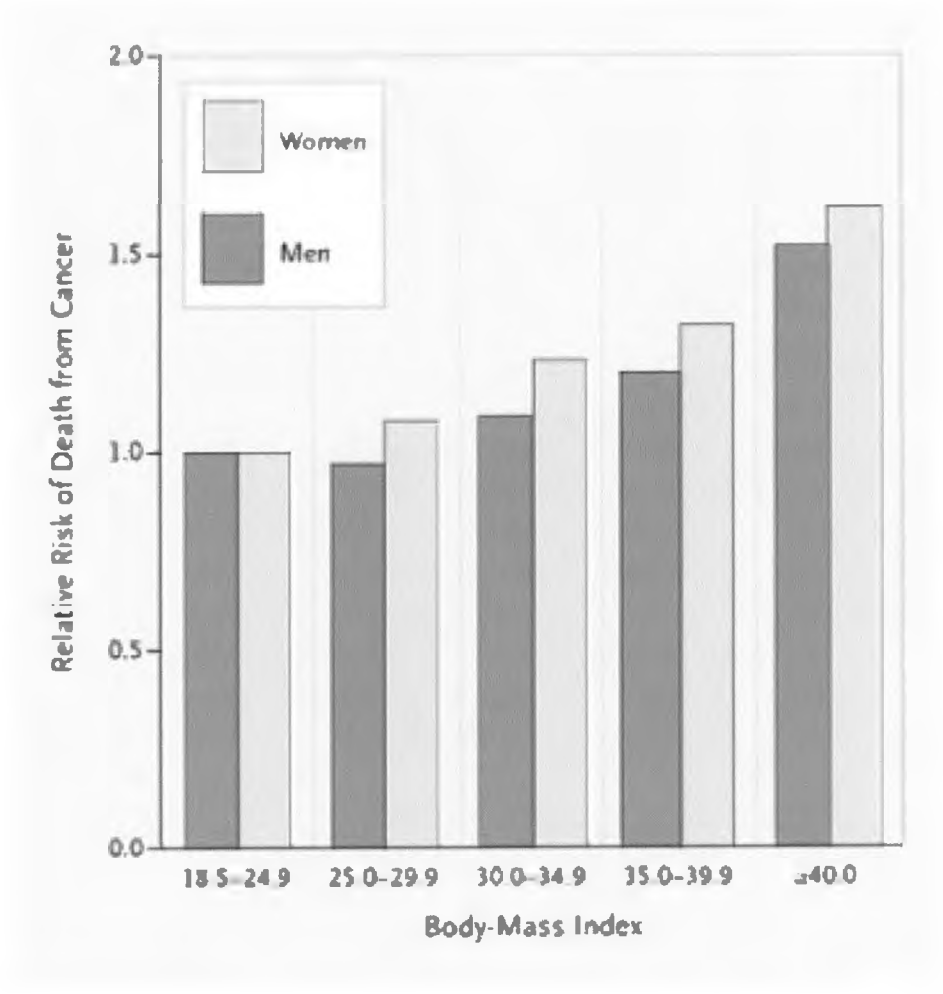


Figure 2.5 contribution of over weight and obesity to mortality from cancer in the USA. Adapted from Adami and Trichopoulos, 2003.

2.2.6. Gallstones

Gallstones are concretions in the gallbladder or bile duct, composed chiefly of a mixture of cholesterol, calcium bilirubinate and calcium carbonate, occasionally as a pure stone composed of just one of these substances. The high production of cholesterol by the liver results in the bile becoming oversaturated and precipitation of cholesterol may occur. Studies suggest that specific dietary factors (fatty food and refined sugars) may be responsible. The probability of gallstone formation in women increased 2.7 fold in women with severe obesity (BMI more than 40) compared with women of BMI less than 24.9, while in men the probability increased 2.3 fold for the same categories (Khare et al., 1995).

2.2.7. Osteoarthritis

Overweight or obese people are under a high risk of developing osteoarthritis (Cicuttini et al., 1996; Hochberg et al., 1995; Carman et al., 1994; Felson et al., 1988). Gaining weight is associated with increased mechanical stress of weight bearing joints. A study (Marks and Allegrante, 2002) has shown that at least two-thirds of osteoarthritis patients were overweight at the time of their surgery, while the National Health and Nutrition Examination Survey (NHANES) found that 43% of the osteoarthritis subjects were overweight (cited in Marks and Allegrante 2002).

2.2.6. Respiratory system problems

Studies on animals have shown that obesity affects the respiratory system by reducing total lung volume and functional residual capacity. Abdominal obesity causes pressure on the diaphragm, decreasing the ability of the lung to expand during inspiration, while accumulation of fat on the chest reduces the chest cavity.

2.2.9. Sleep Apnoea

Sleep apnoea is characterized by recurrent episodes of cessation of respiratory airflow caused by occlusion in the upper airway during sleep, with a consequent decrease in oxygen saturation . Many cross-sectional studies have confirmed the relation between increased body weight and the risk of sleep apnea (Young et al., 2002).Significant sleep apnoea is present in about 40% of obese individuals (Vgontazas et al., 1994),and 70% of sleep apnea patients are obese. An increase of body weight of 10% in one study was associated with a 6 fold increase in the risks of developing of sleep apnoea (Peppard et al., 2000). The exact mechanisms underlying the effects of obesity on the risk of sleep apnoea are still unclear. It may be related to the effect of fat deposition on airway anatomy or changes in upper airway function.

2.2.10. Obesity and women`s reproductive function;

2.2.10.1. Menstrual abnormalities and Infertility

Obesity is related to menstrual abnormalities, infertility and, miscarriage. A study by Hartz et al., (1979) has shown that obesity was associated with menstrual abnormalities including cycles greater than 36 days, irregular cycles, and virile hair growth with facial hair. Obesity in pre-menopausal women is associated with menstrual abnormalities inducing amenorrhea (Willett et al., 1985). One study, found that the risk of subsequent ovulatory infertility was paralleled with increasing BMI at the age of 18 years, even in women at BMI levels lower than considered to be obese (Rich- Edwards et al., 1994).

2.2.10.2. Polycystic Ovary Syndrome (PCOS)

Polycystic ovary syndrome (PCOS), is the association of hyperandrogenism with chronic anovulation in women without specific underlying diseases of the adrenal or pituitary glands. PCOS is associated with obesity, particularly abdominal obesity (Franks, 1995). Insulin resistance is considered to be pivotal in the expression of PCOS, and is highly associated with abdominal obesity. Direct or indirect reduction in insulin concentrations increased ovarian activity (Pettigrew et al., 1997). Reduction in weight has been found to significantly improve outcomes for obese subjects. In one study of obese infertile women who lost an average 10.2 kg/m2 in a 6 month exercise and diet program the majority resumed spontaneous ovulation and achieved pregnancy (Clark et al., 1998).

2.2.10.3. Pregnancy

Studies have reported that obesity during pregnancy is accompanied by increased hypertension, gestational diabetes, congenital malformations, and morbidity for both the mother and the child (Johnson et al., 1987). Obesity has been demonstrated to substantially reduce fertility in the general population and to greatly reduce pregnancy rates during ART (Norman and Clark, 1998; Wang et al., 2000).

The importance of fat distribution

Many factors are involved in the control of body fat distribution including; gender, age , total body fatness and ethnicity (Björntorp, 1991). When fat is located predominantly in the upper body (the shoulders, face, arms, neck, chest and upper portion of the abdomen). This pattern has been termed android, central, male, or upper body segment. It resembles the shape of an apple, and is found frequently in men (Albright and Stern, 1998). When fat tissue accumulates predominantly in the lower body, it is termed gynoid and this pattern is found more frequently in women (Albright and Stern, 1998). It is known that women have more fat than men even at the same relative body weight index (Sjöström et al.,1972; Arner et al., 1991), and this sex difference is caused by greater subcutaneous adipose tissue (Kelly et al., 2000). This gender difference is seen already in the first year of life and possible even prenatally (Karlberg et al., 1968 cited in Krotkiewski et al., 1983). Studies have shown that elderly individuals tend

to accumulate excess abdominal or visceral fat (Brochu et al., 2001; Kohrt et al., 1993).

The importance of fat distribution has been gaining much attention because of the obvious relationship between accumulation of fat in certain area (abdominal fat in particular) and elevating the risk of many diseases such as hypertension (Cassano et al. ,1990; Folsom et al., 1990; Folsom et al. ,1993; Croft et al.,1993) type 2 diabetes (Kissebah and Peiris ,1989; Chan et al., 1994; Carey et al., 1997; Banerji et al., 1997) cardiovascular disease (Ohlson et al., 1985; Rimm et al., 1995; Folsom et al.,1998; Rexrode et al. ,1997; Megnine et al., 1999), stroke (Folsom et al., 1990), breast cancer (den Tonkelaar et al., 1992; Kaaks et al., 1998; Sonnenschein et al., 1999), and risk of death (Kissebah and Krakower 1994; Lapidus et al.,1984).

The importance of fat distribution was highlighted in the 1940`s when Jean Vague noticed that subjects with an android body type have an increased risk of developing certain diseases compared to gynoid-type subjects (cited in Seidell and Flegal, 1997). It was Krotkiewski et al. (1983) who predicted that visceral fat may be of particular importance for metabolic aberrations because of its unique position and relationship to the portal circulation. Different fat depots vary in their responsiveness to hormones that regulate lipolysis (Reynisdottir et al.,1994).

The high rate of turnover of free fatty acids in the visceral fat depot has an important physiological consequence because of the direct link between the visceral depot and the liver through the portal vein (Björntorp, 1991). Increased absolute intra-abdominal fat increased the risk of certain diseases (Kahn, 1993). However, there is still considerable controversy regarding the relative importance of abdominal fat.

Another important issue is the differences in body composition between different ages and between different population groups. Children have more water in their bodies, thicker subcutaneous fat, and less minerals and potassium in the fat free mass than adults. Hydration status also changes with maturation (Lohman, 1992). South Asians in the UK were found to have more abdominal fat compared with Europeans (McKeigue et al., 1991). Asian Indians in the USA were found to have high fat relative to body mass index and muscle mass (Banerji et al., 1999). These differences were associated with the known complications of obesity, such as high blood pressure, higher triglycerides and lower HDL-cholesterol and higher fasting and post prandial glucose levels.

Insulin Resistance

Insulin resistance is the impaired ability of insulin endogenous and exogenous to control hepatic glucose production and to enhance glucose clearance, mainly into skeletal muscle (Walker, 1995). Insulin resistance is the hallmark of the metabolic (or dysmetabolic) syndrome. Insulin resistance also impairs other

biological actions of insulin, including lipid and protein metabolism, vascular endothelial function and gene expression. The demands for insulin increase as cells become insulin resistant. The body will overcome this by secreting more insulin by beta-cells and reduced first- pass clearance of insulin by the liver. This increased demand on the pancreatic beta cells may lead to progressive loss of beta cell function, secondary to exhaustion of their secretory capacity. Decreasing insulin sensitivity or insulin resistance also occurs in the normal population (Walker, 1995) and is not a prerequisite for the development of type 2 diabetes (Yki-Jarvinen, 1995; Groop et al., 1993).

The preliminary effect of high insulin production is weight gain, while long exposure to high insulin promotes hypertension by impairing sodium balance, harm to the kidneys, damage to the vascular system, cardiovascular disease and may cause type 2 diabetes (Reaven, 2002; Brett et al., 2000; Suzuki et al., 2000). Insulin resistance is common in obese patients. Paradoxically, this syndrome is also detected in lipodystrophic patients and genetic mouse models that completely lack adipose tissue (Moitra et al., 1998; Petersen et al., 2002).

There are many patients with insulin resistance who do not develop diabetes. Reasons include PCOS (Franks, 1995), essential hypertension (Cosford, 1999) and medications like glucocorticoids (Dessein et al., 2004) which can also, cause insulin resistance. The early presence of insulin resistance preceding the onset of

type 2 diabetes in obese patients is the best predictor of whether or not an individual will become diabetic in the future (Warram et al., 1990).

Tumour necrosis factor (TNF) α, which is one of many adipocyte products, may play a role as mediator of insulin resistance in infection, tumour cachexia, and obesity. Studies in cultured cells have demonstrated that many cytokines can modulate glucose transport, and TNF α in particular causes the decreased expression of the insulin sensitive glucose transport GLUT4. Using TNFα to treat 3T3-L1 cells for several days leads to inhibition of GLUT4 transcription and expression causing insulin resistance without a depletion of lipid content or a change in other fat specific genes such as lipoprotein lipase (Qi and Pekala, 2000).

Studies have described two mechanisms to explain the way that TNFα causes insulin resistance. Both a defect in the ability of the insulin receptor to autophosphorylate and a loss of its ability to phosphorylate its major substrate, insulin receptor substrate-1 (IRS-1), on the tyrosine residues has been reported. Also, TNFα has the ability to induce serine phosphorylation of IRS-1, which in turn inhibits the insulin receptor from phosphorylating this substrate (Qi and Pekala, 2000).

There is a strong negative correlation between insulin sensitivity and body weight, BMI and waist circumference. The changes in systemic metabolism that

lead to insulin resistance have been reported to develop in an attempt to prevent further weight gain (Eckel, 1992).

The relation between insulin resistance and fat distribution

Many studies have been performed to determine whether overall obesity or obesity in a specific depot is a more important determinant of insulin resistance (Walker, 1995; Arner, 1997; Ross and Hudson,1996; Carey et al. 1996) .The mechanisms underlying the relationship between visceral adipose tissue and subcutaneous adipose tissue and insulin resistance are obscure .

Controversy among scientists about the must important fat depot toward the aetiology of insulin resistance syndrome continues. Many researchers report that visceral adipose tissue (VAT) is a strong determinant of insulin resistance (Brocho et al., 2000; DeNino et al., 2001; Despres et al. ,1989; Ross et al.,2000; Arner, 2003; Arner, 2001; Albu et al., 1997; Park et al., 1991; Rendell et al., 2001; Kissebah and Krakower,1994) .According to the portal theory, free fatty acid release by visceral fat is higher than subcutaneous fat, where antilipolytic hormones, such as insulin have a more pronounced effect but the lipolytic catecholamines have a less pronounced effect . Because visceral fat drains into the portal vein, rapid visceral lipid metabolism results in the delivery of high free fatty acid concentrations to the liver. This in turn leads to stimulation of gluconeogenesis, increased triglyceride synthesis and inhibition of insulin

clearance, which ultimately results in the development of dyslipidaemia, hyperglycaemia and hyperinsulinaemia.

The deposition of fat in visceral fat cells is controlled by many factors. The androgens and estrogens , beside the peripheral conversion of Δ^4-androstenedione to estrone in fat cells are considered to be important factors in body fat distribution (Bray , 2003). As fat cells get bigger to reach maturity, their secretion of peptides and free fatty acids will increase .These products play a major role in insulin resistance and inhibit of the transformation of new preadipocytes (Mohammed-Ali et al., 1998; Fried et al., 1998). Danforth has hypothesized that failure to differentiate new adipocytes in the subcutaneous depots leads to increased fat accumulation in other tissues and fat depots such as visceral fat, skeletal muscle, liver and perhaps pancreatic beta cells (Danforth, 2000). The deposition of fat in non-adipose tissues is an important risk factor of insulin resistance and insulin secretory dysfunction. However, the mechanism explaining this is still not understood (McGarry and Dobbins, 1999).

Debate continues among studies regarding the importance of visceral fat in men and women toward insulin resistance. Corry found that the visceral fat depot in women is a constant feature of the development of insulin resistance syndrome, while in men this syndrome can be present without visceral fat (Corry, 2001). Others reported similar correlation between abdominal fat and insulin resistance in men and women (Wagenknecht at al., 2003; Goodpaster et al., 1999).

Differences among populations in body fat composition may also play an important role in insulin resistance. Asian Indians living in the USA, have more fat compared with Caucasians with same BMI and have a 60% higher risk of insulin resistance than Caucasians (Laws et al., 1994). African Americans and Hispanics were found to be more insulin resistant compared with the Caucasians (Wagenknecht et al., 2003).The relation between visceral fat and insulin resistance syndrome may be weak or less clear in non-Caucasian societies, as the accumulation of fat as visceral fat differs among different populations (Perry et al., 2000; Gautier et al., 1999). Paradoxically, studies in South Africa have shown that black obese women have a higher degree of acquired insulin resistance than white females, despite having a smaller amount of visceral fat (van der Merwe et al., 2000).

Some researchers have suggested that abdominal subcutaneous adipose tissue is largely responsible for the established association between abdominal obesity and insulin resistance (Abate et al., 1996; Kelley et al., 2000). It has been suggested that a resolution of the above controversy may be achieved by subdividing the subcutaneous abdominal fat depot into two layers, using the fascia superficialis. The visceral adipose tissue can also be subdivided into two layers according to the anatomical characteristics (Kelley et al., 2000).

Thus the subcutaneous depot has been divided into superficial and deep compartments, and the visceral fat divided into intraperitoneal and extraperitoneal depots (Cited in Ross, 2002), presuming that adipocytes within the deep subcutaneous compartment are more metabolically active compared to the superficial adipocytes. Assuming that the liberation of non-esterified fatty acids adversely effect insulin action, it follows that the deep compartment would be the stronger predictor of insulin resistance (cited in Ross, 2002). This has been shown in a cohort of lean and obese men and women. These studies lend support to a previous study which reported that posterior subcutaneous fat (analogous to deep subcutaneous fat) is associated with insulin resistance independent of VAT.

Lipoatrophy, is characterized by a paucity of adipose tissue and interestingly is associated with insulin resistant diabetes (Reitman et al., 2000) stressing that both increased and decreased white adipose tissue mass can have profound effects at extra adipose sites. Therefore understanding, the cellular and molecular basis of adipose tissue growth in physiological and pathophysiological states is very important.

Due to the interaction among a large number of factors contributing to the development of insulin resistance, it is exceedingly unlikely that levels of any one factor will enable us to explain the development of insulin resistance. Thus, the

controversy regarding the identification of all the factors related to the development of insulin resistance will continue.

Adipose tissue

Adipose tissue is an areolar tissue specialized as a connective tissue. Its traditional function was thought to be a passive depot, storing and releasing lipid as triglycerides under the influence of various hormones such as insulin, epinephrine, glucagon and Adrenocorticotropic hormone (ACTH). This process is a very important part of the energy metabolism (Salans et al., 1973).However this definition is restrictive as scientists found that the adipose tissue can play an important role as an endocrine and paracrine organ (Mohamed -Ali et al., 1998;Trayhurn and Beattie 2001). Adipose tissue is found in mammals in two different forms, white adipose tissue and brown adipose tissue with different physiological roles. The presence, amount and distribution of each vary depending upon the species or strain, age, sex, environmental and nutritional conditions. However, most adipose tissue in human is white (Cinti, 2002).

Functions and Distribution

Mammalian white adipose tissue, serves three functions : heat insulation, mechanical cushion, and most importantly, to store and mobilize energy. The adipose cells withdraw fatty acids, glycerol and glucose from the circulation or synthesize them from glucose and store them as triglyceride. The glucose mostly used to generate the ATP necessary for triglyceride synthesis and esterification.

Under different circumstances ,when cells require energy such as during periods of fasting and stress, the adipocytes meet these needs by liberating the free fatty acids and glycerol via hydrolyzing the triglyceride, and the cells take on a wrinkled appearance. Body heat insulation, is the responsibility of subcutaneous adipose tissue. Subcutaneous adipose tissue conducts heat only one third as readily as other tissue, and it found exactly below the skin (Summers et al., 2000; Runback et al., 2001).

In the infant and young children there is a continuous subcutaneous layer of fat, over the whole body, In the adult this thins out in some regions but persists and grows thicker in certain sites of predilection. These differ in the two sexes and are largely responsible for the characteristic differences in the body form of males and females (Lohman, 1992).

Brown adipose tissue is some times mistaken for a type of gland, which it resembles more than white adipose tissue. The brown colour of this tissue is derived from the cell rich vascularization and densely packed mitochondria. It varies in colour from dark red to tan, reflecting lipid content. Brown fat is most prominent in newborn animals. In human infants it comprises up to 5% of body weight, then diminishes with age to virtually disappear by adulthood. Brown fat is of particular importance in neonates, small animals in cold environments, and animals that hibernate, because it has the ability to dissipate stored energy as heat. In contrast to other cells, including white adipocytes, brown adipocytes express mitochondrial uncoupling protein, which gives the mitochondria an ability

to uncouple oxidative phosphorylation and utilize substrates to generate heat rather than ATP. In adult mammals, the main bulk of adipose tissue is a loose association of lipid – filled adipocytes, which are held in a framework of collagen fibres. About 60 to 85 % of the weight of white adipose tissue is lipid, with 90 to 99% triglyceride. Adipose tissue contains stromal- vascular cells including fibroblastic connective tissue cells, leukocytes, macrophages, and preadipocytes which contribute to structural integrity (Albright and Stern, 1998).

White adipose tissue can be classified into two major divisions, subcutaneous, which constitute about 80% of the total body fat and visceral, which constitute 10% of the total body fat, with other depots such as orbital, retroperitoneal and perirenal fat. About 95% of triglycerides in the body, are presented in adipose tissue (Arner, 2001).

Expansion of white adipose tissue occurs increasingly after birth as a result of both hyperplasia and hypertrophy. The ability of the body to generate new fat cells occurs even at a late age (Gregoire et al., 1998) .The increase in the cells number occurs primarily by mitotic activity in precursor cells .The developmental sequences of adipose tissue in humans is less well defined .In contrast to most neonates, the human neonate is born relatively fat .Two periods of hyperplastic growth probably occur during the third trimester of pregnancy and just prior to a puberty. Contrary to earlier belief, hyperplastic growth can also occur to adulthood in both humans and rats (Albright and Stern, 1998).

Studies on animals have shown that an exposure to hormones may affect the size of adipose cells. Growth hormone (GH), for example is able to reduce adipocyte volume and this may be due to a direct action on lipid metabolism by decreasing lipogenesis and increasing lipolysis (Lee et al., 1990). Another study showed that the chronic administration of GH *in vivo* reduced both carcass fat and the rate of lipid accretion in the carcass (Campbell et al., 1989).

Adipose tissue development

The transition from egg to the determination and conversion of adipocyte precursor cells into mature adipocytes occurs in a series of stages as depicted in figure 2.6. A single fertilized egg gives rise to nearly 200 different cell types that make up the multiple developmental lineages and multicellular organism. The full developmental program of preadipose tissue from fertilized egg is unknown. However the pluripotent fibroblasts (stem cells) are known to have mesodermal origins (Cornelius et al., 1994) and can differentiate into committed preadipocytes, cartilage, bone or muscle tissue.

In humans preadipocytes begin to differentiate into adipose tissue during late embryonic development , with a majority of the differentiation occurring shortly after birth (Burd et al., 1985).This enables the newborn to cope more efficiently with intervals between nutrient intake (MacDougald and Lane ,1995). Rat and mouse preadipocytes do not begin conversion into adipose tissue until after birth

(Ailhuad et al., 1992). All species have the ability to differentiate preadipocytes throughout their life spans in response to the body's fat storage demands.

Adipose cells first appears about the fifteenth week of human gestation and continue to divide and appear in large numbers until about 23 weeks and then divide more slowly through the remainder of gestation. During the first two years of life, adipose tissue mass grows by an increase in both size and number, initially, by cell enlargement and then after age 6-12 months by an increase in both size and number. Cell number increases slowly from two years of age until close to the onset of puberty, and during adolescence, there is another sharp elevation in cell number that accounts for a spurt in the growth of adipose tissue mass. Thereafter the mass of adipose tissue of a non-obese adult maintaining constant body weight remains stable as do adipose cell size and number (Salans et al., 1973; Björntorp , 1974).

The growth and development of adipose tissue in rats is well documented. During the first 4 weeks after birth the growth is hyperplastic, then from 4-14 weeks adipocyte hyperplasia and hypertrophy occur. After 14 weeks the growth in the adipose tissue is predominantly hypertrophic (Ailhaud et al., 1992). Hyperplasia and hypertrophy can be altered, when nutritional or other stresses are imposed during the preweaning period of growth. Protein restriction during postweaning period may severely limit the rate of adipocyte proliferation and differentiation (Tulp et al., 1979). Adult obesity is usually, but not always associated with

hyperplasia and hypertrophy. Thus, adult rodents can increase their cell number under some circumstances (Faust et al., 1978).There also appear to be circumstances in which the number of adipocytes can increase during adult hood in humans (Hirsch, 1976).

Studies on pigs have shown that the increase of weight between first and second month of age is due to a sharp increase in cell number, while a combination of hypertrophy and hyperplasia is the reason behind weight elevation between the second and fifth months , and after five months of age the increase of weight is mainly because of hypertrophy .The pigs, like other animals, reach a point in their growth at which muscle growth decreases concomitantly with an increase in fat deposition (Anderson and Kauffman ,1973).

Figure 2.6 Adipose tissue development, adapted from McCusker R. site about adipogenesis. on:/classes.aces.uiuc.edu/AnSci312/Adipose/Adipolect.htm.

Adipocyte determination

Determination is the irreversible commitment of a cell to a pathway of differentiation. Determination always precedes differentiation. The process of determination causes stability, heritability restrictions in a cells developmental potential, which is an important principle of development. However, there are two kinds of determination. When the cell holds all the information that it needs, determination in this case is called autonomous determination. While when a cell depends on instructions from neighbours to direct its differentiation , determination is called interactive determination. Specialising and organising cells into tissues are the responsibility of the genes, which are present in all cells. The fertilized egg, and its early daughter cells are totipotent but, as development proceeds, these cells give rise to progressively more specialised cells which have a less extensive developmental repertoire but can still develop into multiple cell types. In the end, the cells become restricted to their ultimate fate, meaning that they become committed to a particular type of differentiation. This commitment or determination involves a gradual reduction of development options that ultimately renders the cells capable of becoming only a single cell type. This restriction of developmental fate occurs gradually and some cells in a lineage become committed long after others. For example most adult tissues contain some cells that can give rise to various differentiated progeny, and thus by definition are not fully committed (Ham and Veomett, 1980; Lodish et al., 1995).

Determination most likely involves the expression of one or few regulatory genes that control the subsequent expression of many other genes in the hierarchy. These restrictions of gene expression can be considered to be developmental (decisions). These decisions can occur without any apparent change in the phenotype of the cells. Therefore one can not establish when a cell has become committed until after the determination has occurred (Gregoire et al., 1998).

The ability of PPAR activators to determine cell linage raises other implications for lipid differentiation factors in disease processes. Differentiation which has gone awry in response to excess lipid derivatives may explain the formation of ectopic tissue: cartilage, bone, fat, and even marrow, in atherosclerotic lesion. Lipids may also have a role in osteoporosis in which bone stroma is replaced as fat. Stromal cells represent about 1% of the cells in the bone marrow. These immature mesenchymal precursor cells can differentiate into either adipocytes or osteoblastic cells. The decision between the two lineages is regulated, in part by PPARγ with activation inhibiting osteoblastic in favour of adipogenic differentiation of these stromal cells (Parhami et al., 1999). Osteoporosis also results from excess osteoclastic resorption relative to osteoblastic formation of bone and this is also regulated by lipids. Monocytes may differentiate along the macrophage and osteoclast lineage, and this choice is also influenced by PPAR activators (Mbalaviele et al., 2000).

Adipocyte differentiation

Adipocyte differentiation is the process whereby a relatively unspecialised cell acquires specialized features of an adipocyte. One of the end results of cellular differentiation is the production of cells that are capable of performing a particular specialized function .There are two factors affecting differentiation, first the communication between individual cells or between cells and second the extracellular environment, but these do not reverse a cells commitment. Adipocytes or fat cells are fully differentiated cells and are incapable of mitotic division. New fat cells, which may develop at any time within connective tissue, arise as a result of differentiation of more primitive cells. Although fat cells, before they commence to store fat, resemble fibroblasts, it is likely that they arise directly from un differentiated mesenchymal cells that are present within the body, commonly as pericytes in relation to small blood vessels. Cells usually have some differentiated characteristics even though they have not yet become fully committed (Gregoire et al., 1998).

The differentiation of a cell is defined by the proteins that it synthesizes and by its shape, both characteristics that establish its function. Because cells produce a broad range of proteins, not all classes of protein establish whether a cell is differentiated. Some proteins are present in all cells , for example mitochondrial proteins or glycolytic enzymes , or are common to several cell types, for example

, the collagens produced by various fibroblasts or the cytokeratins produced by various epithelia. The proteins that establish differentiation of a cell type are those that enable cell to carry out a unique function. Thus in adipocytes the protein enables the cell to produce and store lipid is the one responsible differentiation. Cell differentiation arises, in part, from controlling the synthesis of these "specialization" proteins (Ham and Veomett, 1980).

The first sign of adipose tissue differentiation is the appearance of lipid droplets in the cytoplasm. This is a much easier and more sensitive criterion than measuring enzymatic or chemical changes in whole culture, because of asynchrony of participating cells and the easy microscopic identification of rare cells whose adipose differentiation precedes that of the rest of the population. It has been detected that a positive feedback loop happens between C/EBP α and PPARγ which, responsible for maintenance of terminal differentiation (Gregoire et al., 1998; Samuelsson et al., 1991; Umek et al., 1991). Any kind of molecular disruptions, may effect C/EBPα or PPARγ before adipocyte differentiation, may stop the process. Cells will not reach maturity or become insulin sensitive in term of glucose transport. Many of the genes essential for maintenance of a well differentiated adipocyte phenotype require C/EBP α including: insulin receptor, insulin receptor substrate-1, fatty acid synthase, fatty acid binding protein, and PPARγ (Gregoire et al., 1998; Webster et al., 1994;Lin and Lane ,1992).

The morphological changes, that occur during differentiation, organize the cells in characteristic shapes. The new shape enables the cell to function effectively. The circular shape of the adipocytes enables them to store the maximal limit of lipid. However, the extremist case of specialization occurs in the mammalian erythrocyte, or red blood cell. The cells lose even their nucleus ,and acquired a flattened biconcave membrane-bound bag of haemoglobin (Alberts et al., 1994). Extracellular matrix proteins may play a critical role in modulating adipocyte differentiation by permitting the morphological changes and adipocyte specific gene express ion that associated with differentiation (Gregoire et al., 1998). The distinct shapes result from different arrangements of the various cytoskeletal components inside the cell, including microtubules, and intermediate filaments. The shape of the cell may even control the production of specific proteins. For example, milk production mammary epithelial cells can synthesize milk proteins only when allowed to assume cuboidal shape, and chondrocytes can produce cartilage only when allowed to round up.

Studies showed that the majority of stromal cells present in human adipose tissue are able to undergo adipose differentiation under serum-free chemically defined culture conditions (Djian et al., 1983). Some differentiated cells may no longer be able to under go cell division. Tissues that contain cells in such a terminally differentiated state may also contain undifferentiated stem-cell populations that are able to divide .These stem cells will subsequently differentiate , leading to the terminally differentiated cells for example red blood

cells , plasma cells,. Other tissues are not capable of producing new cells because they consist solely of terminally differentiated cells, for example, neurons, cardiac myocytes (Ham and Veomett, 1980).

Studies have shown that the capacity of preadipocytes to differentiate and accumulate lipid deteriorates with ageing (Hauner et al., 1989; Kirkland et al., 1990; Kirkland et al., 1993). Preadipocytes collected from old rats and humans accumulate less lipid and have lower lipogenic enzyme activities when exposed to variety of conditions that induce differentiation than preadipocytes from younger individuals (Kirkland et al., 1993; Hauner et al., 1989). Consistent with their declined capacity for differentiation, preadipocytes isolated from old rats shown a decreased expression of several differentiation–dependent genes compared with preadipocytes cultured from young rats (Kirkland et al., 1993).These differences were not caused by inherent differences in basal expression of these genes in undifferentiated preadipocytes , rather they reflect a differences in the extent of expression induced during the differentiation process .Thus whereas factors extrinsic to fat tissue (such as diet , activity, and hormonal milieu) contribute to declining fat mass during senescence , decreased capacity for lipid accumulation also involves age-related processes intrinsic to the individual preadipocytes and adipocytes in fat tissue (Djian et al. ,1983).

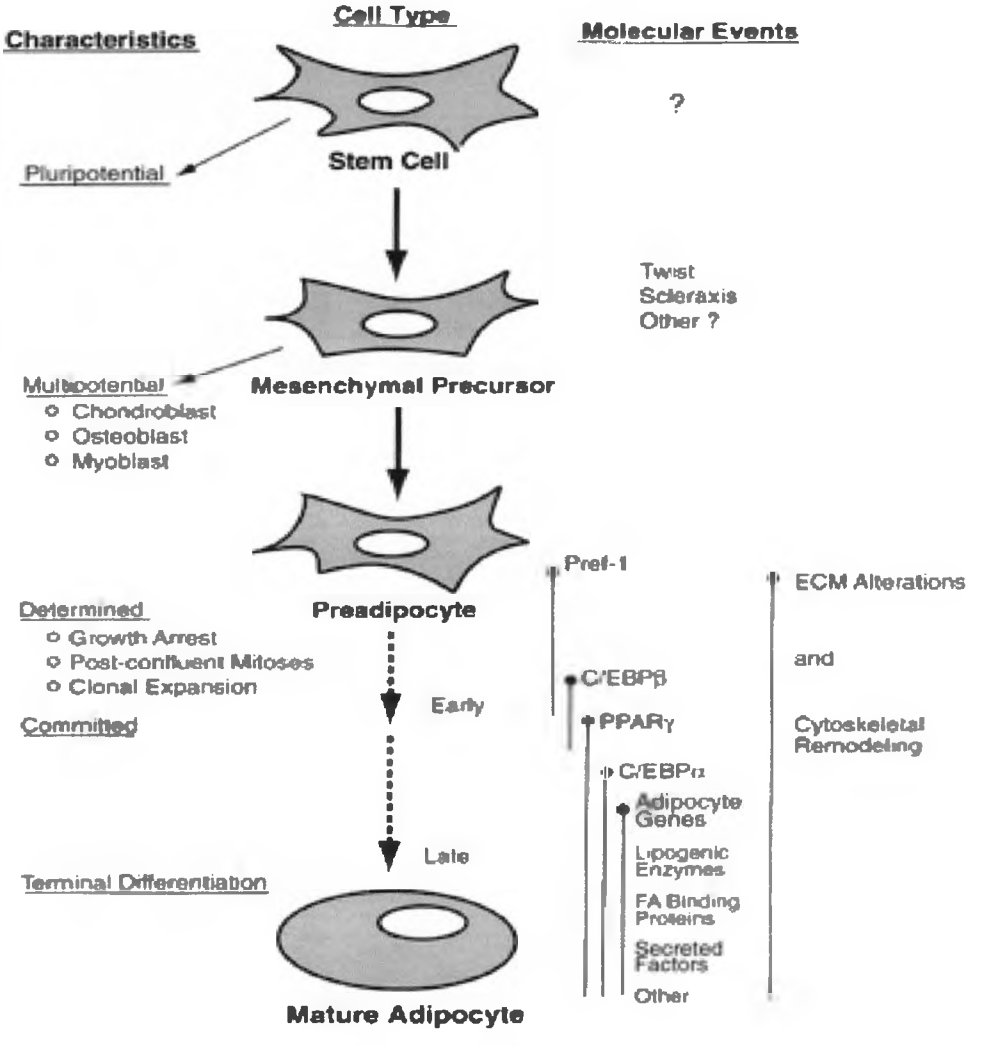


Figure 2.7 adipocyte determination and differentiation. Adapted from Gregoire et al., 1998.

The importance of Cell Shape;

The first hallmark of the adipogenesis process is the dramatic alteration in cell shape as the cell converts from fibroblastic to spherical shape .These morphological modifications are paralleled by changes in the level and type of extracellular matrix components and the level of cytoskeletal components (Gregoire et al., 1998). More recently, findings indicated that these events are the key for regulating adipogenesis as they may promote expression of critical adipogenic transcription factors, including CCAAT/enhancer binding protein α(C/EBP α) and PPARγ. Mediation of the proteolytic degradation of the stromal ECM of preadipocytes by the plasminogen cascade is required for cell-shape change, adipocyte-specific gene expression, and lipid accumulation (Selvarajan et al., 2001).

The alterations in cell shape play a major role during developmental process of the cell. Beside its importance in differentiation, cell shape can help to determine which proteins are synthesized. As the adipose conversion progresses, the 3T3-L1 cells withdraw their processes and change their shape from highly extended to nearly spherical (Carraway and Carraway, 1989).

Cell shape is determined by two factors, the first is the interaction with adjacent surfaces, either other cells or the extracellular matrix, and second is the cytoplasmic framework of fibrillar structures, the cytoskeleton. The cytoskeleton is made up of polymers of fibril forming proteins and various accessory proteins organized together into microfilaments, intermediate filaments and microtubules.

The accessory proteins affect filament assembly or link the filaments to one another or to other cell components. The cytoskeleton is not a permanent structure but changes rapidly in response to cellular events .Indeed the characteristic shape and the intermediate filament proteins of epithelial cells can change to those of mesenchymal cells during normal development. Various external factors, transduced by variety of signalling systems at the surface and within cells result in changes in cell shape (Cunningham et al., 1987).

Origin of 3T3-L cells

The in vivo study of preadipocyte differentiation is difficult. Fat tissue within animals consists of approximately one third adipocytes, while in humans it is about 50% (Cornelius et al., 1994). The remaining percentages of human and animals fat tissue is a combination of small blood vessels, nerve tissue, fibroblasts and preadipocytes in various stages of development (Geloen et al., 1989). It is difficult to distinguish preadipocytes from fibroblasts and the inability to align preadipocytes at similar developmental stages confounds details in vivo studies. Despite these difficulties, preadipocyte primary culture has been used (Lodish et al.,1995) however, such primary cultures present a number of problems. First, the contamination of preadipocytes with other kinds of cells increases the difficulty of isolating pure preadipocytes. Second, due to the low percentage of preadipocytes of total adipose tissue, a large amount of adipose tissue is needed in order to get enough preadipocytes. Furthermore, primary cultures have a limited life span in culture (Ntambi and Young-Cheul, 2000).

Therefore, the molecular control of adipogenesis has largely been studied by using in vitro models .There are advantages and disadvantages to the use of a cell line to study preadipocyte differentiation. A cell line derived from cloning is a homogenous population of cells that are all at the same stage of differentiation. This allows for definitive responses to treatments. The ability to passage these cells has added another important facility to the study of adipogenesis by providing a stable source of preadipocytes .The disadvantage is that the molecular events that constitute adipogenesis in a cell line are not necessarily the same as those in a human preadipocyte. Furthermore, the ability of a preadipocyte cell line to differentiate often falls with increasing passage number (Ntambi and Young-Cheul, 2000).

Cells of clonal lines can be classified into three groups. First are the totipotent embryonic stem cells (or ES cells). Second, the multipotent or pluripotent stem cells following myogenic, chondrogenic or adipogenic pathways (C1, TA1, 30A5, azaCyd, 10T1/2, Bal/c 3T3, 1246, RCJ3.1 and CHEF/18 fibroblasts). The third class, is the unipotent preadipocytes (3T3-L1, 3T3-F422A, 1246, Ob1771, TA1 and 30 A5), and these cell lines have undergone determination to the adipose linage, and can either remain as preadipocytes or undergo conversion to adipose tissue (Ailhaud, 1997). The models most frequently used for studies of adipocyte development are subclones of the 3T3 cell line and the ob 17 cell line (Kim and Moustaid-Moussa, 2000).These clonal cells possess the ability to differentiate into adipose cells in the presence of adipogenic factors after becoming confluent

and reaching a state of growth arrest. On the basis of studies on these cell lines two stages have been identified during the adipose differentiation process. The first step is the commitment of the cells to differentiation process, which requires growth arrest and is characterized by the expression of early markers of differentiation such as lipoprotein lipase (Ailhaud,1992; Amri et al., 1986).The second step represents the terminal differentiation of the cells, including the acquisition of the adipocyte phenotype .

The 3T3-L1 and 3T3 –F422A culture lines which originated from disaggregated Swiss 3T3 mouse embryos are the most commonly used culture models. The 3T3 line was evolved from the disaggregated cells of late foetuses of the mouse. It is an excellent model system for studying the behaviour of fibroblasts and molecular events responsible for the differentiation of preadipocytes into adipocytes .These cells undergo differentiation, when subjected to a differentiation regimen consisting of insulin, dexamethasone, and isobutyl-methylxanthine (Wang et al., 1992).

In normal development, preadipose cells lose their ability to grow when they mature; but if they are kept in the growing state under culture conditions there is no obvious reason why they should not become established cell lines, like other mesenchymal cell types of rodents. Though, under certain conditions, the appearance of 3T3 cells may resemble that of endothelial cells, they are fibroblasts by functional criteria. When the growth of 3T3 cells is arrested, a

certain proportion will convert to adipose cells. This is true for each of a considerable number of randomly chosen clones isolated from 3T3 stock stored frozen for over a decade (Green and Kehinde, 1974).

Endocrine and Paracrine effect of the adipocyte

Mature adipocytes, the main cellular component of white adipose tissue, are uniquely equipped to function in energy storage and balance under tight hormonal control. However, with the realization that adipocytes secrete factors known to play a role in, insulin sensitivity, immunological responses, vascular diseases, and appetite regulation, a much more complex and dynamic role of white adipose tissue has emerged. In addition to proteins involved in lipid and lipoprotein metabolism, cytokines, and growth factors, adipocytes also synthesize factors involved in the regulation of food intake and energy homeostasis. Adipocyte derived factors include leptin, adipsin, acylation stimulating protein, agouti, angiotensinogen, prostaglandins, adiponectin, resistin, TNFα, macrophage migration inhibitory factor, and PPARγ (Kim and Moustaid-Moussa 2000; Rosen et al., 2000; Frühbeck et al., 2001) (See Figure 2.8).

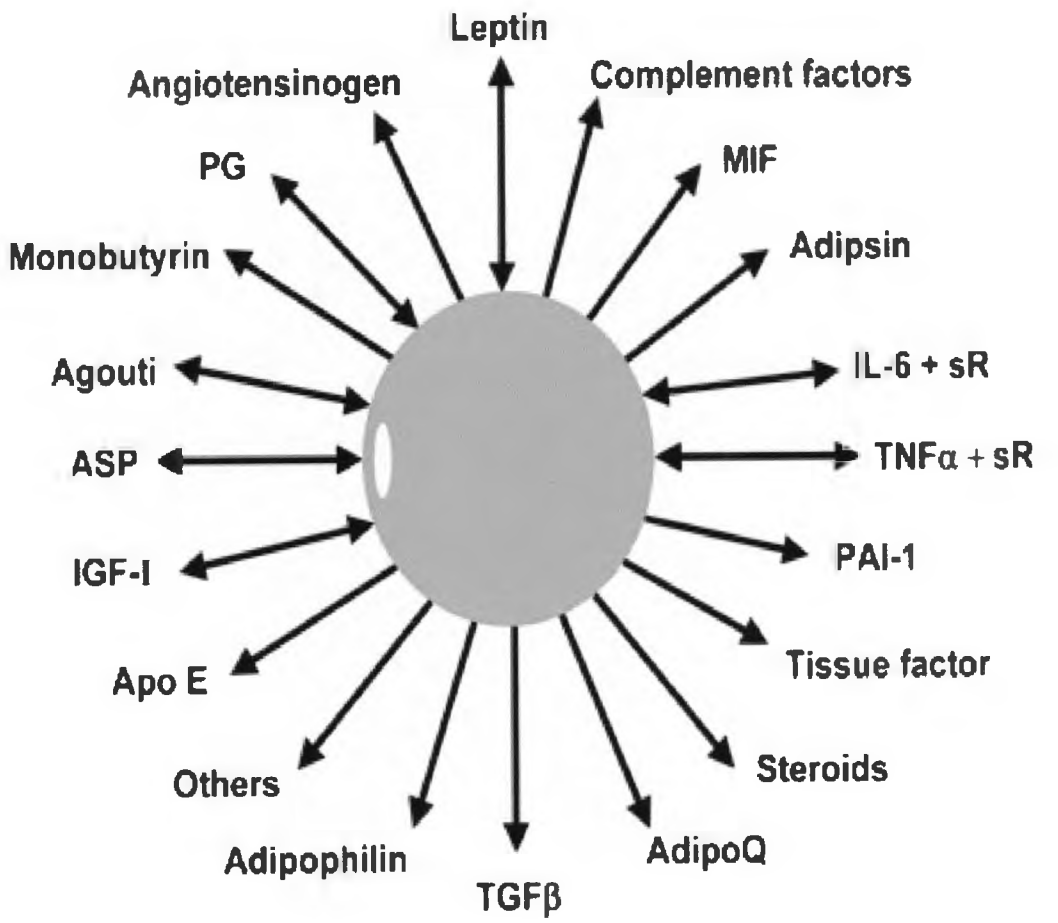


Figure 2.8 Adipocyte products, adapted from Frühbeck et al., 2001

Alkaline Phosphatase:

Alkaline phosphatase is an enzyme made in many tissues. The enzyme present in all tissues of the body especially at or in the cell membranes, and it exist at high concentrations in intestinal epithelium, kidney tubules, bone (osteoblasts), liver, and placenta. ALP, like many other isoenzymes works in alkaline environment, and it catalyses the hydrolysis of phosphatase esters releasing an alcohol and inorganic phosphate. As the enzyme has low specificity, it can hydrolyse a wide range of substrates. Although, the precise role of ALP is not yet clear, it appears that the enzyme is associated with lipid transport in the intestine and with the calcification process in bone (McComb et al., 1979).

The optimum activity of alkaline phosphatase is at about pH 10 *in vitro* thus the enzyme itself is inactive in the blood. Alkaline phosphatase acts by hydrolysing phosphorus (an acidic mineral) bonds, creating an alkaline hither main importance of measuring alkaline phosphatase is to check the possibility of bone or liver disease. Since the mucosal cells that line the bile system of the liver are the source of alkaline phosphatase, the free flow of bile through the liver and down into the biliary duct and gallbladder are responsible for determining the enzyme level in the blood.

It was thought before that ALP reaching the liver from other tissues (especially bone) was excreted into the bile and that the elevated serum enzyme activity found in the hepatobiliary disease was the result of failure to excrete the enzyme through the bile. It is now clear that the liver secretes more ALP in the case of

hepatobiliary disease. Thus, blocking the bile duct with stone for example enough to increase the level of the enzyme in the blood serum more than three fold. When the liver, bile ducts or gallbladder system are not functioning properly or are blocked, this enzyme is not excreted through the bile and alkaline phosphatase is released into the blood stream. Thus the serum alkaline phosphatase is a measure of integrity of the hepatobiliary system and the flow of bile into the small intestine (Eliakim et al., 1991).

Temporary increase of the enzyme is found during bone fracture healing, growing children, and third trimester of pregnancy (McComb et al., 1979).
The relationship of alkalinity to bone development warrants further discussion because its plays a major role in the prevention and reversal of osteoporosis. Calcium comes out of solution and crystallizes in an alkaline environment, but is dissolved in an acidic environment. The osteoblasts by creating a local environment of alkalinity via alkaline phosphatase help build bone Its also implies that in order to slow bone loss, one can not be in an acidic state Studies have shown that giving potassium bicarbonate is just as effective as calcium in correcting osteoporosis. Therefore in an acidic state, the body will compensate by increasing the bone alkaline phosphatase levels. The optimal range for alkaline phosphatase depends on the age .A growing adolescent will have a much higher alkaline phosphatase level than a full grown adult because her/his osteoblasts are laying down bone very rapidly (see table 2.4).

Adults	U/l
Men (n=221)	40-129
Women(n=229)	35-104
Children	**U/l**
aged 1 day	<250
aged 2-5 days	<231
aged 6 days to 6 months	<449
aged 7months to 1year	<462
aged 1year to 3 years	<281
aged 4 to 6years	<269
aged 7 to 12 years	<300
aged 13 -17 years(males)	<390
aged 13-17 years(females)	<187

Table 2.4 shows ALP normal levels in men, women, and children.

Alkaline phosphatase isozymes

Alkaline phosphatase (ALP) is a zinc and magnesium –containing metalloenzyme (EC 3.1.3.1) that hydrolyzes phosphate ester with a high pH optimum. It is found in most species from bacteria to humans and also as free enzyme in natural waters and sediments. In mammals, it is a glycoprotein attached to the membrane by a glycosylphosphatidylinositol (GPI) anchor (McComb et al., 1979).

In mammals, there are four different ALP isozymes are currently known, each encoded by at least one separate gene. Three of the four are tissue specific alkaline phosphatase: the placenta, the placenta like germ cells, and intestinal isozymes .The fourth isozymes, known as liver/bone /kidney isozyme, is tissue non-specific alkaline phosphatase and has physical and biochemical properties that clearly distinguish it from the other ALP isozymes (Moss, 1982; Harris, 1989).

Intestinal alkaline phosphatase is a late development in the alkaline phosphatase gene family, appearing first in mammals and before the appearance of placental ALP in primates. All these intestinal ALPs are compact genes contained within 5-6 kb. There are fetal and adult forms of human intestinal ALP (Eliakim et al., 1991, Young et al. 1981).

Most species express just a single intestinal ALP. The multiple forms of mRNAs encoding human Intestinal ALP are due to differences in polyadenylation, and

only one protein is produced .At least two protein isozymes have been reported in two species, the cow and the rat .The rat intestine produces two isozymes of intestinal ALP , but unlike the near identity of isozymes in the other species these share only 79% identical amino acids (Young et al., 1981).

Placental alkaline phosphatase (EC3.1.3.1) is a dimeric sialoprotein that is expressed in the membrane of the syncytiotrophoblast of the human placenta During pregnancy, Placental ALPs is synthesized after the 12^{th} week and is shed into the maternal circulation. It is serum concentration is correlated with its placenta growth, increases as pregnancy proceeds and reaches its maximum at the last three months. Placental ALPs activity in plasma may be used as an indicator of placental function (Fishman and Sie.,1971, McComb et al., 1979)..

Role of alkaline phosphatase

Up to date there is little biochemical evidence for alkaline phosphatase function ,except its role in bone mineralization(via its role in the metabolism of calcium phosphates) (Yoon et al.,1989;Beertsen and van den Bos 1992;Fedde et al.,1999) and dephosphorylation of adenosine nucleotides (Gallo et al.,1997).The ALPs have been postulated to be involved in a range of other processes , including cell adhesion, vitamin B transport, and cell signalling (Rindi et al.,1995;Muler et al.,1991,Hui et al.,1993).On the other hand humans with mutations in the tissue non specific ALP isozyme have defective bone mineralization , infantile hypophosphatasia (Henthorn et al.,1992).

ALP is a marker of osteoblast linage, and hypophosphatasia, a recessive human disease with multiple bone disorders, has been linked to mutations in ALPL, the tissue non specific ALP (Henthorn et al., 1992).

The tubules are sites for deposits of calcium and magnesium phosphates in the form of luminal concretion bodies, or spherites (Wessing and Eichelberg, 1978). These bodies have traditionally been considered to be sites of storage extraction of phosphates of calcium, magnesium, and other metals. As these spherites pack into the initial segments of the anterior tubules, it is possible that the deposits are laid down in the initial segment and then pass though the tubule to be excreted in the urine. Alkaline phosphatase on the apical surface of the lower tubule might allow for the selective reclamation of calcium when needed. This might explain the semilethal nature of the mutations; the severity of the disruption of calcium metabolism might depend on the nature of the diet.

Alkaline phosphatase activities in the vertebrate brain are generally lower than those in the liver and kidney (McComb et al., 1979). However, in the neuronal tissue, intense alkaline phosphatase activity has been located in the neuronal cell bodies and processes in some parts of central (CNS) and peripheral nervous systems (PNS) of many animals such as mouse and rat (Sood and Mulchandani, 1977). The enzyme has been localized on the outer surface of plasma membranes of nerve cell bodies and dendrites (Mori and Nagano, 1985). It has been shown that alkaline phosphatase is associated with synaptic vesicles

isolated from bovine cerebral cortex (Zisapel and Haklal, 1980). It has suggested that ALP has a function related to particular features of the reactive neurons. All these facts shown that ALP may play some role in transmembrane transport and in cell differentiation in the nervous tissue (Mahmood et al., 1992). .

Inhibition of alkaline phosphatase

Inhibitors can be divide into two classes, reversible and irreversible types. Reversible inhibition implies that the activity of the enzyme is fully restored when the inhibitor is removed from the system in which the enzyme acts by some physical separative process, such as dialysis, gel filtration, or chromatography. An irreversible inhibitor, on the other hand, combines covalently with the enzyme so that physical methods are ineffective in separating the two.
Competitive inhibition by metal ions can arise when two metal ions compete for the same binding site on the enzyme. Thus Ca^{+2} is an inhibitor for some enzymes that depend on Mg^{+2} activation (Stryer 1980). Whilst divalent ions like Mg^{+2}, Co^{+2}, Mn^{+2} are activators of ALP (McComb et al., 1979).

Levamisole is a strong non-competitive inhibitor of liver, bone, kidney alkaline phosphatase whilst having little effect on the intestinal or placental form (van Belle, 1976). This inhibitor has been used over a wide range of concentrations to inhibit ALP. Thus, 5mM levamisole causes maximum inhibition of ALP in human serum (Chan and Kellen, 1975), whilst ALP purified from human bone can be inhibited by concentrations between 1-100uM (van Belle, 1976), 100uM causing

nearly 100% inhibition. Levamisole however, also has many other effects including immunomodulatory and anthelminthic properties (Amery and Bruynseels, 1992) and can act as a nicotinic and adrenergic receptor agonist (Hsu, 1980). The related compound, L-P-Bromotetramisole has been found to be more potent than levamisole as an inhibitor of alkaline phosphatase (Brogers et al., 1975). The stereospecificities of these alkaline phosphatase inhibitors have been demonstrated biochemically as well as cytochemically in a variety of tissues and species. It has been suggested that both these compounds inhibit ALP activity by blocking dephosphorylation of the phosphoenzyme (van Belle, 1976). Phenylalanine has been used to inhibit placental and intestinal alkaline phosphatase enzyme, the inhibition being approximately 80%, whilst there is little effect on the liver or bone enzymes (Fishman et al., 1963). L-homoarginine is a particularly powerful inhibitor of the bone and liver enzymes whilst the intestinal and placental enzymes are less effected (Fishman and Sie, 1970).

It has been shown that Phe Gly Gly inhibits intestinal ALP, but not tissue non-specific ALP. Intestinal ALP is well known as a tissue specific alkaline phosphatase (Doellgadt and Fishman, 1977). Histidine, has also been used as a tissue non-specific alkaline phosphatase inhibitor (Fishman and Sie, 1971). The mechanism by which these amino acids block ALP activity is uncertain, although it has been suggested that like levamisole the Phe containing inhibitors may block dephosphorylation of the enzyme (van Belle, 1976).

Alkaline phosphatase activity has been inhibited by okadaic acid. Kinetic analysis has demonstrated that okadaic acid acts as a non-competitive inhibitor of alkaline phosphatase from E.coli. The same inhibition pattern was obtained for the human placental and the calf intestinal enzyme (Mestrovic and Pavela-Vrancic 2003).

Aims of study

The main aims of this study were to:

1. Determine whether ALP is expressed in 3T3-L1 cells and human preadipocytes
2. Determine whether ALP is involved in the adipogenic process by use of ALP inhibitors
3. Determine whether ethnic differences exist in adipogenesis in white and black South African females

CHAPTER THREE

MATERIALS AND METHODS

3.1. Murine 3T3-L1 Cell Culture Work

3.1.1. Preparation of DMEM and Trypsin Solution

The substances in table 3-1 were mixed together. The pH of the solution should be 7.1-7.2. The medium was filter-sterilised before use using a 0.22micron sterile syringe filter and was stored at 4°C until use.

Table 3-1: Murine 3T3-L1 Culture Medium (DMEM)

Substances	Amount added
Dulbecco's Modified Eagle Medium (Gibco Invitrogen Corporation)	1.35g
Sodium bicarbonate (Merck)	0.37g
Foetal Bovine Serum (Gibco BRL)	10.0ml
Penicilin (10000U/ml)/Streptomycin (10000ug/ml) (BioWhittaker)	1.0ml
Sodium pyruvate (100mM) (Gibco Invitrogn Corporation)	1.0ml
L-Glutamine (200mM) (Gibco Invitrogen Corporation	1.0ml
Distilled water	87ml
Total Volume	**100ml**

Trypsin solution: This was used for passaging the 3T3-L1 cells. A 10x trypsin stock solution (Sigma) was diluted 10 fold with phosphate buffered saline (PBS), pH 7.2 . Trypsin was then filter sterilised and allocated in 1ml aliquots to cryo-tubes and frozen at –20 °C, until needed.

3.1.2. Transformation Medium

Transformation medium was used for initiating the conversion of the 3T3-L1 cells into adipocytes. It was prepared using the DMEM described in table 3.1 This was supplemented with the following;

3- Isobutyl-1-methylxanthine (IBMX) (Sigma)

A 25 mg aliquot of IBMX was dissolved in 1ml of warm methanol. From this, 0.1ml was added to every 50 ml of DMEM to give a final concentration of 0.5mM.

Dexamethasone (Sigma)

A 1mg measure of dexamethasone was dissolved in 1 ml 99% ethanol (BDH), and then this was diluted to 20ml with PBS. A 0.1ml aliquot of this solution was added to every 50ml of DMEM to give a final concentration of 0.22uM.

Insulin (U100 Actrapid insulin, Novo-Nordisk)

A 0.13ml aliquot of insulin was added to every 50ml of DMEM to give a final concentration of 1.7uM.

After the addition of all 3 reagents to the DMEM the medium was filter sterilised and used immediately.

3.1.3. Freezing and Thawing Cells

To create a stock of frozen 3T3 L-1 cells, the confluent cells (grown in 25 cm^2 flasks) were rinsed with sterile PBS and detached by incubating with 1ml of trypsin for 5-10 minutes at 37 °C. The reaction of trypsin was stopped with DMEM containing 10% foetal bovine serum (FBS), and the cell suspension was centrifuged at 4000 g for 3 minutes . The supernatant was discarded and the cell pellet was resuspended with 2ml of freezing medium , which consisted of the DMEM described in table 3.1 supplemented with 10% dimethyl sulphoxide (DMSO; Sigma). This solution was then dispensed into cryo-vials, 1ml per tube and left overnight in a polystyrene container at -70 °C. The following day the vials were transferred to liquid nitrigen.

Cells were re-cultured by removing vials from liquid nitrogen storage and placing in a 37 °C water bath. Cells thawed quickly with shaking and were transferred to a 25cm^2 tissue culture flask containig 5ml of DMEM supplemented with 20% FBS. Cells were incubated overnight in a 37 °C incubator in a 95% air 5% carbon dioxide atmosphere. Cells were checked for growth and medium replaced with fresh medium. If cells were seen to be growing well DMEM-20% FBS was replaced with DMEM containing 10% FBS after 3-4 days ie medium in table 3.1.

3.1.4. Culturing and Transforming the 3T3-L1 Cells

Transformation medium was added to cells in 25cm^2 flasks once they were confluent .Only cells that had been passaged less than 10 times were used for transformation .After 3 days incubation with transformation medium , the medium was replaced with DMEM containing all ingredients in table 3.1, plus 1.7uM insulin. Cells were maintained for a further 3 days in this medium after which it was replaced with normal DMEM (table 3.1). Medium was then changed every third day.

3.1.5. Alkaline Phosphatase Inhibitors Used with 3T3-L1 Cells

Inhibitors of ALP activity were added to 3T3-L1 cells at the same time as adipogenesis was initiated with transformation medium. Cells were cultured up to 11 days in the presence of these inhibitors and medium was changed every third day (see 3.1.4). ALP activity and adipogenesis were measured at base line and 3, 7, and 11 days after addition of transformation medium. With the inhibitor Phy Gly Gly, these variables were measured only on day zero, 7, and 11. The inhibitors used were:

Levamisole (Sigma)

Levamisole. (0.048g) was dissolved in 2ml PBS and shook until solution was clear. Solution was filter sterilized using a 0.22 micron filter, then 100ul was allocated in each 5ml of tissue culture medium, to give a final concentration of 2.0 mM.

L-phenylalanyl-glycyl-glycine (Phe-Gly-Gly) (Sigma)

A 0.056 g measure of Phe-Gly-Gly was dissolved in 20ml of culture medium, and shook in order to ensure that it was fully dissolved. The medium was then filter sterilized, and 5ml allocated into 25cm^2 culture flasks containing confluent 3T3-L1 cells. The final concentration was 20 mM.

Histidine (Merck)

Histidine (0.465g) was dissolved in 60ml of culture medium and shook , then the medium was filter sterilized and 5ml allocated into 25 cm^2 culture flasks containing confluent 3T3-L1 cells .The final concentration was 50 mM.

The concentrations of the three inhibitors, used were taken from the literature and were confirmed by comparing the effects of different concentrations of each inhibitor on ALP activity or level of adipogenesis. The same concentrations were used for both 3T3-L1 cells and human preadipocytes. The concentrations used were: levamisole 2.0 mM (van Belle, 1977, Price, 1993); histidine, 50 mM (Fishman and Sie, 1971); Phy Gly Gly, 20 mM (Doellgast and Fishman ,1977).. For levamisole, ALP activity in 3T3 –L1 cells was measured after 7 days of transformation (see section 3.3, and 3.4) over a range of concentrations (0.5 mM, 1 mM and 2 mM) on two separate occasions. For histidine, adipogenesis in 3T3-L1 cells was assessed visually after 7 days of treatment with 25 mM, 50 mM and 100 mM of histidine. Phe.Gly.Gly was assessed in a similar manner to histidine using concentrations of 10, 20, 40 and 80mM.

3.2. Measurement of Adipogenesis

Adipogenesis was measured using the oil red O technique. This technique depends on the ability of the lipid droplets in the mature adipocyte to collect the red stain. Oil red O (Sigma) was prepared (Ramirez-Zacarias et al., 1980) by dissolving 420 mg oil red O in 120 ml of absolute Isopropyl alcohol (BDH). The solution was then gently shaken at room temperature overnight .The solution was then filtered twice through a Whatman filter paper then 90 ml of distilled water was added and working solution was left overnight at 4°C.

Intracytoplasmic lipids were quantitated according to the method of Ramírez-Zacarías et al. The 3T3-L1 cells were fixed by addition of glutaraldehyde to a final concentration of 3% for 2 hours. Fixation solution was then replaced by 500µl of 60% isopropyl alcohol. After 5 min, isopropyl alcohol was evaporated and preadipocytes were stained by complete immersion in a working solution of 300µl oil red O for 2 hours. The stain solution was removed and cells rinsed in 500µl of 60% isopropyl alcohol for 5 s. The dye was extracted from the cells by adding 700µl of 60% isopropyl alcohol and shaking for 2 h. The extracted dye was quantitated spectrophotometrically by measurng the absorbance at 510 nm.

3.3. Extraction of Alkaline Phosphatase from Cells

Table 3-2. Alkaline Phosphatase Cell Extraction Solution

Substance	Amount used
Tris-HCL (Sigma)	157.6mg
Triton x-100 (Sigma)	1ml
Phenylmethylsulphonyl fluoride (Sigma)	35mg
Distilled Water	100ml

The solution was pH'ed to pH 7.2. Extraction solution was aliquotted in to Eppendorf tubes (1ml per tube) and kept at –20 °C.

Cell extracts of 3T3-L1s were isolated at baseline and 3, 7 and 11 days after initiation of adipogenesis using the method of Merchant-Larios et al. (Merchant-Larios et al., 1985).Thus, tissue culture medium was removed from the cells and 0.5 ml of ice cold extraction solution was added. The flasks were shaken to detach the cells and the suspension transferred to an Eppendorf tube and centrifuged for 10 minutes at 15,000 g. The supernatant was removed and immediately analysed for alkaline phosphatase activity using an automated colorimetric assay (see section 3.4). The protein content of the supernatant was analysed using the Bradford method (Bradford, 1976) see section 3.5. Alkaline phosphatase activity was calculated as mU of activity per mg protein.

3.4. Measurment of alkaline phosphatase activity

Alkaline phosphatase activity was measured in the Chemical Pathology routine laboratory at the Johannesburg General Hospital, Parktown using a commercial kit (Roche) on an autoanalyser (Modular, from Roche). The assay was performed by a medical technologist. Each assay run contains internal control samples and data was only accepted if controls gave readings within the range specified by the kit manufacturers (Roche). Alkaline phosphatase was measured in human serum samples and cell extracts (see section 3.3). Alkaline phosphatase extraction solution was analysed using this assay method and gave a zero reading on the autoanalyser. The extraction solution was also shown not to interfere with the assay by diluting human serum with the extraction solution and then measuring alkaline phosphatase activity on the autoanalyser.

3.5. Measurment of cell protein

The protein content of 3T3-L1 and human preadipocyte cell extracts was measured using the Bradford method (1975). The protein reagent was prepared by dissolving 100mg of G-250 (Sigma) in 100ml of 85% phosphoric acid (Merck) then 50ml of 95% ethanol (Merck) was added to the mixture and 850ml of distilled water was also added . The solution was shaken unil the G-250 was completely dissolved.

The albumin standard was prepared by dissolving 1 mg of albumin (Sigma) in 1ml distilled water. The albumin standard curve was prepar from this stock solution as described in table 3.3.

Table 3- 3. Albumin standard curve for Bradford protein assay

Volume of albumin stock (ul)	Volume of distilled water (ul)
0	1000
20	920
40	960
60	940
80	920
100	900
120	880

The above standards were vortexed and then 300ul was taken from each and mixed with 2.7ml from the protein reagent. At the same time 50ul from each sample was added to 950ul of distilled water and vortexed .Then 300ul was taken and added to 2.7ml of protein reagent. All samples were vortexed and then absorbance measured in disposable cuvettes.

3.6. Culture of human preadipocytes

3.6.1.Subjects

3.6.2. Breast Reduction Subjects

Human adipose tissue samples were obtained from the mammary adipose tissue of 15 white women of mean BMI 24.7± 1.3 (range 22.2-26.1) and mean age 43.0±4.6 (range 35-51). Tissue was also obtained from 13 black females of

mean BMI 25.1±1.6 (range 22.0-26.7) and mean age 45.7±5.0 (range 38-55). Subject details are given in table 1 of Appendix 1. All subjects were undergoing elective surgical mammary gland reduction. All subjects were healthy. All women were free of disorders of carbohydrate and lipid metabolism, as assessed by history and clinical examination. Ethical approval for use of the adipose tissue was obtained from the University of Witwatersrand, Faculty of Health Sciences Human Ethics Committee. Informed consent was obtained from each patient before surgical intervention. Adipose tissue was processed immediately after removal, and was transferred to the laboratory in sterile Hanks balanced salt solution (Gibco) supplemented with 25mM HEPES (Sigma), 100 U/ml penicillin, 100ug/ml streptomycin (BioWhittaker), 3% bovine serum albumine (Sigma) and pH of 7.2. This medium is called "human adipocyte isolation medium".

3.6.3. Abdominal Subcutaneous Adipose Tissue Subjects

Human abdominal subcutaneous tissues samples were obtained from 13 black women of mean BMI 38.±7.4 (range 29.3-53.5), and mean age 46.5 ± 6.5 (range 35-58). Subject details are given in table 2 of Appendix 1. All women were free from diseases, and disorders of carbohydrate and lipid metabolism, as assessed by history and clinical examination. These subjects had been volunteers in previous studies and fasting blood glucose levels had shown them to be non-diabetic. Ethical approval for use of the adipose tissue was obtained from the University of Witwatersrand, Faculty of Health Sciences Human Ethics Committee. Informed consent was obtained from each volunteer.

A small incision biopsy was performed under local anaesthesia (2% lignocaine). The skin incision was approximately 2cm in length and the procedure performed in theatre under sterile conditions and with the availability of cauterisation to control small bleeding points. A total of 5-10g of subcutaneous fat was removed from the torso of each patient from a site to one side of the umbilicus and placed in sterile isolation medium. As described in section 3.6.2. Preadipocyte isolated was peformed immediately.

3.6.4. Isolation of Preadipocytes and Mature Adipocytes from Human Subcutaneous Abdominal and Mammary Gland Adipose Tissue

Human fat tissue was collected from the theatre in sterile human adipocyte isolation medium. After excision of blood vessels, adipose tissue was minced into small pieces using sterile scissors, then the sample was washed with isolation medium and centrifuged for 5 minutes at 380g. Tissue was then decanted into isolation medium (0.7g tissue per ml medium) supplemented with 0.75mg/ml collagenase (Roche) and digested 1 hour at 37 centigrade with constant shaking. Suspended cells were then filtered through a 250micron metal filter and the product spun for 10 minutes at 380g. This gives rise to a pellet of stromavascular cells containing the preadipocytes and a layer of mature adipocytes which float on top of the medium due to their high fat content. The mature adipocytes were taken and frozen in liquid nitrogen, and the solution over the stromavscular pellet thrown away,while the pellet was resuspended in 100ml of red cell lysis buffer (0.154M ammonium chloride [Univar], 10mM potassium bicarbonate [Merck],

0.1m M EDTA [Sigma] and 10% fetal bovine serum [Gibco]) and allowed to settle for 10 minutes at room temperature followed by 10 minutes centrifugation at 380g. The cell pellet was resuspended in human preadipocyte tissue culture medium. This medium was DMEM-F12 medium (BioWhittaker) containing 15mM HEPES and 2mM glutamine, supplemented with 10% fetal bovine serum (Gibco) and 100 U/ml penicillin, 100ug/ml streptomycin (BioWhittaker). The cells were then filtered through a 20micron nylon mesh and aliquotted into 6-well tissue culture plates (Nunc). The preadipocytes were then cultured overnight and the next day were washed using DMEM-F12 medium.The medium was changed every third day until cells were confluent. At this point, adipogeness was initiated by adding the transformation medium. This medium is the same as the human preadipocyte tissue culture medium described above with these additions:

Hydrocortisone (Sigma)

1mg was dissolved in 0.5ml of absolute ethanol and 0.5ml chloroform and 90ul of this mixture was added to 3.8ml of PBS. From this solution 100ul was added to 50ml of DMEM-F12 medium , to give a final concentration of 100 nM

3, 3, 5-Triiodo-1-thyronine (Sigma)

1 mg was dissolved in 1ml of 1M sodium hydroxide. From this solution 100ul was taken and added to 1ml PBS. Then, 14ul was taken from this solution and added to 1ml PBS. Finally, 25ul was taken from this solution and added to 50ml DMEM-F12, to give a final concentration 1 nM.

IBMX (Sigma)

25 mg was weighed and dissolved in 1ml of warm methanol, then 110µl was taken from this solution and added to 50ml of DMEM-F12, to give a final concentration of 0.5 mM.

Insulin (U100 Actrapid insulin, Novo-Nordisk)

A 0.13ml aliquot of insulin was added to every 50ml of DMEM to give a final concentration of 1.7µM.

3.6.5 Inhibitor studies on human preadipocytes

Adipogenesis and ALP activity were measured on day zero and following 12 days of cell growth in transformation medium. Adipogenesis and ALP activity were measured using the same techniques previously described for the 3T3 L1 cells (see sections 3.2, 3.3 and 3.4). Inhibitors were added to the cells on day zero through to day 12, with medium changed every three days. ALP activity was also measured in human preadipocyte cell extracts in the presence and absence of levamisole. This was performed on 5 different cell extracts obtained from mammary tissue of white females. The extracts were obtained after the cells had been grown in human transformation medium for 12 days in the absence of ALP inhibitors, and as described in section 3.2. The cell extracts were divided into 2 aliquots of 250ul each and to one extract 4ul of PBS was added and to the other 4ul of a levamisole solution (gives final concentration of 2.0 mM) made up as follows: 0.045g of levamisole dissolved in PBS (see section 3.1.5).

3.7 Localisation of Alkaline Phosphatase Activity

The 3T3-L1 and human preadipocytes were cultured on cover slips in small petri dishes under the same conditions described before, and adipogenesis was intiated using the previously described methodology (see sections 3.1.4 and 3.6.4). Cells that had been grown in differentiation medium for at least 7 days were used for the subcellular localization of alkaline phosphatase .Tissue culture medium was aspirated off cells which were then washed two times with phosphate buffered saline solution (PBS), pH 7.4. Cells were then fixed for 15 minutes at room temperature with a 3% formaldehyde / PBS solution and then washed three times with PBS.

Alkaline phosphatase detection was carried out with ELF 97 endogenous phosphate detection kit (Molecular Probes). The ELF 97 substrate was diluted 1:20 in ELF-97 buffer, vortexed and filtered through an ELF 97 spin filter. An 80ul aliquot of substrate solution was applied per coverslip and the reaction monitored on Carl Zeiss Axiovert 100 M fluorescence microscope. The signal was visualized with Hoechst/DAPI filter set with excitation of 365 nm and emission > or = 400 nm. After 15 minutes, once sufficient reaction product had formed, the cover slips were mounted on microscope slides in ELF 97 mounting medium . Omission of substrate produced no labelling .Images were digitally captured using Carl Zeiss Axiovision software, version 2.0.5.

Dr Clem Penny performed the image capture of the stained cells with help from Mr Aus Ali.

3.8 The Effect of BMI on ALP and Liver Enzyme Activity in Serum

The finding that ALP was expressed in human preadipocytes led us to question whether serum ALP levels may not be associated with body fat mass. Total serum ALP level was therefore measured in 101 African subjects of varying BMI. Ethical approval for the collection of serum samples was obtained from the University of Witwatersrand, Faculty of Health Sciences Human Ethics Committee. Informed consent was obtained from each volunteer. .Only subjects who had no known liver pathology or bone fractures within the last 2 years were included in the study. Bone fracture and liver disease can lead to elevated serum ALP levels (Price, 1993) and liver disease can also lead to elevated serum levels of albumin, bilirubin, alanine aminoransferae (ALT), total protein and gamma glutaryltransferase (GGT). These enzyme levels were therefore also measuerd in serum to ensure that none of the volunteers had any liver pathology which may lead to elevated ALP levels. Elevated serum liver enzyme concentrations are also associated with obesity (Golik et al., 1991), particularly abdominal obesity. Therefore, this study also analysed the association of liver enzyme levels with BMI and waist-to-hip ratio.

ALP in the serum is mainly derived from the liver, bone and intestine (Price, 1993) and the assay used to measure serum ALP cross reacts with all 3 of these forms of the enzyme. A bone-specific ALP ELISA is available and this assay was also used to measure bone ALP. The value derived from the bone specific assay was subtracted from that obtained from thre total ALP assay to

give an estimate of the liver ALP levels in the serum. Intestinal ALP makes up less than 15% of the total ALP in serum (Price, 1993).

A small study was undertaken to test the effect of food intake on serum total ALP levels. This was performed in order to determine whether non-fasting blood samples could be used for the measurement of serum ALP levels in the larger study. A total of 12 (10 females) volunteers were used and ALP levels were measured in serum after an overnight fast and 60 minutes after breakfast, both blood samples being drawn on the same day. The serum total ALP level (mean ± SD) was 61.4 ± 17.6 U/l before breakfast and 60.8 ± 17.1 U/l after breakfast ($p=0.43$). Thus, food intake had no effect on serum ALP levels and therefore in the larger study, non-fasting blood samples were used to determine serum levels of total ALP.

Weight and height were measured in 101 African subjects using a stadiometer (Modern Scale Co., Johannesburg). Waist-to-hip ratio (WHR) was measured standing by taking waist circumference as the midpoint between the lower rib margin and the iliac crest and hip circumference as the widest circumference of the buttock. A 5ml blood sample was taken from the antecubital vein of the forearm from each subject. The sample was immediately centrifuged and the serum removed and aliquotted into 2 tubes, one for liver function tests and the second for bone-specific ALP measurement. Serum samples were stored at $-20^{o}C$ until assayed. Ethical approval for blood sampling was obtained from the University of Witwatersrand, Faculty of Health Sciences Human Ethics Committee. Informed consent was obtained from each volunteer.

All following assays were measured in the Chemical Pathology routine laboratory using a commercial kit (Roche) on an autoanalyser (Modular, from Roche).

3.8.1. Alanine aminotransferase(ALT) measurement

ALT enzyme catalyzes the following reaction;

α-ketoglutarate +L-alanine→L-glutamate+pyruvate

The increase in pyruvate is determined in an indicator reaction catalyzyed by lactate dehydrogenase(LDH) as follows;

Pyruvate+NADH+H^+ ↔L-lactate+NAD^+

NADH is than oxidized to NAD. The rate of the photometrically determined NADH decrease is directly proportional to the rate of formation of pyruvate and thus the ALT activity.

3.8.2. Albumin (ALB) measurement

At the pH value of 4.1 albumin displays a sufficiently cationic character to be able to bind with bromcresol green (BCG), an anionic dyestuff, to form a blue –green complex.

Albumin+BCG → albumin BCG complex

The colour intensity of the blue-green colour is directly propotional to the albumin concentration and can be determined photometrically

3.8.3. Gamma-glutamyl-transferase (GGT) measurement

This reaction depends on the ability of gamma-glutamyltransferase to transfer the γ-glutamyl group of L- γ-glutamyl-3-carboxy-4-nitroanilide to glycylgycine. The amount of 5-amino-2-nitrobenzoate liberated is propotional to the GGT activity and can be determined photometrically. The reaction below describe the whole process;

L- γ-glutamyl-3-carboxy-4-nitroanilide+ glycylgycine→ L- γ-glutamyl-glycylglycine+5-amino-2-nitrobenzoate.

3.8.4. Total bilirubin (TBil) measurement

Indirect bilirubin is liberated by the addition of a detergent , and the total bilirubin is then coupled with 2,5-dichlorophenyl diazonium tetrafluoroborate under strongly acidic conditions (pH 1-2) to form the corresponding azobilirubin, as described below:

Bilirubin+diazonium ion→ azobilirubin

The photometrically determined colour intensity of the red azo-dye is directly proportional to the total bilirubin concentration.

3.8.5. Total protein (TP)

Divalent copper reacts in alkaline solution with protein peptide bonds to form the characterstatic purple-colored biuret complex. Sodium potassium tartrate prevents the precipitation of copper hydroxide and potassium iodide prevents autoreduction of copper.

Protein + Cu^{+2} →Cu-protein complex

The colour intensity is directly proportional to the protein concentration which can be determined photometrically.

3.9. Statistical Analysis and Data Presentation

The statistical analysis for all experiments was performed using the Statistica package (Statsoft Inc., Tulsa, Oklahoma, USA). Differences within treatment groups were compared using a paired Students t-test. The effects of removing or changing concentrations of the constituents (ie insulin, IBMX and dexamethasone) of the transformation medium were analysed using ANOVA. The inhibtor studies on 3T3-L1 cells were performed a minimum of three times for each inhibitor. In human preadipocytes levamisole treatment was performed on mammary cells from 9 black and 8 white females. All other inhibitors were tested on a minimum of four subjects of each ethnic group. Preadipocytes from some patients were sufficient to perform studies with more than one inhibitor. Comparisons of means within subject groups were performed by Students paired t-test whilst differences between ethnic groups and between preadipocyte-isolation sites were compared using Students non-paired t-test..
Relationships between anthropometric variables and liver function were analysed by Pearson correlation and multiple regression analysis. Data that was not normally distributed was either log transormed or converted to reciprocals, or square roots according to the degree of skewness. Differences between groups (males versus females, lean versus overweight versus obese) were compared

using ANCOVA and post hoc comparisons were performed using the Tukey honest significant difference (HSD) test for equal N numbers. Data is presented for ALP inhibitor studies as histograms and is also presented in numerical form in Appendix 2, in an unadjusted form. This data was also expressed as a percent of the data obtained on day 0 (set at 100%). The percentage values have only been given for the 3T3-L1 levamisole data (see section 4.1.2) as this was only experiment in which significant trends were uncovered by expressing data as percentages. In all other experiments there were no significant differences in observed trends for data expressed unadjusted or as percentages.

CHAPTER FOUR

RESULTS

Introduction

The initial aim of this research project was to determine whether ALP is expressed in 3T3-L1 cells and human preadipocytes, and if so what role does it play, if any in intracellular lipid accumulation. An additional aim was to assess whether ethnic differences existed in adipogenesis rate in white and black South African females. When it became clear that ALP was expressed in human preadipocytes, additional aims were added to the project. These were to determine whether ALP was expressed in different adipose tissue depots (mammary and subcutaneous abdominal) in humans and was the level of activity different in the two ethnic groups under investigation. Additionally, since ALP is found in human serum and it has been assumed that the principal tissue sources for serum ALP were liver, bone and intestine we decided to investigate whether adipose tissue also contributed to the ALP present in the circulation.

The data in the Results section has been set out in the following order:

1. Results from 3T3-L1 cells: measurement of presence of ALP activity before and during adipogenesis; the effects of ALP inhibitors on lipid accumulation; effect of varying concentrations of transformation medium components i.e. insulin, IBMX and dexamethasone on lipid accumulation and ALP activity; intracellular localisation of ALP.
2. Results from human preadipocytes: ALP activity in mammary tissue preadipocytes from black and white females and effects of ALP inhibitors on

cytoplasmic lipid deposition; intracellular localisation of ALP; ethnic differences in ALP activity and rate of intracellular lipid accumulation; detection of ALP activity in subcutaneous abdominal adipose tissue preadipocytes isolated from black females and effect of ALP inhibitors; comparison of ALP activity and rate of intracellular lipid accumulation in the 2 fat depots.

3. Measurement of ALP activity in human serum: differences in ALP and other liver enzyme serum levels in lean, overweight and obese subjects; gender differences in liver enzyme levels in serum; correlation between BMI and total, bone-specific and total-bone specific ALP activity in human serum; correlation between level of liver enzymes present in human serum and selected anthropometric variables.

The results for all the preadipocyte experiments are shown in this chapter as histograms and are also shown in Appendix 2 as means ± SEMs.

4.1. 3T3-L1 study results

As discussed in section 3.1.5, the concentration of ALP inhibitors used in the following experiments was determined by reviewing the available literature and confirming by performing ALP assays or assessing adipogenesis over a range of different inhibitor concentrations. With levamisole, ALP activity was assessed and results were as follows: without levamisole, 91.7±11.1 mU/mg cell protein (mean ±SD) ; 0.5 mM levamisole, 76.7±23.5 ; 1 mM levamisole, 64.1±16.8 ; 2.0 mM levamisole , 49.0±9.0. For histidine, 25 mM caused minimal inhibition of adipogenesis in 3T3-L1, whilst 50 mM and 100 mM both caused an approximate

80% inhibition of adipogenesis. Phe Gly Gly at 10, 20, 40, and 80 mM cause no inhibition of adipogenesis in 3T3 L1 cells.

4.1.1. Alkaline phosphatase activity is present in 3T3-L1 cells and increases during the differentiation process

Alkaline phosphatase activity was detectable in non-differentiated 3T3-L1 cells at a level of 1.11 ± 0.62 mU/mg protein (mean ± s.e.m. of 4 experiments). After 3, 7, 11 and 17 days treatment with differentiation medium the levels of alkaline phosphatase activity were: 10.94 ± 2.89 (n=3), 17.16 ± 6.03 (n=4), 22.97 ± 9.19 (n=4) and 31.2 ± 0.73 mU/mg protein (n=2) respectively and the level of alkaline phosphatase activity was significantly different from the level in non-differentiated cells from day 3 onwards ($p<0.05$ for all).Adipogenesis assessed using oil red O ,also increased over the 17 day period, and was significantly different from the level on day zero from day 7 onwards ($p<0.01$ for all).Figure 4.1 shows the parallel relationship between ALP activity and adipogenesis in 3T3-L1.

4.1.2. Effect of levamisole treatment

In the presence of levamisole, ALP activity in the 3T3-L1 cells tended to be lower on days 7 and 11 compared to levels in cells not treated with the inhibitor, but mean values were not significantly different. Likewise, ALP activity was higher on days 3, 7 and 11 compared to activity on day 0, but these differences were not statistically different. This lack of significance is a result of the high standard errors. Therefore, data was also expressed as a percentage of the ALP activity obtained on day 0, which was set at 100%. This lowered the standard errors, and

significant differences were then obtained for ALP activity in absence and presence of levamisole on days 7 (898±96% vs. 615±102%, respectively; P=0.002) and days 11 (1150±136% vs. 531±31%; P=0.01).

Triglyceride content of 3T3-L1 cells in the absence of levamisole was significantly higher on days 7 and 11 (both $P<0.05$) compared to day zero. This was not the case on day 3. Statistically significant differences between treatments were observed on day 7 and day 11 (both $P<0.05$), but not on day 3. Levamisole is therefore inhibiting both ALP activity and adipogenesis (see figure 4.2A,B).

4.1.3. Effect of Phe Gly Gly treatment

Differentiation in the presence of Phe-Gly-Gly did not lead to any significant changes in either alkaline phosphatase activity or level of adipogenesis (see figure 4.3A,B). Significant differences were found for ALP activity when treated ($P<0.05$ and $P<0.005$) and non-treated (both $P<0.05$) cells on day 7 and day 11, respectively, were compared with day zero. Significant differences were also found for lipid accumulation when non-treated cells on day 7 ($P<0.05$) and day 11 ($P<0.005$), were compared with day zero. Only treated cells on day 11 ($P<0.005$) showed significantly higher lipid accumulation than cells on day 0.

4.1.4. Effect of histidine treatment

Histidine also inhibited both alkaline phosphatase activity and adipogenesis but to a greater extent than did levamisole. Thus, on days 3 , 7, and 11 histidine treatment completely blocked the rise in ALP activity, whilst in the absence of

histidine, ALP activity was significantly higher than on day zero, after 3 (P,0.05), 7 ($p<0.005$) and 11 ($p<0.05$) days of transformation. Significant differences between treatments were observed on days 3 ($P<0.05$), 7 ($p<0.005$), and 11 ($p<0.05$). Data for the effects of histidine on adipogenesis mirrored those for ALP activity.

Light microscopic analysis of lipid droplet formation in the 3T3-L1 cells in the presence and absence of histidine and using oil red O to stain for triglyceride, demonstrated the strong inhibitory effect of histidine on intracellular triglyceride accumulation (See Picture 4.1A,B).

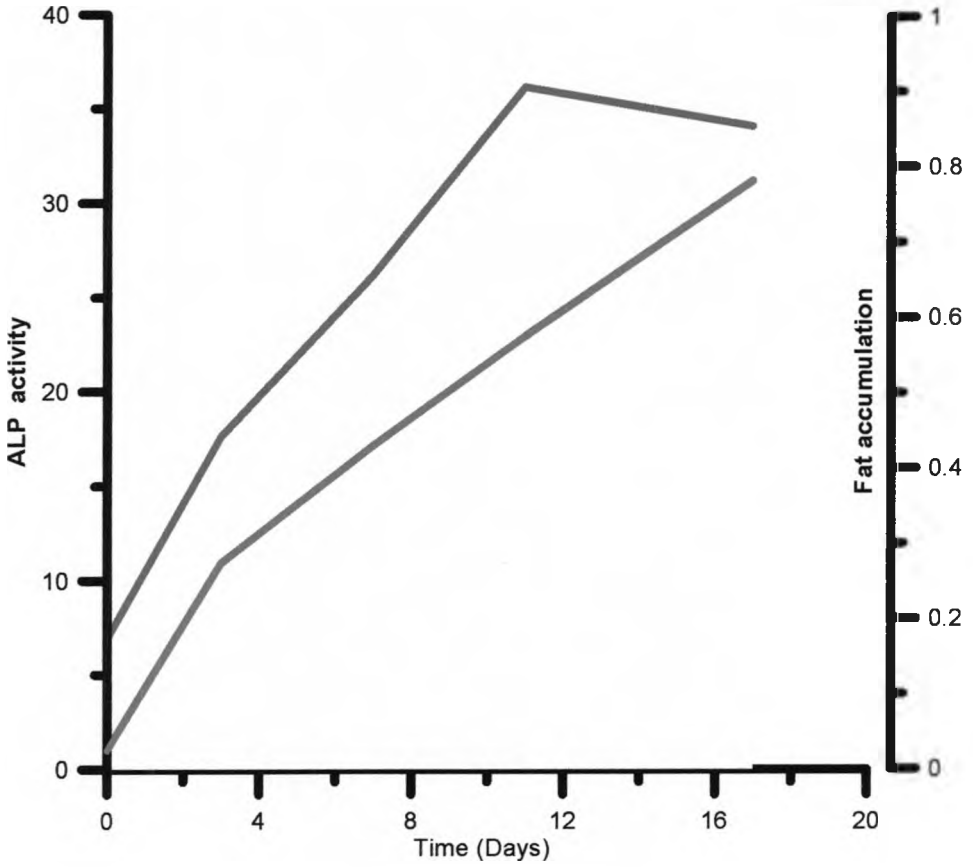
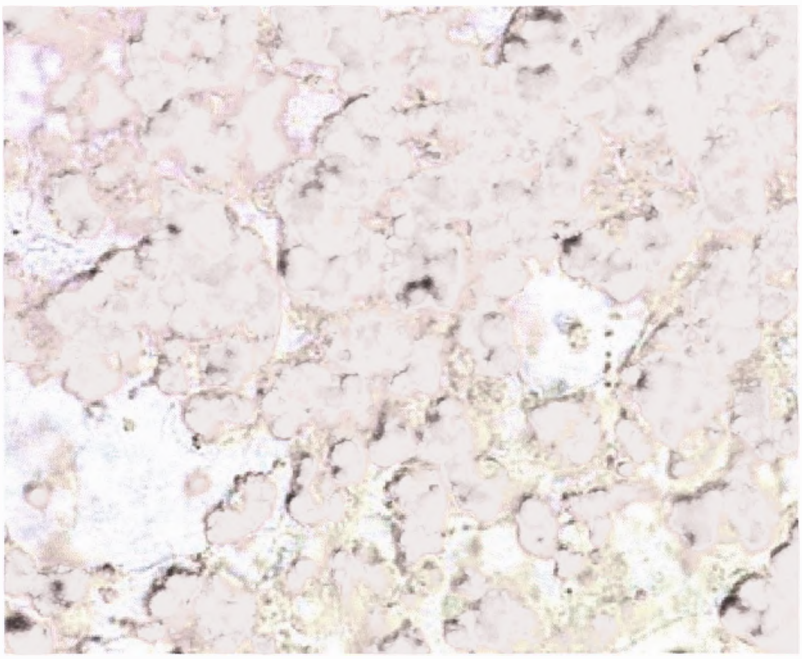
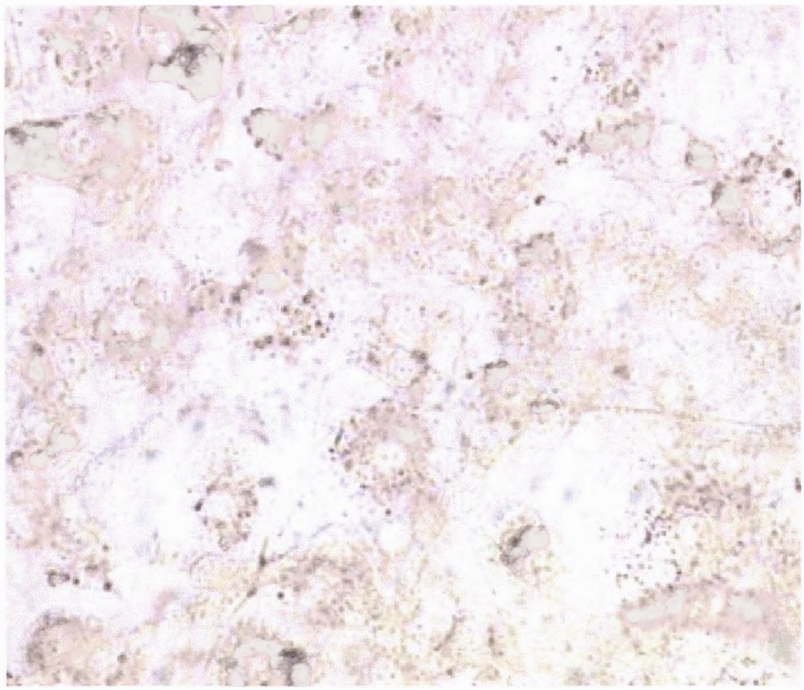


Figure 4.1 The relation between ALP activity and intracellular lipid accumulation in 3T3-L1 cells (red line represents ALP and blue line represents fat accumulation). See text in section 4.1.1 for details of number of times experiment performed and mean ± SEM values.

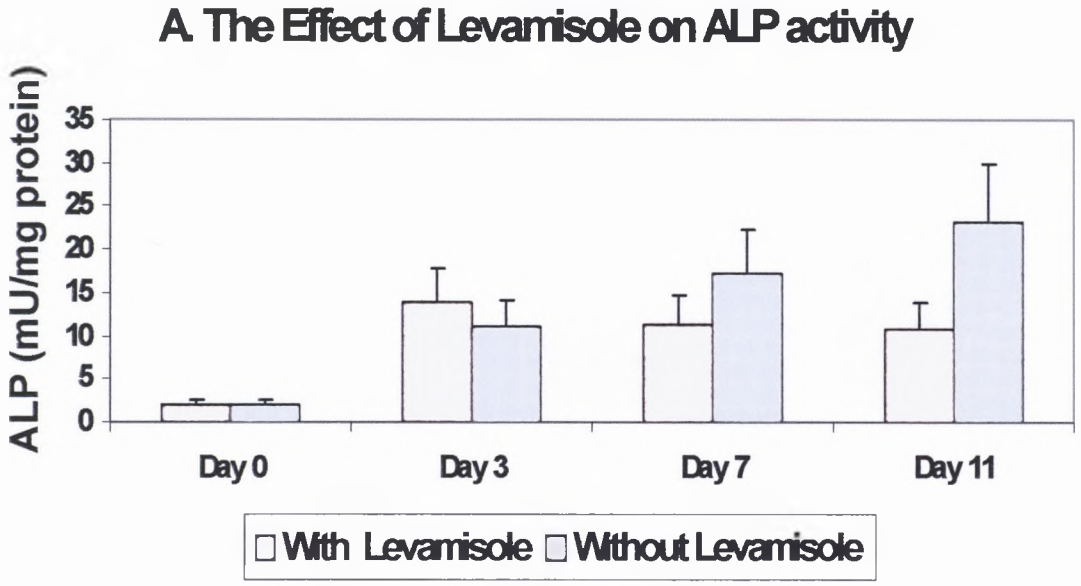
A. Without histidine



B. With histidine



Picture 4.1 A, B shows the effect of histidine treatment on lipid accumulation in 3T3-L1 cells.



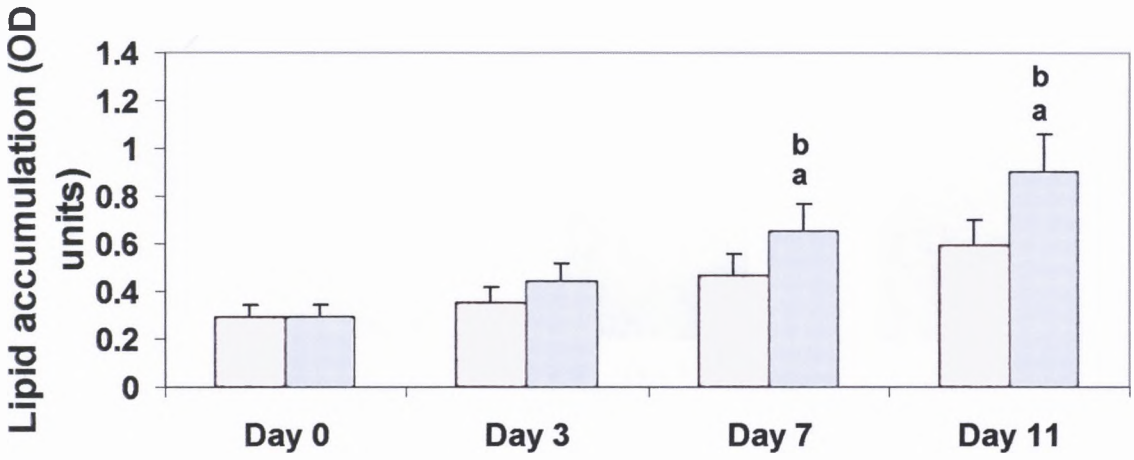
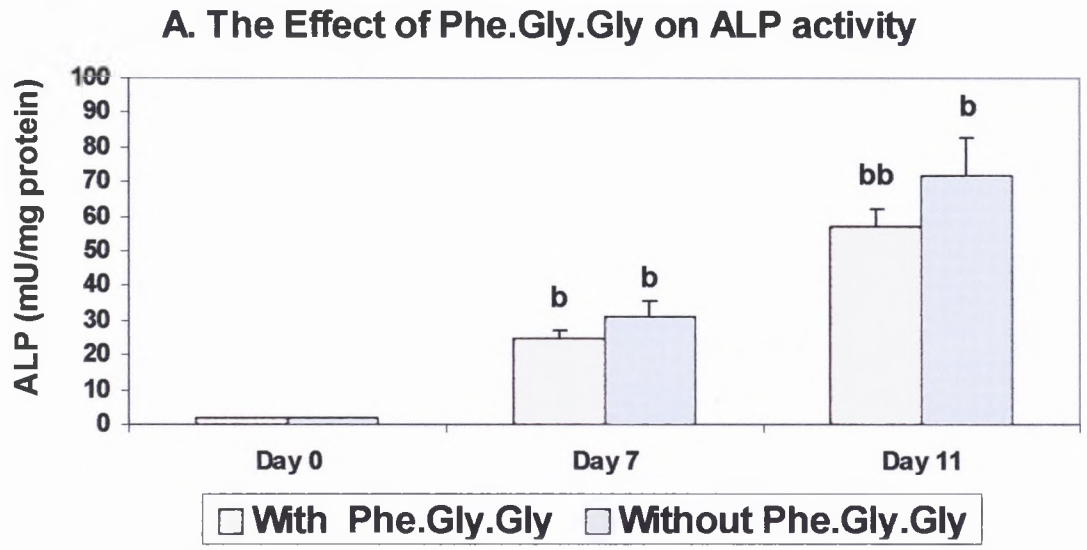


Figure 4.2. Effect of levamisole on: A. ALP activity and B. Lipid accumulation in 3T3-L1 cells. Each bar represents the mean ± SEM of 4 experiments.

[a]P<0.05 Vs treated cells [b]P<0.05 Vs cells on day 0.



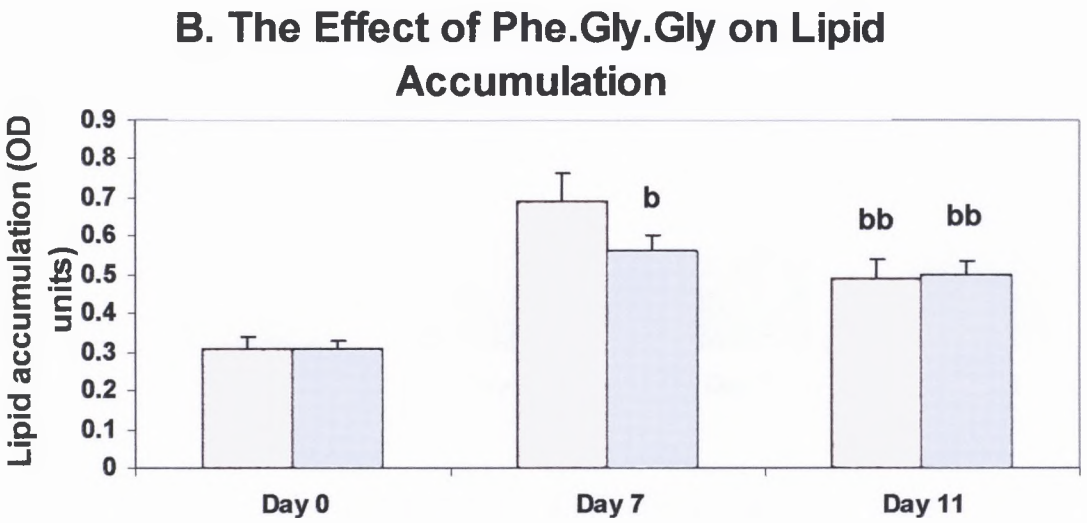


Figure 4.3. Effect of Phe.Gly.Gly on: A. ALP activity and B. Lipid accumulation in 3T3-L1 cells. Each bar represents the mean ± SEM of 4 experiments.

$^{b}P<0.05$, $^{bb}P<0.005$ Vs cells on day 0.

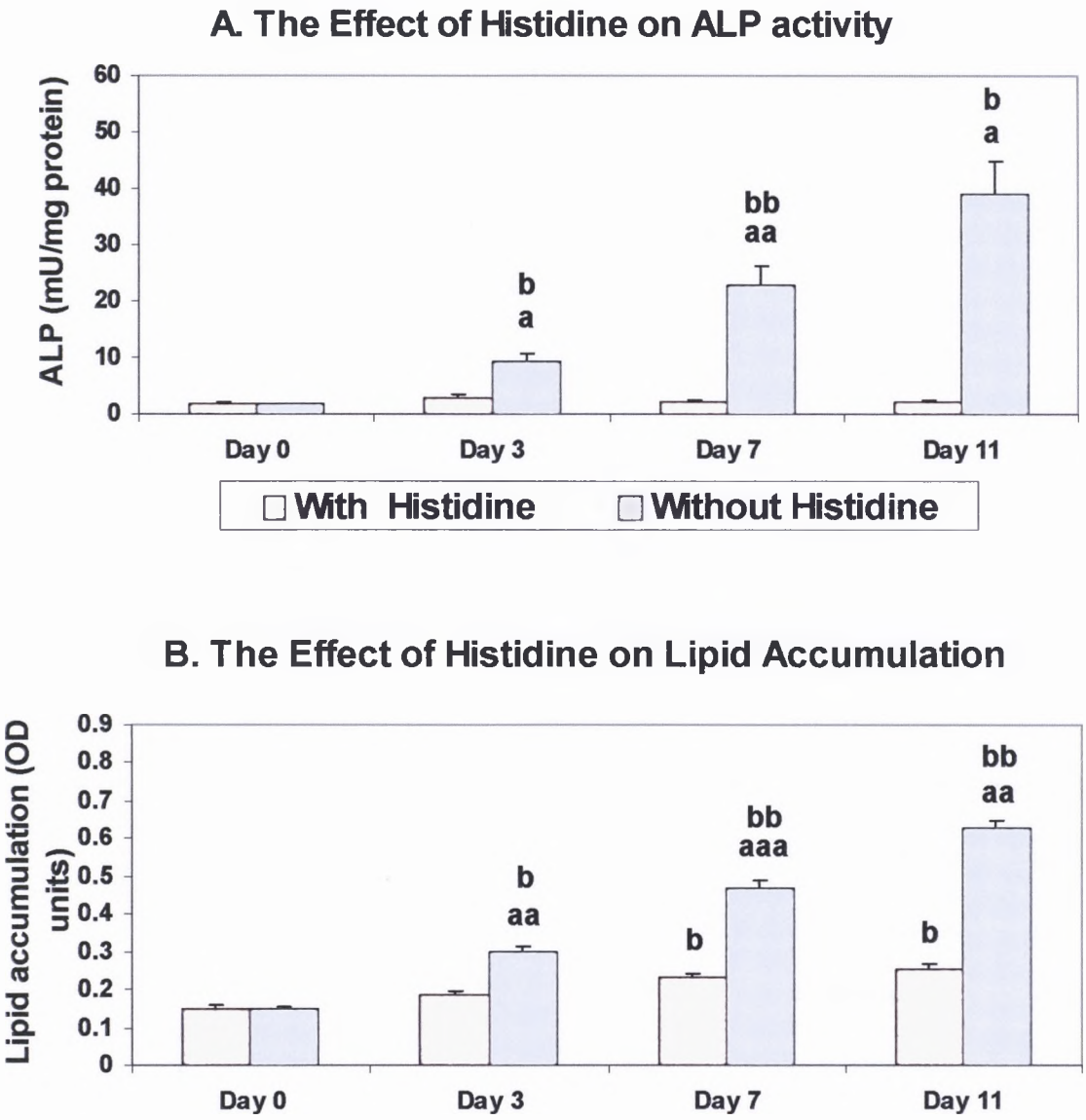


Figure 4.4. Effect of Histidine on: A. ALP activity and B. Lipid accumulation in 3T3-L1 cells. Each bar represents the mean ± SEM of 4 experiments.

$^{a}P<0.05$, $^{aa}P<0.005$, $^{aaa}P<0.0005$ Vs treated cells; $^{b}P<0.05$, $^{bb}P<0.005$ Vs cells on day 0..

4.1.6. The effect of removing IBMX, dexamethasone, or insulin from the transformation medium

These experiments compared four treatments as follows; 0 IBMX (when the transformation media had no IBMX), 0 DEXA (when the transformation media had no dexamethasone), 0 Insulin (when the transformation media had no insulin), and normal transformation media (which contains the normal levels of IBMX, DEXA, and insulin). Differences in ALP activity were significant on day 3($p<0.05$) and day 6($P<0.05$) compared with day zero, when insulin and dexamethasone were removed from the transformation medium, and this was also the case for the normal medium. ALP activity on day 6 was higher compared to that on day 3 for both the normal medium and the medium lacking dexamethasone. This was not the case for the medium lacking insulin. Therefore, the removal of dexamethasone has no effect on ALP activity whereas, the removal of insulin does have an effect after 6 days. The removal of IBMX from the transformation medium resulted in ALP levels on day 3, and day 6 that were not different from that on day zero.

Removal of dexamethasone or insulin from transformation medium had no effect on adipogenesis. Thus, after 6 days of treatment triglyceride accumulation was significantly higher compared to day zero for the normal medium and the mediums lacking insulin or dexamethasone. Removal of IBMX from transformation medium however, blocked adipogenesis. These results mirrored those obtained for ALP activity (see figure 4.5A, B).

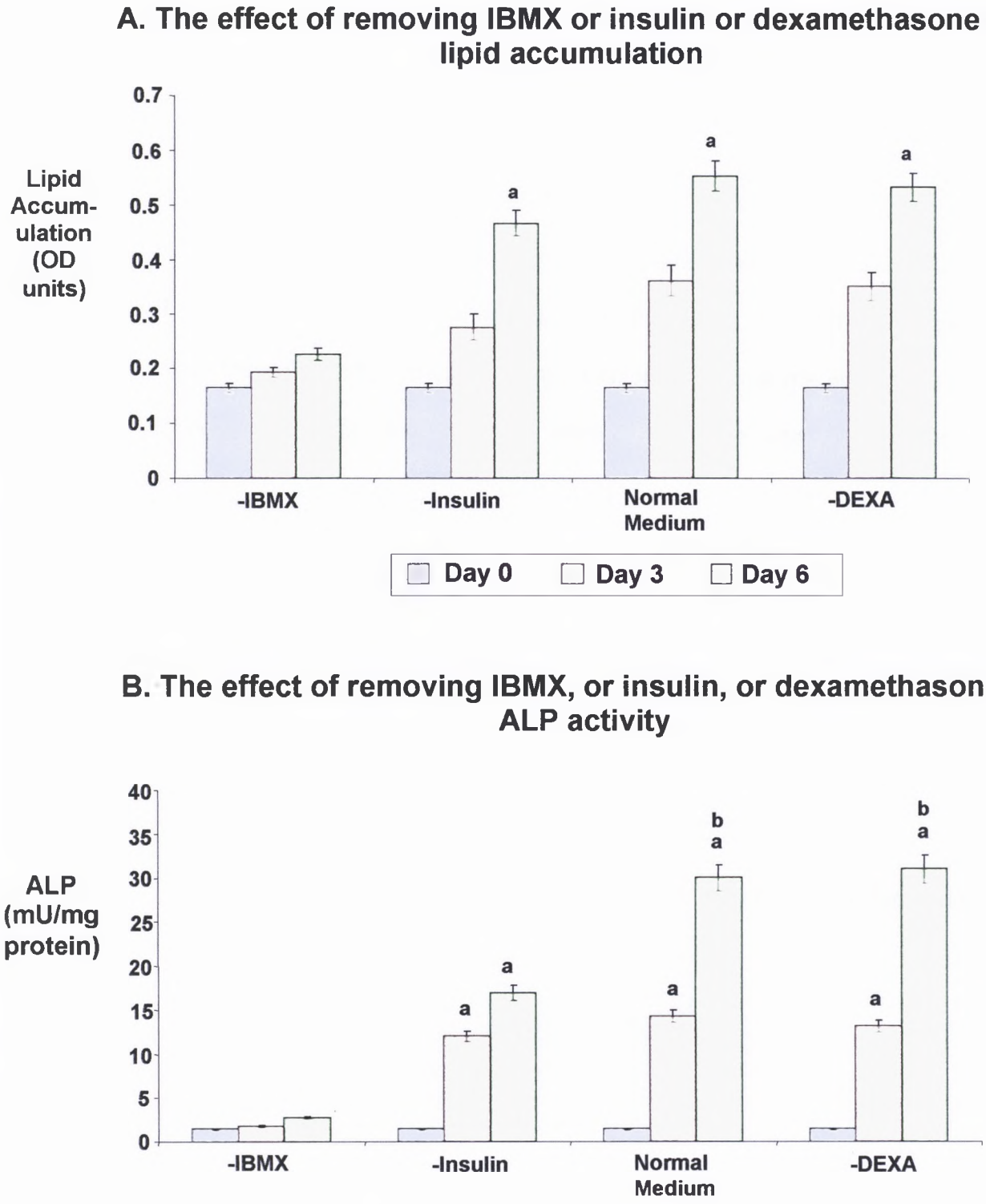


Figure 4.5. The effect of removing insulin, IBMX or dexamethasone on: A. Lipid accumulation and B. ALP activity in 3T3-L1 cells. Each bar represents the mean ± SEM of 3 experiments. $^{a}P<0.05$ Vs day zero, $^{b}P<0.05$ Vs day 3

4.1.7. The dose response effect of IBMX and dexamethasone on adipogenesis and ALP activity

This experiment was performed in order to determine the dose response relationship between IBMX and adipogenesis, and whether this relationship was similar to the dose response effect on ALP activity. The IBMX concentration chosen were 0.17mM, 0.50mM (concentration in normal medium),and 1.5 mM. The dexamethasone concentration were also varied and the concentrations chosen were 0.023uM, 0.07uM, 0.22uM (concentration in normal medium) and 0.66 uM.

ALP activity was significantly higher on day 3 ($P<0.05$) and on day 6 ($P<0.05$) respectively, for medium containing 0.5mM, and 1.5 mM IBMX when compared to medium containing 0.17 mM IBMX. Altering dexamethasone concentrations had no effect on ALP activity. Adipogenesis also increased with increasing IBMX concentrations, but this trend was not as obvious as that seen for ALP activity . Changing dexamethasone concentrations had no effect on adipogenesis (see figure 4.6A, B).

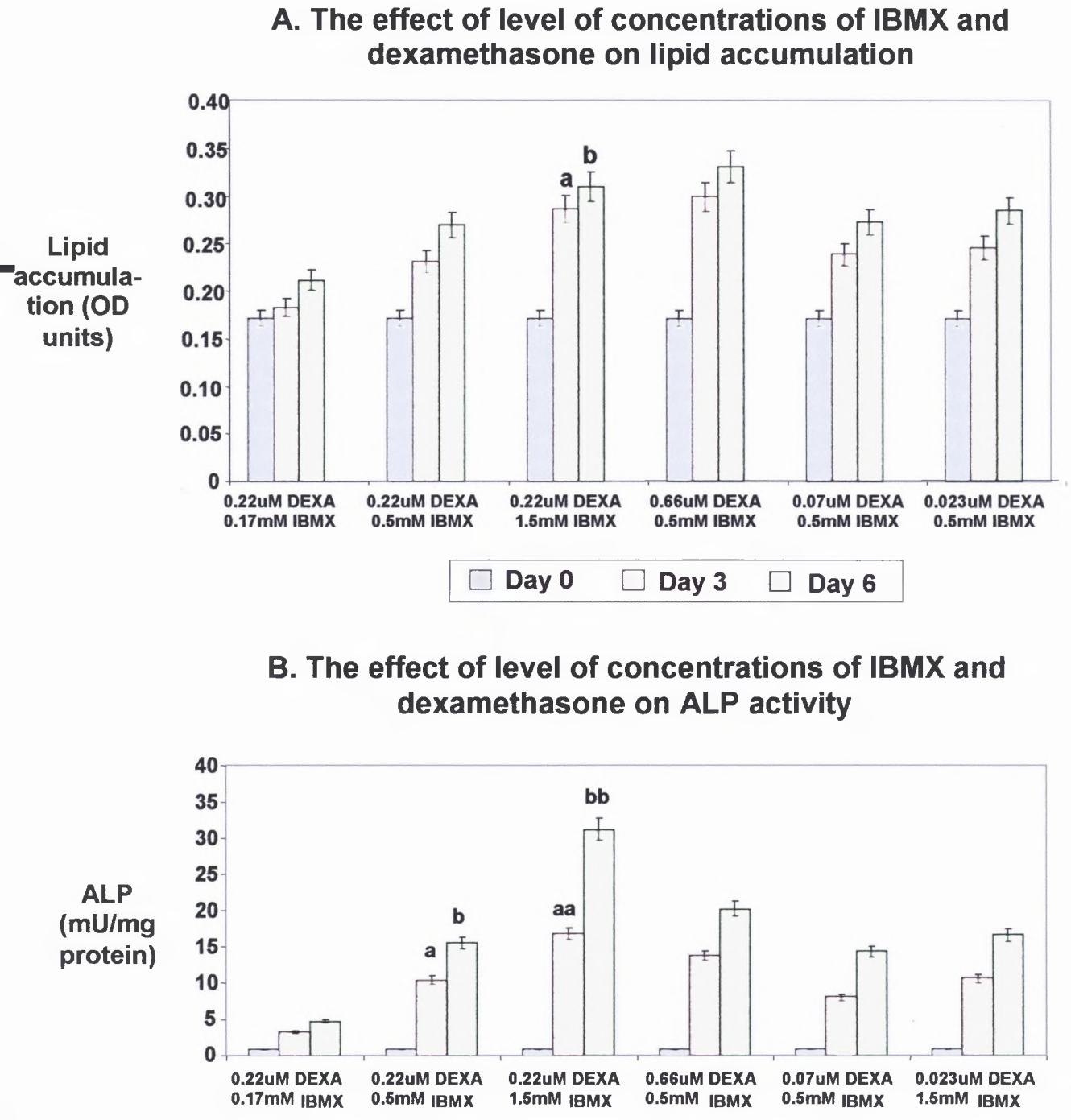
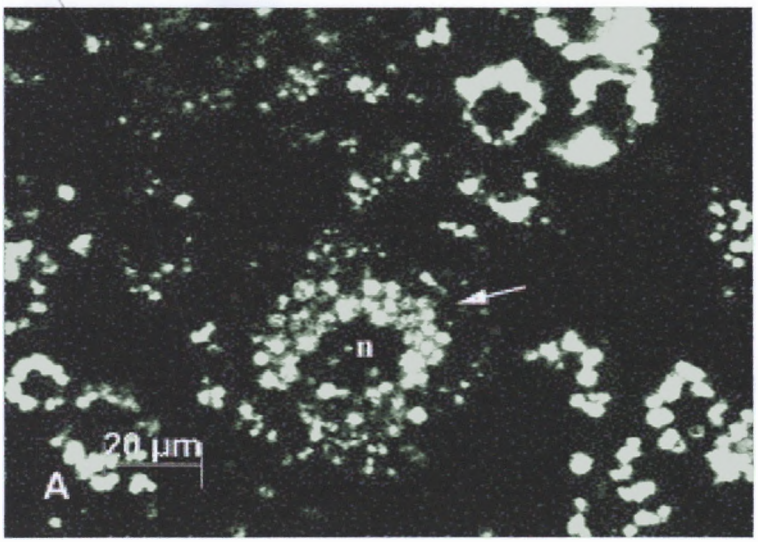


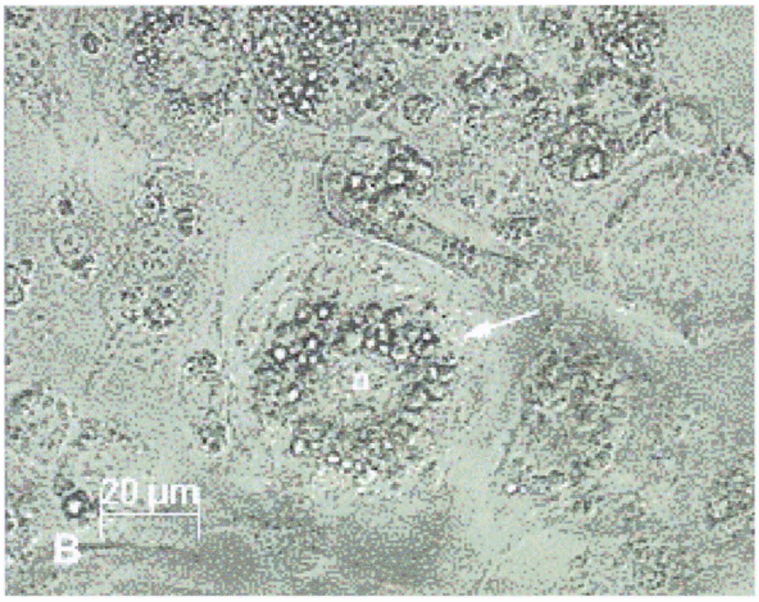
Figure 4.6. The effect of varying concentration of IBMX or dexamethasone on: A. Lipid accumulation and B. ALP activity in 3T3-L1 cells. Each bar represents the mean ± SEM of 3 experiments.

$^{a}P<0.05$. $^{aa}P<0.005$ Vs day 3, 0.17mM IBMX; $^{b}P<0.05$, $^{bb}P<0.005$ Vs day 6, 0.17mM IBMX.

4.1.8. Localisation of ALP activity in 3T3-L1 cells

Using the ELF-97 system for localising ALP activity in intact cells, the fluorescent end product resulting from ALP, cleavage of ELF-97 resulted in fluorescent staining present only on intracellular lipid droplet of 3T3-L1 cells(see picture no 4.2A,B)





Picture 4.2A, B. Fluorescent staining of: A. Alkaline phosphatase activity in 3T3-L1 cells, and B. Normal light microscopy image of 3T3-L1 cells. Images in A and B are the same.

N=nucleus ; arrow indicates lipid droplets.

4.2. Human study results

4.2.1. Breast fat tissue experiments

Anthropometric data for 3 of the 28 human subjects from whom mammary tissue was obtained, were not available (see Appendix 1, table 1). When data from these subjects was removed from the statistical analyses, none of the trends observed were changed.

4.2.1.1. Effect of Levamisole

Alkaline phosphatase activity was detected in non differentiated human preadipocytes collected from the breast during breast reduction operations.

Its activity increased during the 12 day differentiation process.

In White patients the activity of the enzyme was significantly higher on day 12 for the preadipocytes treated with levamisole ($P<0.0005$) compared with non-treated preadipocytes (see figure 4.7A, B). The ALP activity was significantly higher for both treated and non treated preadipocytes ($P<0.0005$), when it was compared with undifferentiated preadipocytes on day zero (number of patients was 9) . These results conflict with our earlier results for 3T3-L1 cells, where the treatment with levamisole inhibited the activity of ALP. In contrast, treatment of human preadipocytes with levamisole caused inhibition of adipogenesis in these same patients. There were significant differences between the preadipocytes treated with levamisole, and non-treated preadipocytes ($P<0.0005$). The adipogenesis results for both treated and non-treated cells were significantly

higher (P<0.0005), on day 12 when compared with undifferentiated preadipocytes on day zero (see figure 4.7A, B).

Similar results were found with preadipocytes collected from Black women. Differences in ALP activity were found on day 12 when treated (P<0.05) and non-treated cells (P<0.05) were compared with ALP activity on day zero. Significant differences were found between treated (P<0.05) and non treated cells on day 12, with ALP activity again being higher in preadipocytes treated with levamisole (Number of patients was 8).

Adipogenesis on day 12 was significantly higher in treated (P<0.005) and non-treated cells (p<0.0005) when compared with adipogenesis on day zero. Significant differences (see picture number 4.3A,B) were also found on day 12 between non-treated cells (P<0.0005) and treated cells (see figure 4.8A,B). Despite levamisole activating ALP activity it was still able to inhibit adipogenesis, as observed in 3T3-L1 cells.

The effect of levamisole treatment on ALP activity in preadipocyte cell extracts was also investigated. Five (5) separate extracts were obtained from mammary tissue preadipocytes cultured for 12 days in absence of ALP inhibitors in human transformation medium. ALP activity was measured in presence and absence of 2.0mM levamisole. The results (means ± SEMs) were as follows:

Treatment	**ALP activity (U/L extract)**
Without levamisole	94.8 ± 30.4
With levamisole	48.8 ± 17.5*

*P=0.025 vs. without levamisole

Levamisole caused a significant (50.4 ± 5.2%) inhibition of ALP activity in the cell extracts.

4.2.1.2. Effect of Phe.Gly .Gly

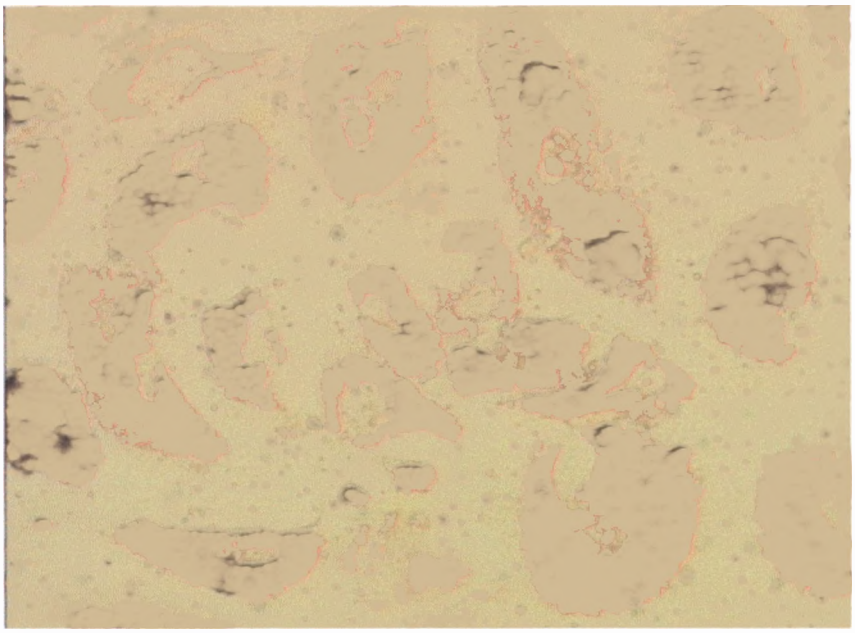
Treating the preadipocytes collected from White and Black females with the tissue specific ALP inhibitor Phe Gly Gly did not effect either adipogenesis (see picture number 4.4A,B) or alkaline phosphate activity (number of patients in these experiments were 4 for each race). The results were significantly higher in cells collected from White ($P<0.05$), and Black subjects ($P<0.05$), when compared with day zero for both adipogenesis and ALP activity respectively(see figures 4.9A,4.9B,4.10A,4.10B) .

4.2.1.3. Effect of Histidine;

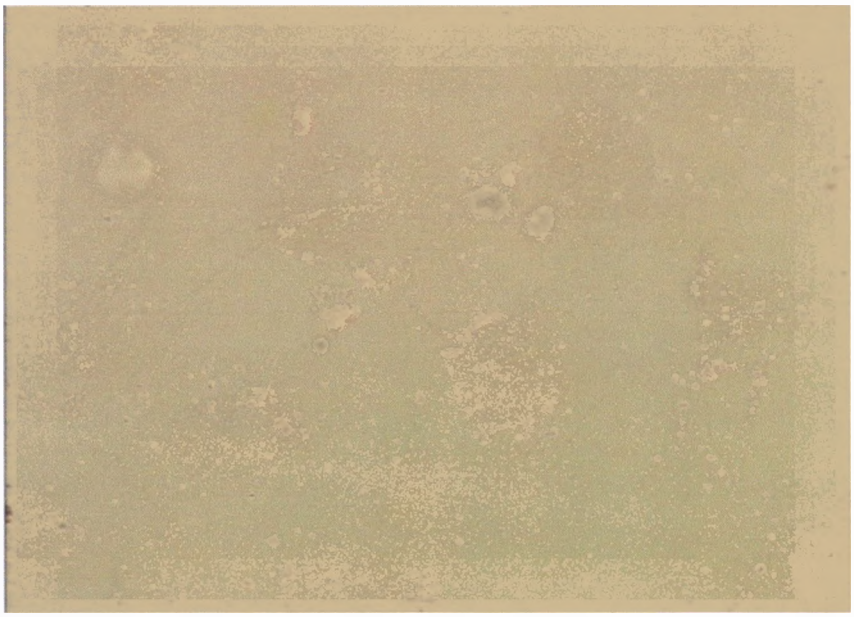
Treating preadipocytes with histidine demonstrated inhibition in the activity of alkaline phosphatase when comparing non-treated and treated cells in white ($P<0.05$),and black subjects ($P<0.0005$) on day 12. ALP activity on day 12 in treated cells collected from black subjects was less than that on day zero ($P<0.005$; see figure number 11B,12B).

Histidine treatment, also caused inhibition of adipogenesis (see figures 11A,12A and picture number 4.5A,B), for both white ($P<0.05$),and black subjects($P<0.0005$). These results were not surprising, as the same effect has been shown with our earlier results using the same inhibitor with 3T3-L1.The number of women in histidine experiment were 4 black women and 4 white women.

A. Without levamisole (control).

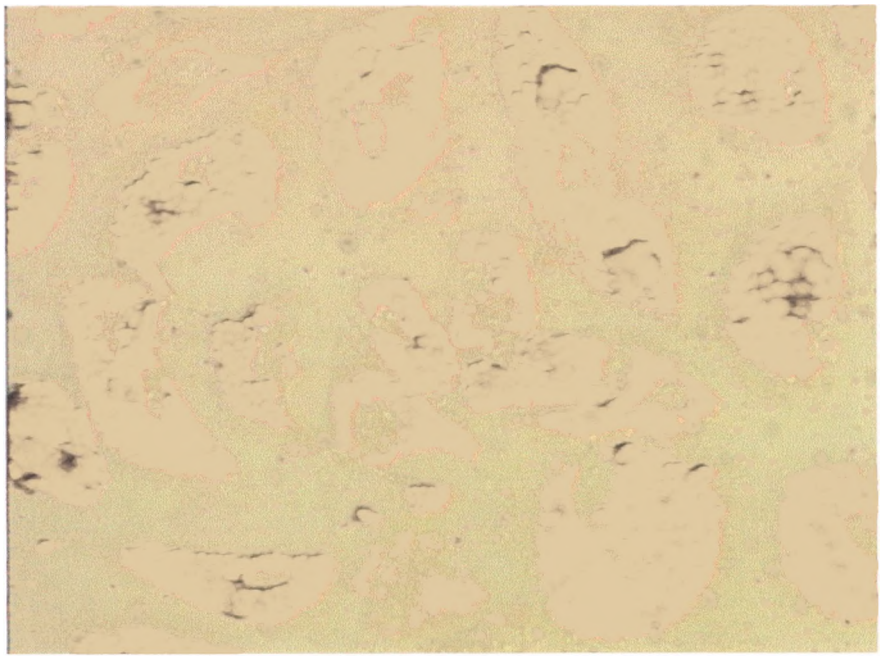


B. With levamisole.

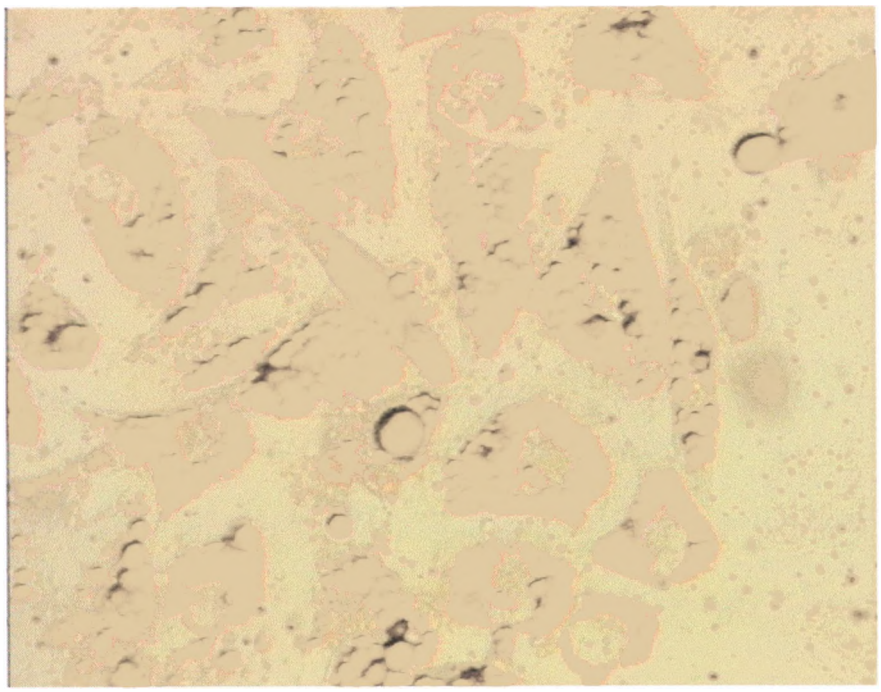


Picture 4.3A,B. The effect of: A. Absence and B. Presence of levamisole on lipid accumulation in human preadipocytes.

A. Without Phe Gly Gly (control).

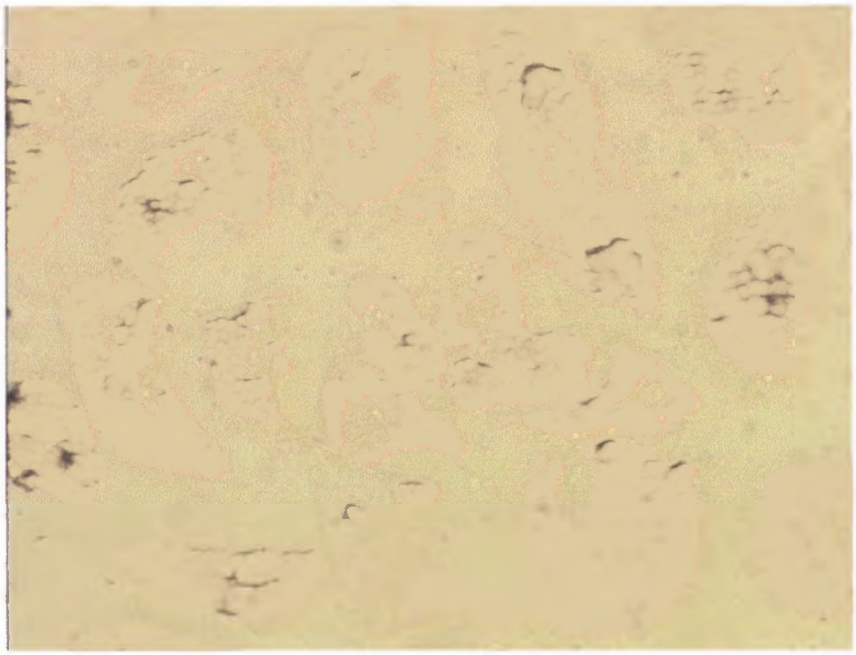


B. With Phe Gly Gly.

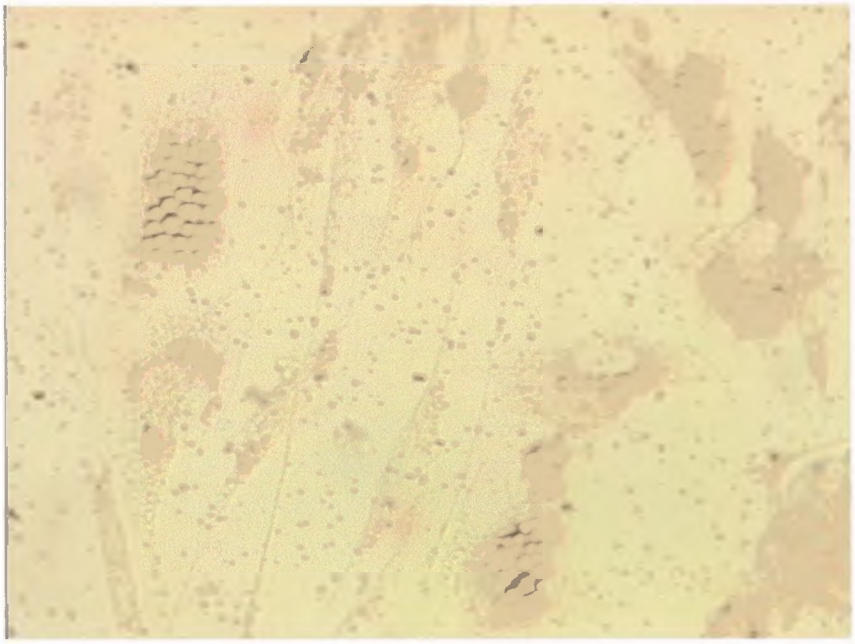


Pictures 4.4A,B. The effect of: A. Absence and B. Presence of Phe.Gly.Gly on lipid accumulation in human preadipocytes.

A. Without histidine (control).



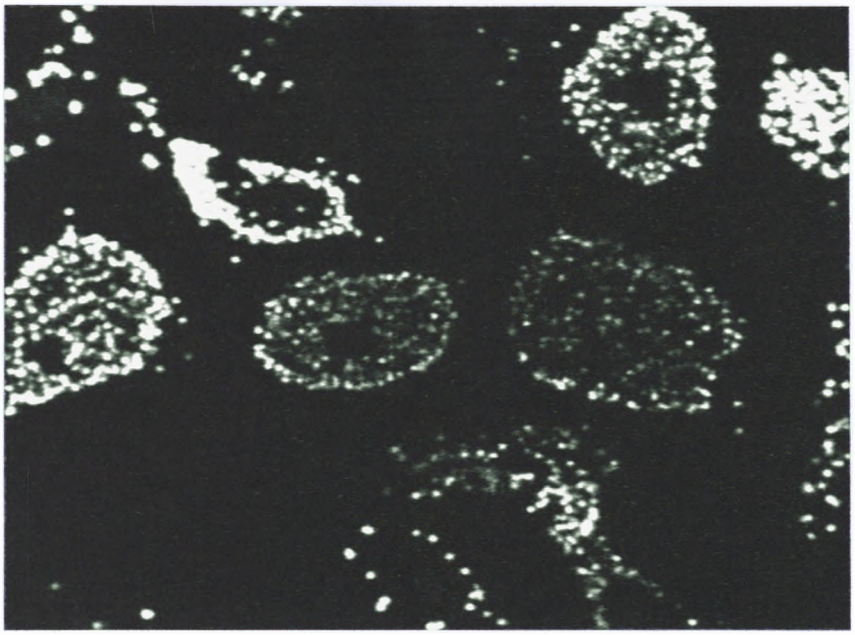
B. With histidine.



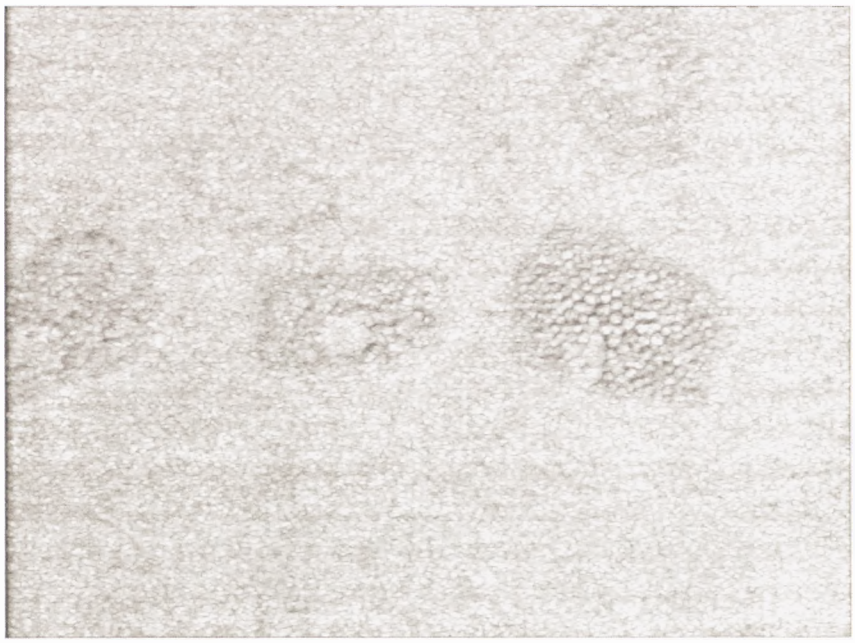
Pictures 4.5A, B. The effect of: A. Absence and B. Presence of histidine on lipid accumulation in human preadipocytes.

Alkaline phosphatase localized on lipid droplets

Immunocytochemical analysis of differentiated human preadipocytes showed that ALP was present on the lipid droplets as shown in picture number 4.6A,B.



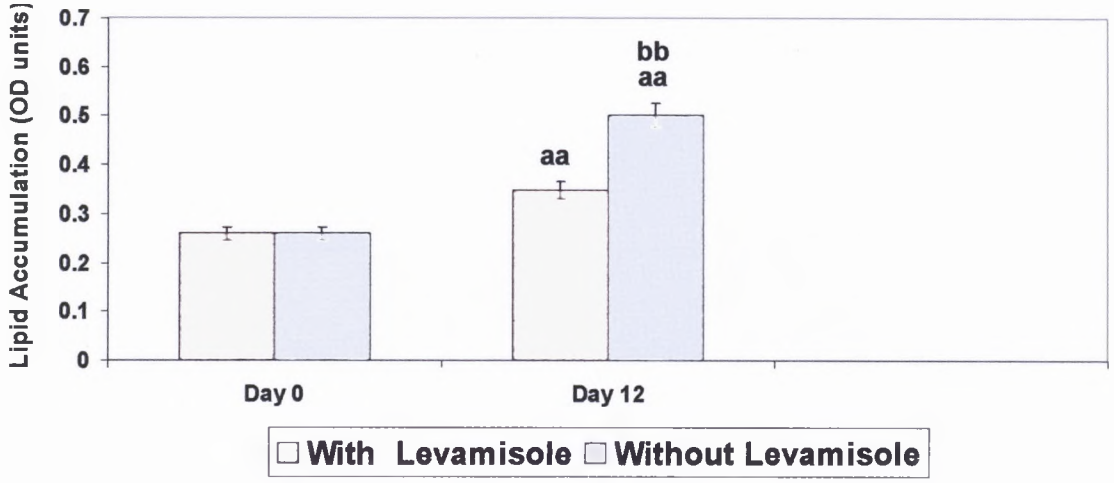
A. Fluorescent micrograph of human preadipocytes stained for ALP



B. Normal light micrograph of human preadipocytes

Picture 4.6A,B. Fluorescent staining (A) of alkaline phosphatase activity in human preadipocytes. Normal light microscopy image of human preadipocytes (B). Images in A and B are the same .

A. Effect of levamisole treatment on lipid accumulation in preadipocytes from mammary tissue obtained from white females



B. The effect of levamisole on ALP activity in preadipocytes from mammary tissue taken from white women

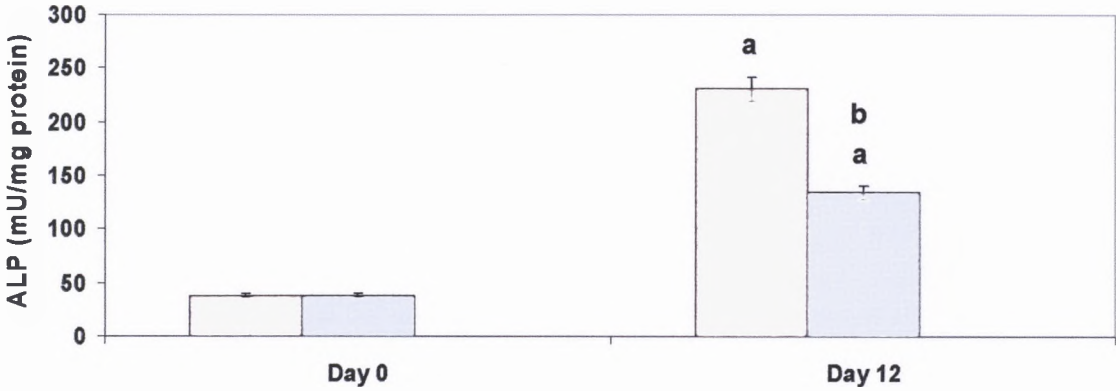
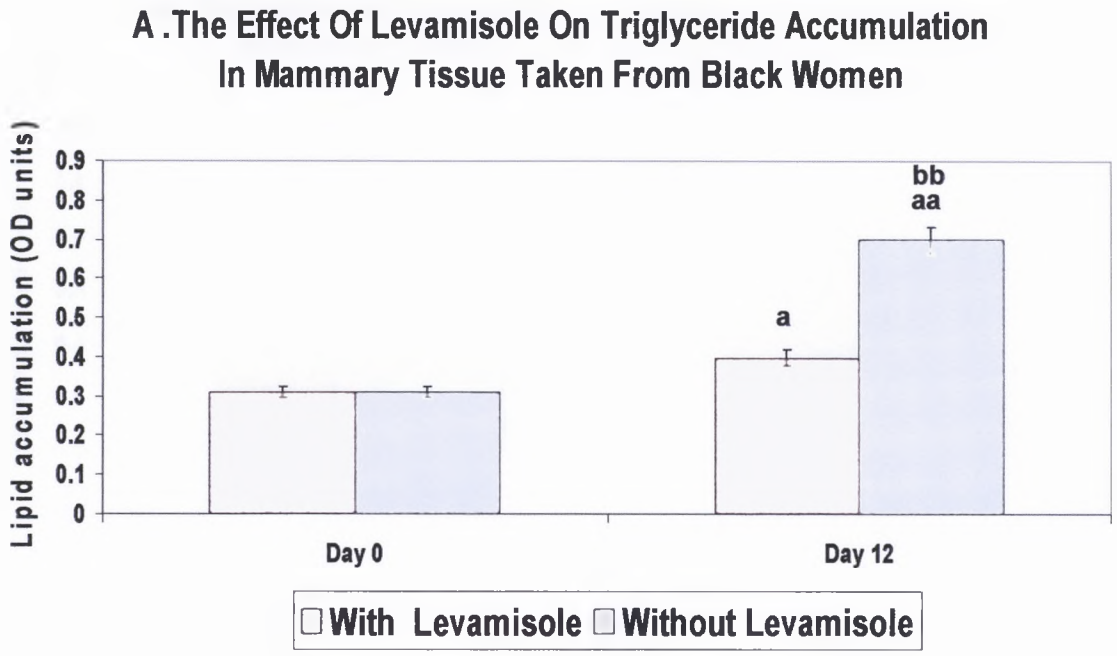


Figure 4.7 A, B. The effect of levamisole on: A. Lipid accumulation and B. ALP activity in preadipocytes isolated from the mammary tissue of 8 white females.

Each bar is mean ± SEM. $^{a}P<0.05$, $^{aa}P<0.005$ Vs day zero and $^{b}P<0.05$, $^{bb}P<0.005$ Vs treated



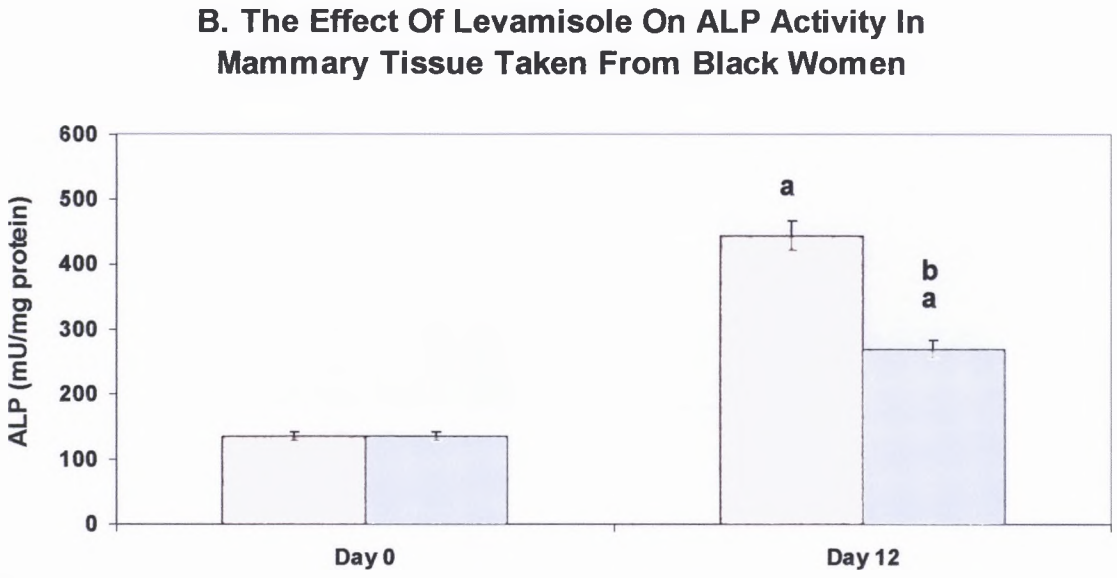
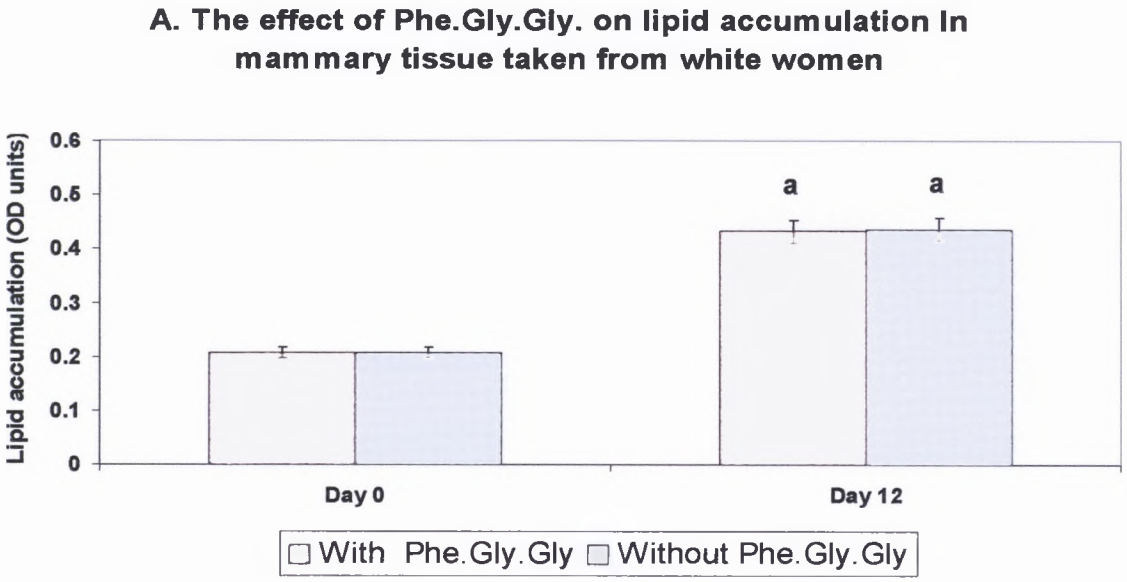


Figure 4.8 A, B. The effect of levamisole on: A. Lipid accumulation and B. ALP activity in preadipocytes isolated from the mammary tissue of 9 black females.

Each bar is mean ± SEM. $^{a}P<0.05$, $^{aa}P<0.005$ Vs day zero; and $^{b}P<0.05$, $^{bb}P<0.005$, Vs treated.



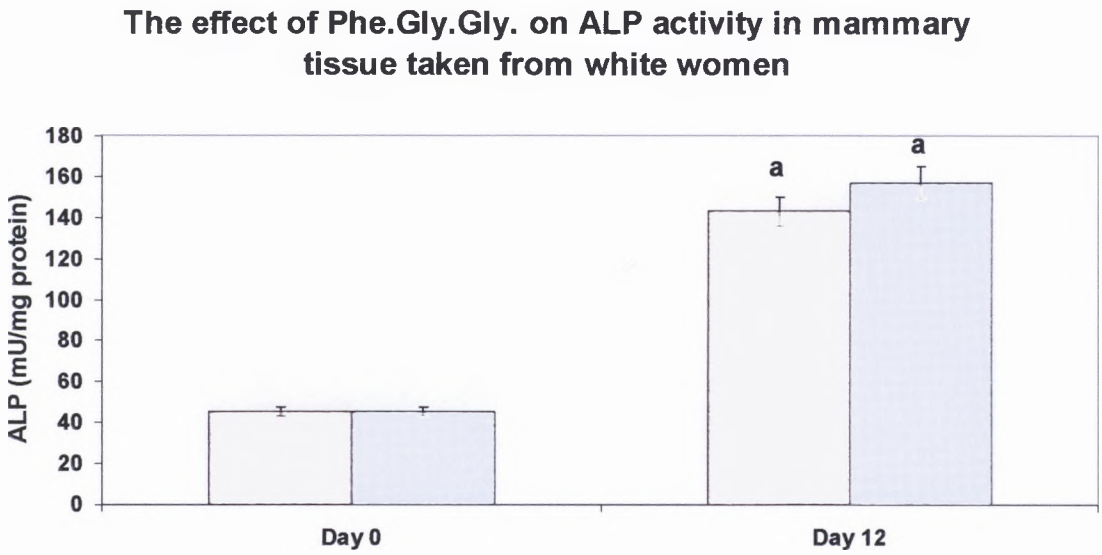
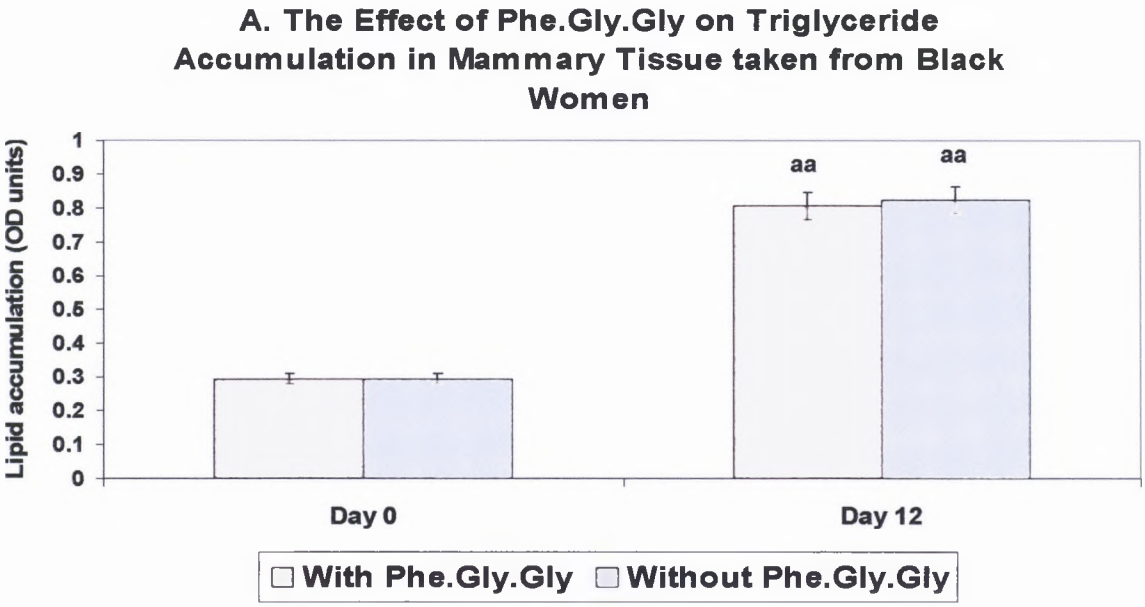


Figure 4.9 A, B. The effect of Phe.Gly.Gly on: A. Lipid accumulation and B. ALP activity in preadipocytes isolated from the mammary tissue of 4 white females.

[a]P<0.05 Vs day zero. Each bar is a mean ± SEM.



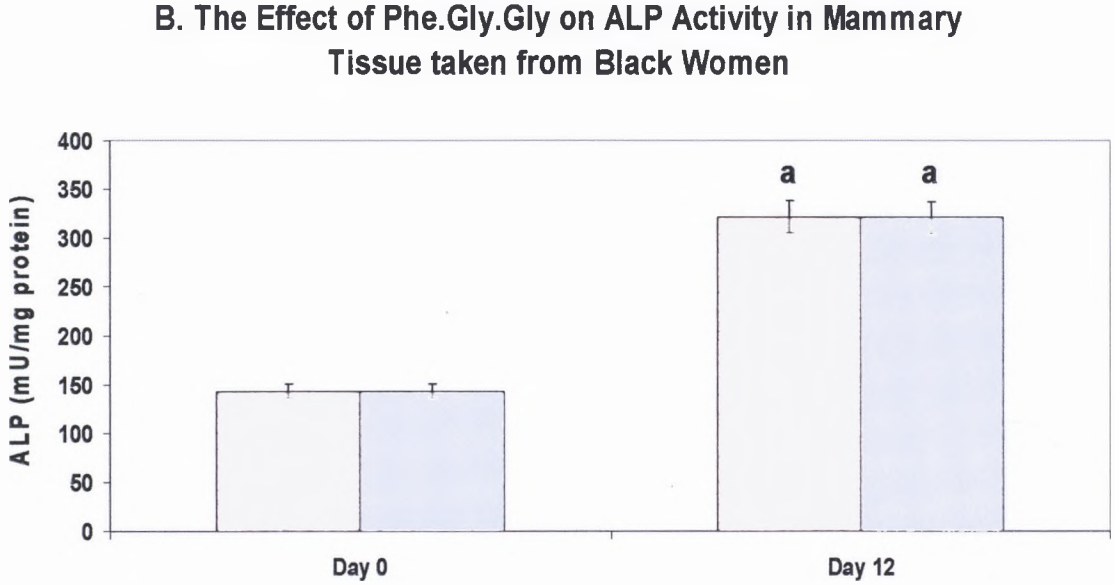
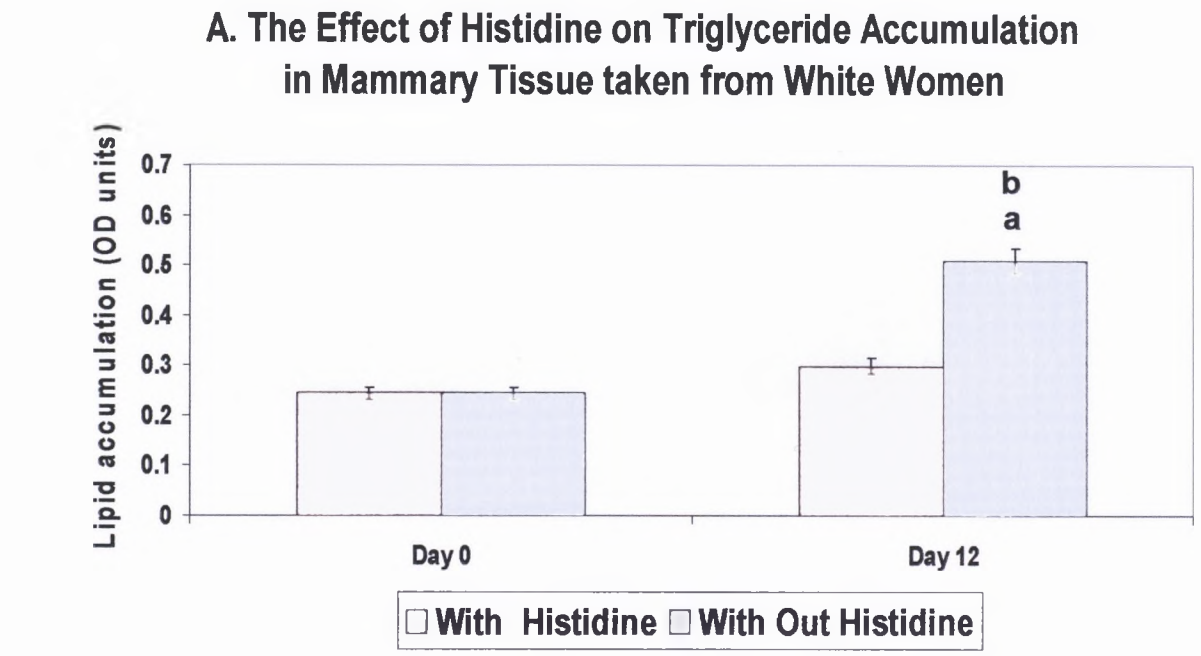


Figure 4.10 A, B. The effect of Phe.Gly.Gly on: A. Lipid accumulation and B. ALP activity in preadipocytes isolated from the mammary tissue of 4 black females.

$^{a}P<0.05$, $^{aa}P<0.005$ Vs day zero. Each bar is a mean ± SEM.



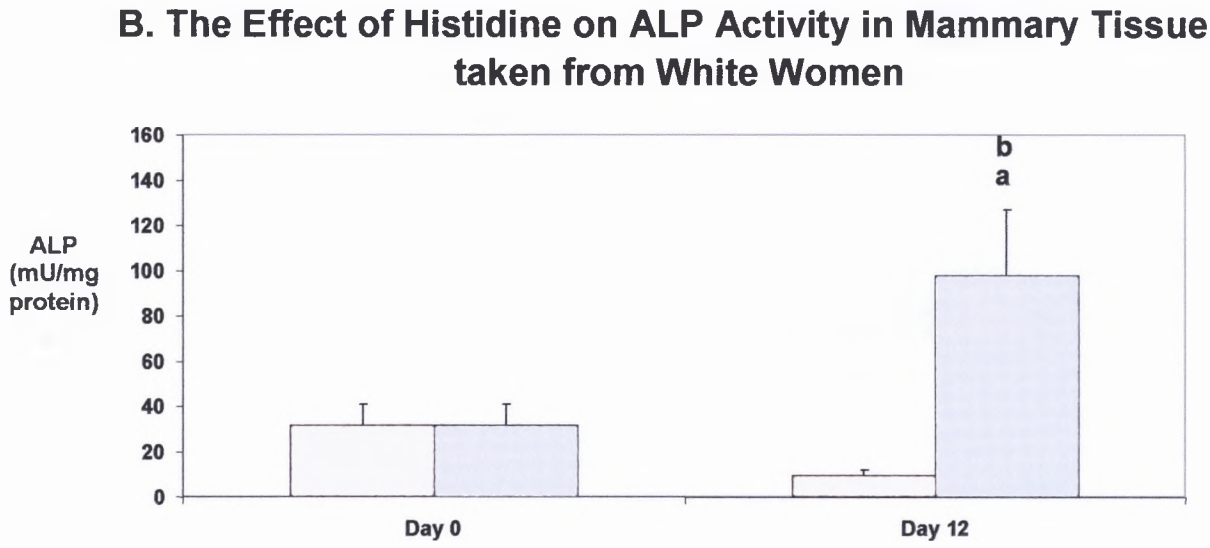
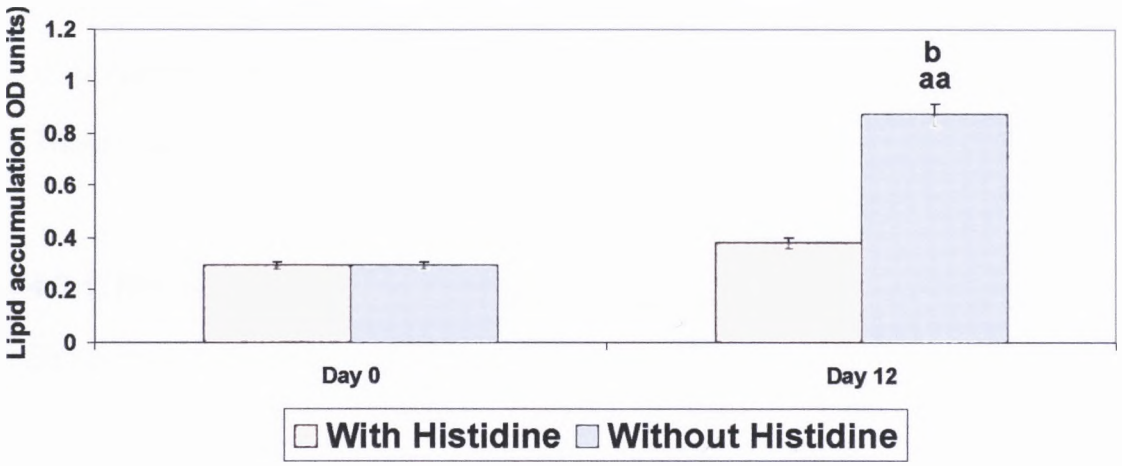


Figure 4.11 A, B. The effect of Histidine on: A. Lipid accumulation and B. ALP activity in preadipocytes isolated from the mammary tissue of 4 white females.

[a]P<0.05 Vs day zero; and [b]P<0.05 Vs treated. Each bar is a mean ± SEM .

A. The Effect of Histidine on Triglyceride Accumulation of Mammary Tissue taken from Black Women



B. The Effect of Histidine on ALP Activity in Mammary Tissue from Black Women

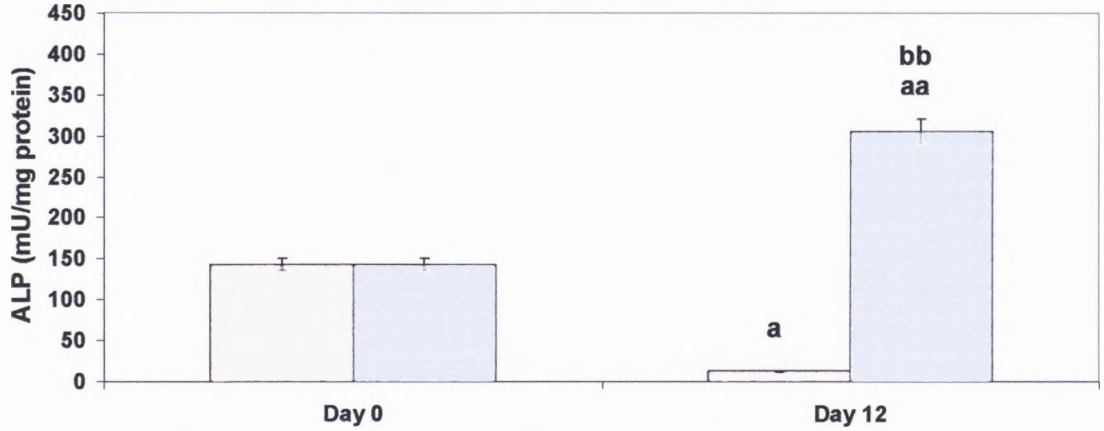


Figure 4.11 A, B. The effect of Histidine on: A. Lipid accumulation and B. ALP activity in preadipocytes isolated from the mammary tissue of 4 black females.

$^{a}P<0.05$, $^{aa}P<0.005$, Vs day zero; and $^{b}P<0.05$, $^{bb}P<0.005$ Vs treated. Each bar is a mean ± SEM.

4.3. Ethnic differences in ALP activity and adipogenesis

Interesting significant differences were observed for ALP activity and adipogenesis between preadipocytes collected from black and white women. These differences are described bellow.

4.3.1. Ethnic differences during levamisole treatment

Significant differences between black and white subjects were seen on day zero for both ALP activity ($P<0.0005$), and adipogenesis ($P<0.005$) as shown in figure 16A,16B. Differences in ALP activity on day 12 were observed when black non-treated preadipocytes ($P<0.05$), were compared with white non-treated preadipocytes. Adipogenesis on day zero was significantly higher in black preadipocytes ($P<0.005$) compared with white preadipocytes, while on day 12 a significant difference was observed when non-treated preadipocytes from black subjects ($P<0.0005$) were compared with white subjects(see figure 13A,B).

4.3.2. Ethnic differences during Phe Gly Gly treatment

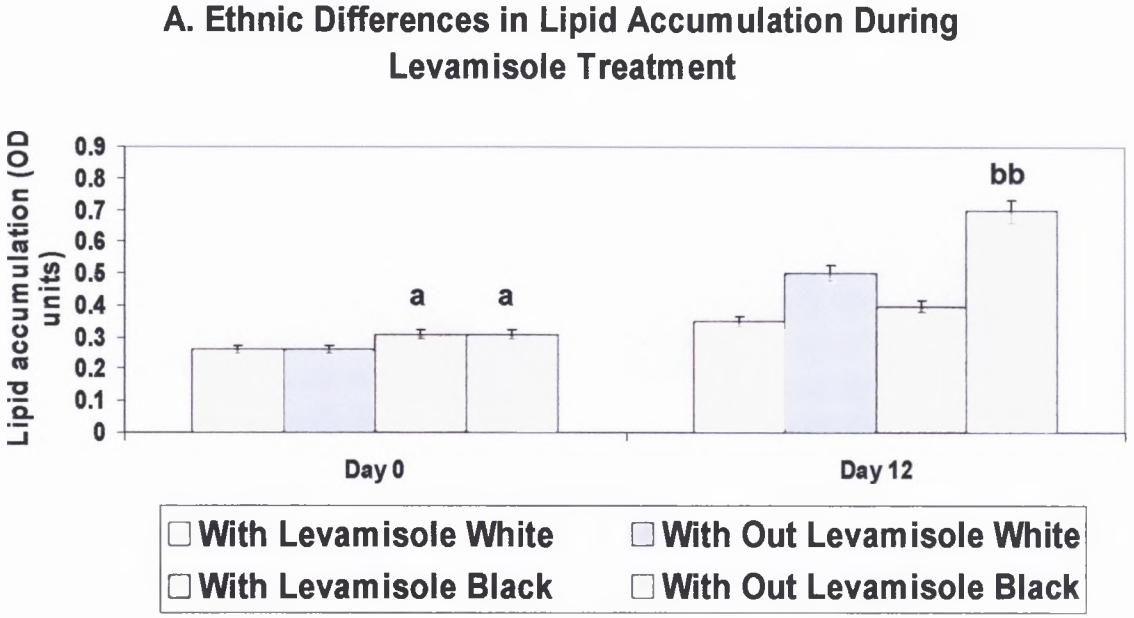
As has been seen before tissue specific ALP inhibitor Phe Gly Gly, did not show any effect on either ALP activity or adipogenesis. However, significant ethnic difference has been shown between white and black subjects. Differences were highly significant in black compared with white subjects on day zero for both ALP activity ($P<0.0005$) and adipogenesis ($P<0.0005$). Again differences were highly significant on day 12 for treated and non treated preadipocytes collected from

black ,when compared with white subjects for both ALP activity ($P<0.005$) and adipogenesis ($P<0.005$ see figure 14 A,B).

4.3.3. Ethnic differences during histidine treatment

Differences in ALP activity were highly significant when preadipocytes from black females were compared with those from white subjects ($P<0.0005$) on day zero. On day 12 differences were only significant when non-treated preadipocytes from black females were compared with non-treated preadipocytes from white subjects ($P<0.005$).

During adipogenesis experiments, significant differences were seen on day zero ($P<0.05$). On day 12 significant differences were observed when non-treated cells collected from black subjects were compared with their counterparts collected from white subjects ($P<.0005$; see figure 15A,and 15B).



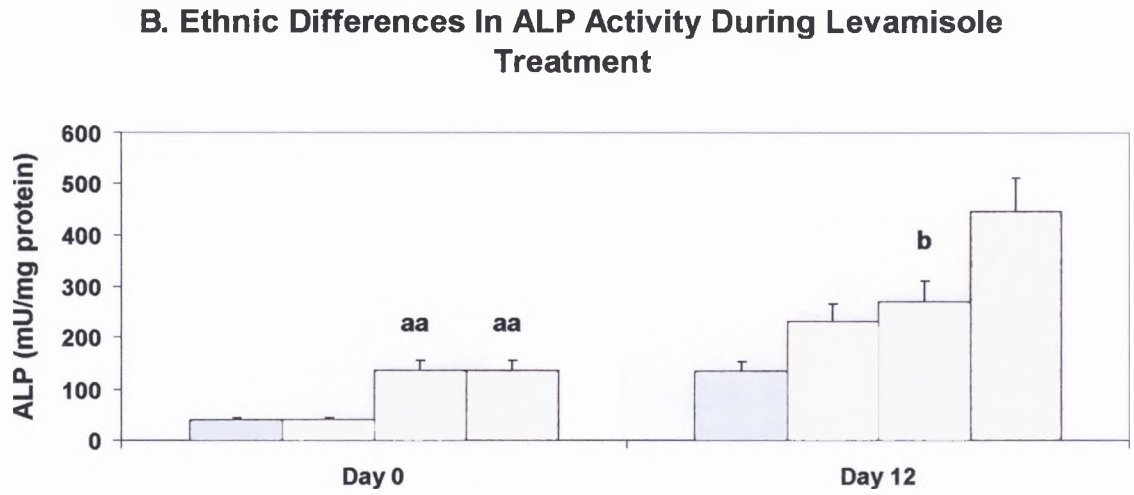
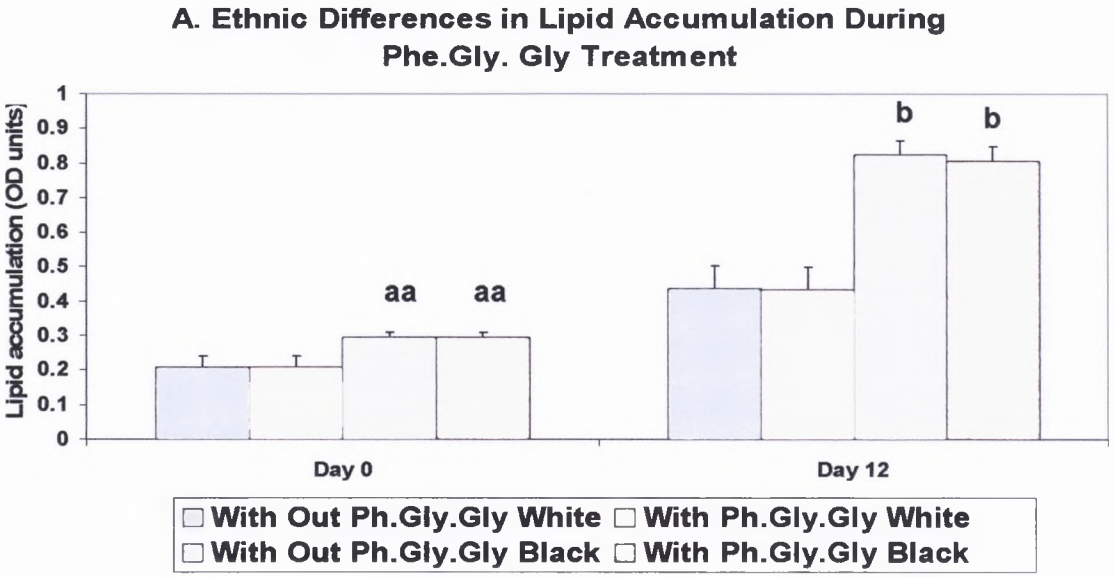


Figure 4.13 A, B. Levamisole effects on: A. Lipid accumulation, B. ALP activity in preadipocytes taken from mammary tissue of 9 black and 8 white females.

$^{a}P<0.05$, $^{aa}P<0.005$, Vs white females, day 0 and $^{b}P<0.05$, $^{bb}P<0.005$ Vs white females day 12. Each bar is a mean ± SEM..



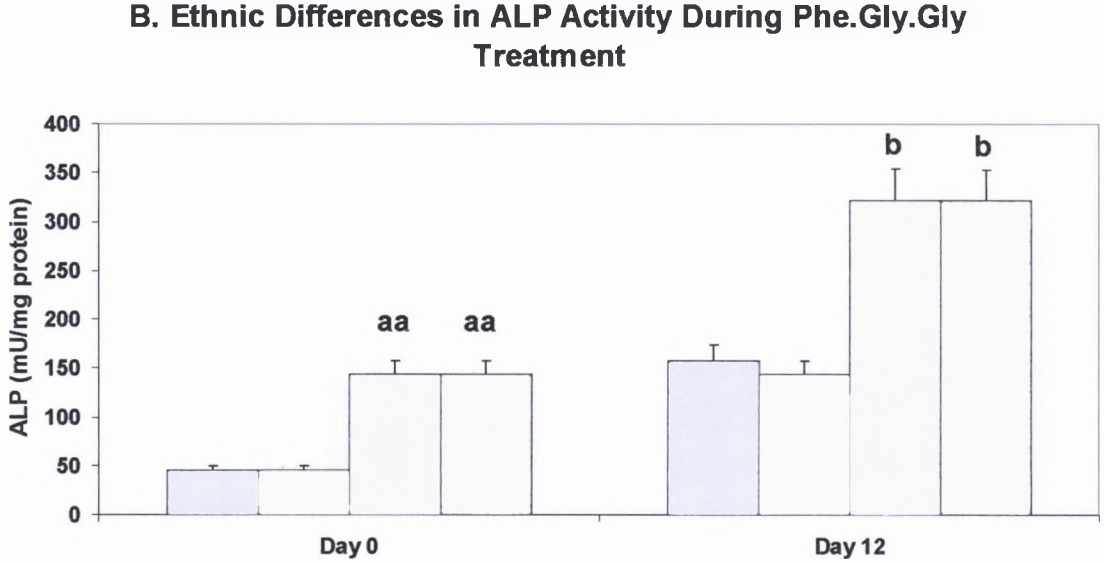


Figure 4.14 A, B. Phe Gly.Gly effects on: A. Lipid accumulation, B. ALP activity in preadipocytes taken from mammary tissue of 4 black and 4 white females.

$^{aa}P<0.005$ Vs white females, day zero and $^{b}P<0.05$ Vs white females, day 12. Each bar is a mean ± SEM.

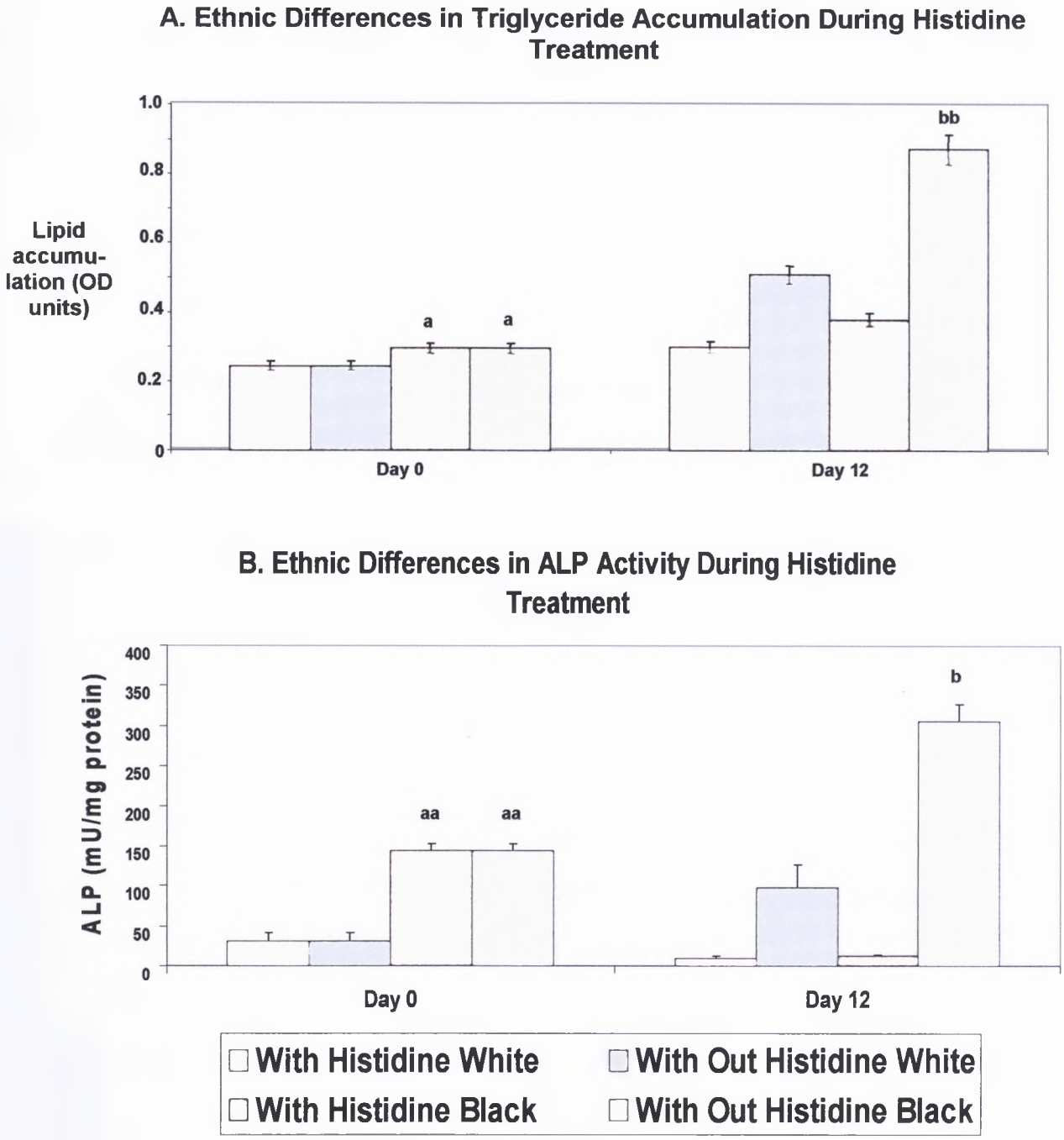


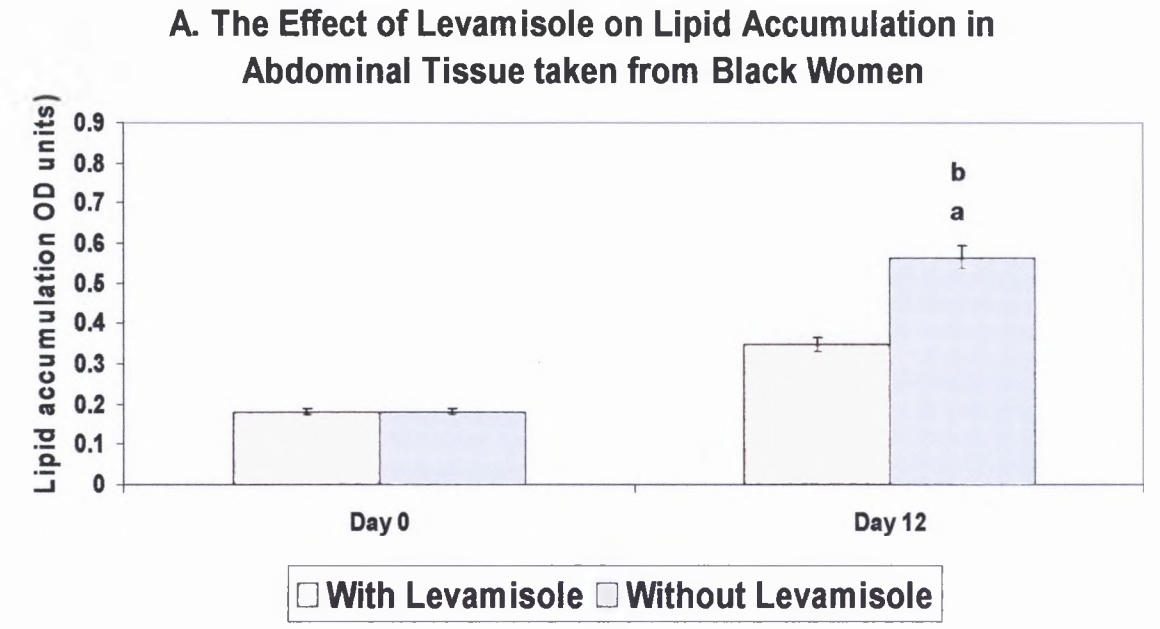
Figure 4.15 A, B. Histidine effects on: A. Lipid accumulation, B. ALP activity in preadipocytes taken from mammary tissue of 4 black and 4 white females.

$^{a}P<0.05$, $^{aa}P<0.005$, Vs white females, day zero and $^{b}P<0.05$, $^{bb}P<0.005$ Vs white females day 12. Each bar is a mean ± SEM.

.4.4. Abdominal subcutaneous fat tissue results;

The effect of levamisole on alkaline phosphatase activity was studied using preadipocytes isolated from 4 obese black women. Preadipocytes treated with levamisole had higher ALP activity compared with non treated preadipocytes when ALP activity was measured on day 12 ($P<0.005$).These results contrast with the inhibition of adipogenesis observed in treated preadipocytes when compared with non treated preadipocytes ($P<0.05$). Significantly higher ALP activity was observed for both treated and non-treated cells on day 12 when compared with ALP activity on day zero($P<0.05$ for both). These results parallel our earlier results collected from breast tissue for both white and black subjects .

Histidine treatment of human abdominal preadipocytes showed inhibition of both adipogenesis(non-treated Vs treated cells $P<0.0005$) and ALP activity(non-treated cells $P<0.0005$) .These results parallel the earlier results collected from breast tissue for both white and black subjects(see figures 4.16A,4.16B and 4.17A,4.17B).



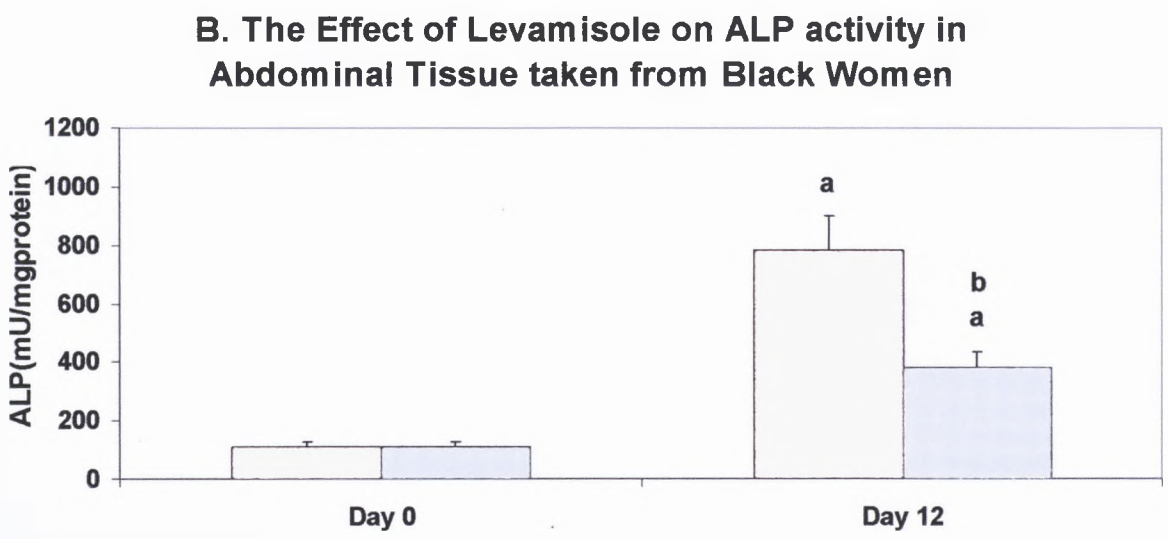
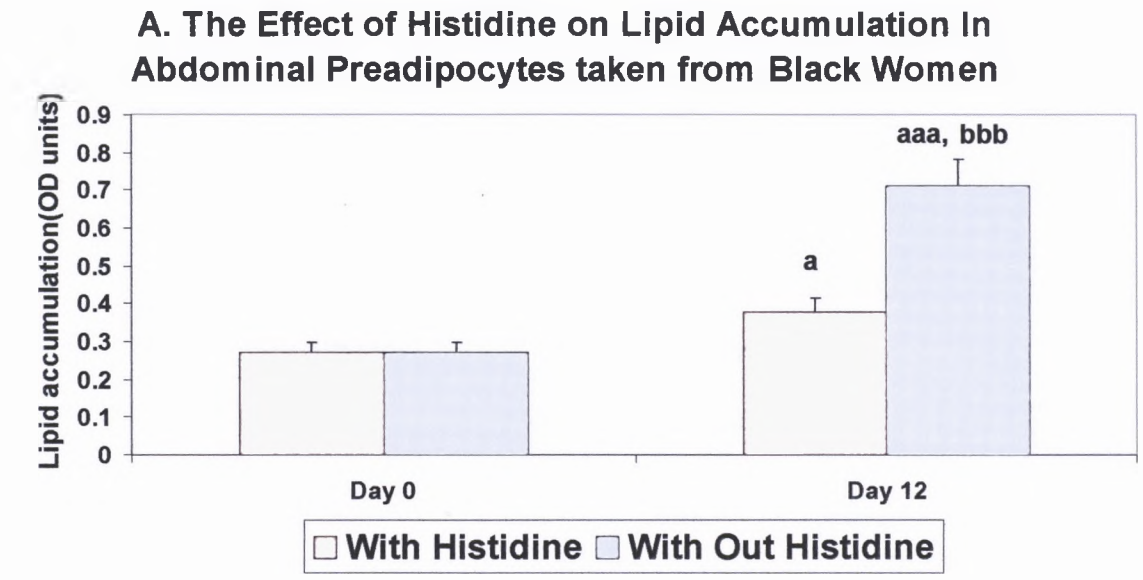


Figure 4.16 A, B. The effect of levamisole on: A. Lipid accumulation and B. ALP activity in preadipocytes isolated from abdominal tissue of 4 black females.

[a]P<0.05 Vs cells on day zero and [b]P<0.05 Vs treated cells. Each bar is a mean ± SEM.



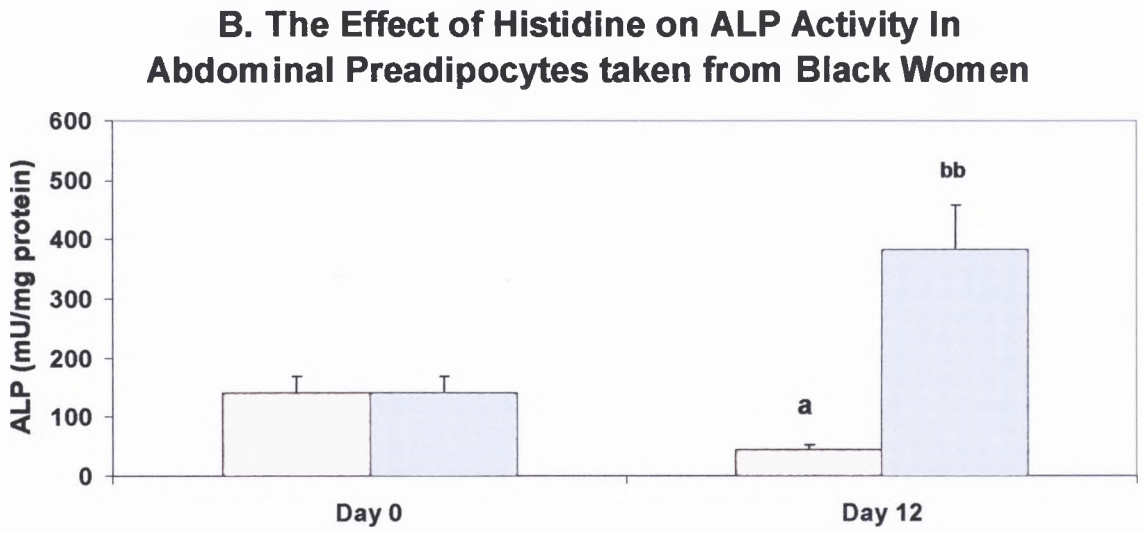


Figure 4.17 A, B. The effect of levamisole on: A. Lipid accumulation and B. ALP activity in preadipocytes isolated from abdominal tissue of 10 black females. $^{a}P<0.05$, $^{aaa}P<0.005$ Vs day zero; and $^{bb}P<0.005$, $^{bbb}P<0.0005$ Vs treated cells. Each bar is a mean ± SEM.

4.5. The effect of the site on adipogenesis and ALP activity

Differences in adipogenesis between black abdominal subcutaneous preadipocytes and black mammary gland preadipocytes were not significant during histidine treatment, but significant differences were observed on day zero between the depots with levamisole treatment. No differences were seen with any of the treatments for ALP activity (see figures 4.18A, 4.18B and, 4.19A, 4.19B). When data was combined from all inhibitor studies and comparison made on day zero and day 12 for treatments without inhibitors , significant differences were only observed for adipogenesis on day zero : mammary preadipocytes, 0.30 ± 0.006 OD units; abdominal preadipocytes, 0.24±0.02 OD units (p=0.003).

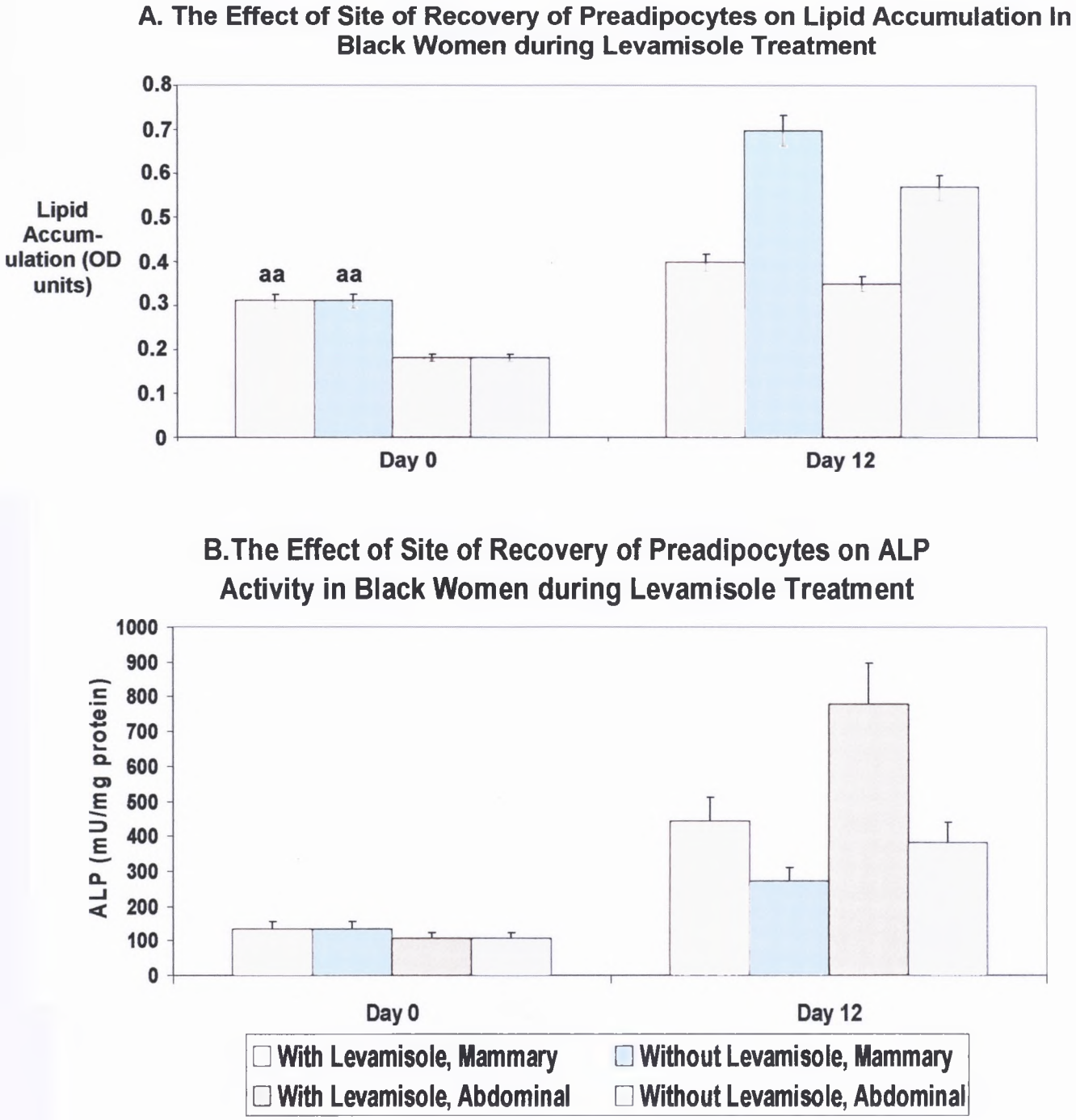
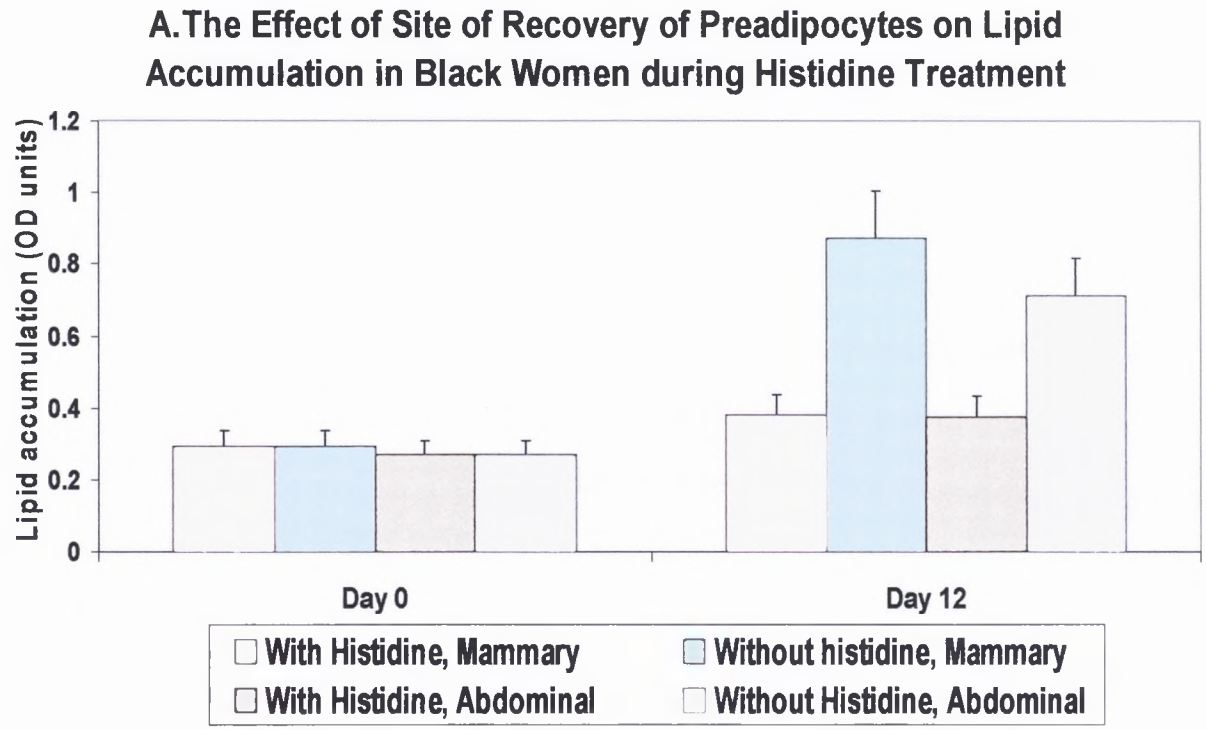


Figure 4.18A, B. The effect of site of recovery of preadipocytes treated with levamisole on: A. Lipid accumulation and B. ALP activity in black females (N=9 for mammary and N=4 for abdominal).

[aa]P<0.005, Vs abdominal on day zero. Each bar represents mean ± SEM.



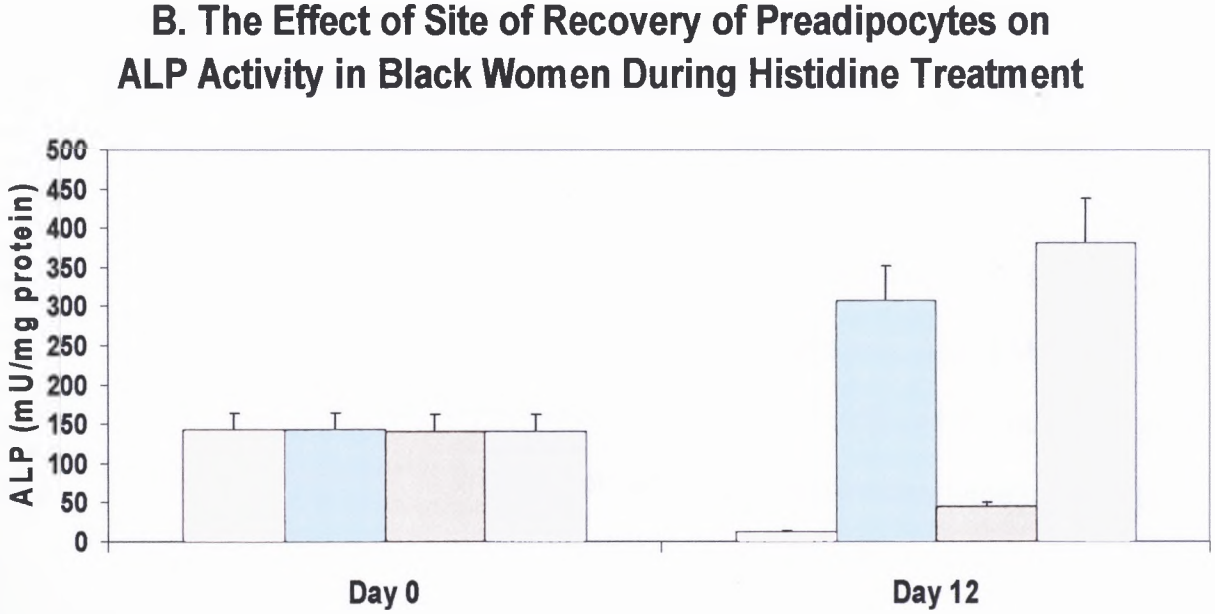


Figure 4.18A, B. The effect of site of recovery of preadipocytes treated with histidine on: A. Lipid accumulation and B. ALP activity in black females (N=4 for mammary and N=10 for abdominal).

[aa]P<0.005, Vs abdominal on day zero. Each bar represents mean ± SEM.

4.6. The effect of weight on ALP activity and liver enzymes

Table 4.1.shows that females were older, shorter, heavier, and had a greater waist-circumference, but had lower waist–hip ratio than males. Table 4.2 demonstrates that females had higher total protein but lower albumin levels than male subjects. Data from table 4.3, shows that with increasing BMI, there was an increase in age weight, waist and hip circumferences, but height decreased.

This table also shows that total ALP, and total-bone ALP levels were higher in obese than lean subjects.

Table 4.4 shows that age correlates positively with all measures of ALP, and with total bilirubin and weakly (P=0.087) with albumin levels. BMI, correlates positively with total-bone ALP, and there is a weak positive correlation with total ALP (P=0.063). There is a strong negative correlation between BMI and total bilirubin (P=0.02), and a weaker negative relationship with albumin levels (P=0.051). Waist-hip ratio, displayed a strong positive relationship with albumin (P=0.003) and ALT (P=0.0001) levels and a weak positive relationship with total bilirubin levels (P=0.07). Multivariate regression analysis, using sex, age, BMI, and waist-hip ratio, as independent variables, demonstrated that ALP measurements correlated positively with age, but not with any of the anthropometric variables. The main determinant of albumin levels was found to be sex (P<0.0001), with a weaker input from waist-hip ratio (P=0.07). ALT levels were predominantly determined by waist hip ratio (P=0.0002) and total bilirubin concentrations demonstrated a weak relationship with waist-hip ratio (P=0.06).

A. Anthropometric data for females

Variables	N Number	Mean
Age	75	41.08 ± 10.062[a]
Height	75	1.575 ± 0.0668[b]
Weight	75	78.874 ± 20.099[b]
BMI	75	31.79 ± 8.055[b]
Waist	75	90.086 ± 15.897[a]
Hip	75	108.88 ± 15.595
Waist-Hip	75	0.8340 ± 0.1450[a]

B. Anthropometric data for males

Variables	N Number	Mean
Age	26	33.961 ± 9.631
Height	26	1.685 ± 0.653
Weight	26	64.33 ± 10.799
BMI	26	22.64 ± 3.610
Waist	26	79.36 ± 9.116
Hip	26	89.22 ± 7.284
Waist-Hip	26	0.89 ± 0.934

Table 4.1: Anthropometric measurements for **A.** Females and **B.** Males
[a]$p<0.005$ Vs males
[b]$p<0.0005$ Vs males

A. Liver function tests for females

Variables	N number	Mean	Median	Minimum	Maximum
Total ALP	74	72.432 ± 22.807	72	33	142
Bone ALP	74	32.97 ± 12.96	30	15	66
Total-Bone ALP	73	40.48 ± 17.22	39	15	121
TOTPR	74	80.229 ± 9.380[a]	79	67	123
ALBUMIN	74	41.229 ± 3.556[b]	42	25	49
ALT	74	13.702 ± 5.730	12.5	5	42
TOTBILUR	74	7.121 ± 3.759	6	2	20
GGT	74	42.067 ± 49.888	25	7	286

B. Liver function tests for males

Variables	N number	Mean	Median	Minimum	Maximum
Total ALP	26	69.807 ± 18.639	65	47	114
Bone ALP	26	33.042 ± 10.07	31	20	55
Total-Bone ALP	26	33.45 ± 9.94	31	21	63
TOTPR	26	75.961 ± 4.617	76	66	87
ALBUMIN	26	45.692 ± 2.222	46	41	50
ALT	26	16.192 ± 5.810	15	10	39
TOTBILUR	26	8.423 ± 4.041	7	2	21
GGT	26	31.961 ± 27.162	22.5	11	138

Table 4.2: ALP and liver enzyme data for **A.** Females and **B.** Males
[a]$p<0.005$ Vs males
[b]$p<0.0005$ Vs males
Data corrected fro age, BMI, and waist-hip ratio.

A. Anthropometric data for different BMI groups

Variables	Lean (n=37)	Overweight (n=22)	Obese (n=41)
Age	35.6 ± 10.3	39.2 ± 9.7	42.7 ± 9.84[aa]
Height	1.63 ± 0.09	1.61 ± 0.06	1.57 ± 0.065[aaa, b]
Weight	58.3 ± 7.7	72.7 ± 6.45[aaa]	92.03 ±16.71[aaa, bbb]
BMI	21.9 ± 1.98	27.7 ± 1.39[aaa]	37.4 ± 6.6[aaa, bbb]
Waist	74.7 ± 8.48	85.5 ± 7.35[aaa]	99.92 ± 12.85[aaa, bbb]
Hip	89.2 ± 7.2	101.8 ± 6.6[aaa]	118.4 ± 13.24[aaa, bbb]
Waist-Hip	0.844 ± 0.14	0.8429 ± 0.09	0.85 ± 0.15

B. Liver function data for different BMI groups

Variables	Lean (n=37)	Overweight (n=22)	Obese (n=41)
Total ALP	66.6 ± 22.2	69.0 ± 21.8	76.5 ± 19.5[a]
Bone ALP	31.3 ± 12.2	32.8 ± 10.4	34.6 ± 13.2
Total-Bone ALP	35.3 ± 18.6	36.3 ± 13.9	42.99 ± 13.3[a]
Total Protein	79.0 ± 9.0	82.1 ± 11.9	77.5 ± 5.3
Albumin	42.7 ± 4.96	42.6 ± 3.7	41.97 ± 2.5
ALT	14.6 ± 6.2	14.8 ± 4.6	13.9 ± 6.2
Total Bilirubin	8.3 ± 4.5	7.7 ± 3.6	6.6 ± 3.25
GGT	25.8 ± 13.97	41.6 ± 41.9	50.6 ± 60.5

Table 4.3: Anthropometric (A) and liver enzyme differences (B) between different BMI groups
[a]$p<0.05$, [aa]$p<0.005$, [aaa]$p<0.0005$ vs lean; [b]$p<0.05$ and [bbb]$p<0.0005$ vs overweight
Data corrected for age and sex.

Table 4.4. Univariate regression analysis of liver function tests .

	Log TALP	Log BALP	Log T-BALP	Sqrt Alb	Log ALT	Log TBilr	Log GGT
Log Age	r=0.33 p=0.001	r=0.27 p=0.006	r=0.39 p<0.0001	r=-0.17 p=0.087	NS	r=-0.20 p=0.044	NS
RecBMI	r=-0.19 p=0.063	NS	r=-0.22 p=0.03	r=0.20 p=0.051	NS	r=0.23 p=0.02	NS
RecWHR	NS	NS	NS	r=-0.29 p=0.003	r=-0.41 p<0.0001	r=-0.18 p=0.07	NS

Rec = recipracal
Sqrt = square root
NS = P>0.10

Table 4.5. Multivariate regression analysis of liver function tests.

Model number	Dependent variable	Independent variable	β-value (p-value)	R-value (p-value)
1	**Log TALP**	Sex Log Age Rec BMI Rec WHR	0.03 (0.81) 0.40 (0.0001) -0.08 (0.48) -0.11 (0.27)	0.44 (0.0004)
2	**Log BALP**	Sex Log Age Rec BMI Rec WHR	0.17 (0.18) 0.32 (0.002) -0.11 (0.35) -0.06(0.55)	0.35 (0.013)
3	**Log T-BALP**	Sex Log Age Rec BMI Rec WHR	-0.03 (0.79) 0.36 (0.0006) -0.09 (0.45) -0.08 (0.40)	0.43 (<0.0001)
4	**Log ALT**	Sex Log Age Rec BMI Rec WHR	0.12 (0.34) 0.002 (0.98) 0.06 (0.62) -0.38 (0.0002)	0.44 (0.0005)
5	**Log TBilr**	Sex Log Age Rec BMI Rec WHR	-0.02 (0.85) -0.15 (0.14) 0.19 (0.11) -0.19 (0.06)	0.33 (0.03)
6	**Sqrt Alb**	Sex Log Age Rec BMI Rec WHR	0.55(<0.0001) -0.05 (0.60) -0.13(0.22) -0.16(0.07)	0.57 (<0.0001)

CHAPTER FIVE

DISCUSSION

Introduction

The increasing worldwide prevalence of obesity (Seidell, 2001) and obesity-related disorders [type 2 diabetes mellitus, hypertension and ischaemic heart disease (Zimmet et al., 2001)] has led to major research interest in the control of adipose tissue mass and how increased adiposity leads to insulin resistance and the associated metabolic disorders. Weight gain and obesity occur when energy intake exceeds energy expenditure, and excess energy is deposited in adipocytes. At the cellular level obesity is characterized by both adipocyte hypertrophy and increased number of adipocytes (Hirsch and Batchelor, 1976; Ginsberg-Fellner and Knittle, 1981). New adipocytes arise in the process of differentiation of preadipocytes. Over the past decade there has been an explosion of knowledge about the intrinsic regulatory mechanisms determining adipocyte differentiation (Darlington et al.,1998; Fajas et al.,1998; Ruesch and Klemm, 1999). It has been determined that adipocyte differentiation is a highly regulated processes that involves sequential activation of several transcription factors, such as C/EBP β, C/EBP α, and PPAR γ and induction of highly specific proteins such as GLUT-4 , FAS , and others (Spiegelman and Flier, 1996; Fajas et al., 1998; Hauner, 1990). It was believed that only early-onset obesity was associated with adipocyte hyperplasia whereas maturity–onset obesity was believed to result solely from adipocyte hypertrophy (Hirsch and Knittle, 1970; Salans et al., 1973). However, studies have shown that an increase in fat cell number appears to be well correlated with severity of human obesity in adult life (Hirsch and Batchelor, 1976). Moreover, specific early differentiation

genes have been reported to be expressed in adipose tissue from very old mice (Kirkland et al. 1990). In addition fat cell precursors, such as stromal-vascular cells collected from adult human adipose tissue can be fully differentiated into mature adipocytes in vitro (Hauner et al., 1989). Furthermore, adipocyte development has been shown to depend on preadipocyte recruitment in vivo. Although the relative contribution of adipocyte hypertrophy versus hyperplasia to human adiposity is unknown, the capacity to generate new adipocytes from precursor cells persists throughout life and clearly plays a role in regulating adipose tissue mass.

The present study demonstrates that alkaline phosphatase plays a key role in the control of preadipocyte intracellular lipid accumulation and therefore this enzyme may be a prime site for the future development of therapeutic interventions for obesity and comorbid diseases.

5.1. 3T3-L1 studies

The 3T3 –L1 cell line is a useful model of cellular differentiation, in which inducers such as dexamethasone in combination with isobutylmethyl xanthine (IBMX), and insulin promote a highly differentiated state in which the cells accumulate lipid. The capacity to stain the cells for lipid with oil red O provides a method for the determination of the major morphological change that occurs during adipogenesis, intracellular lipid accumulation.

5.1.1. The role of alkaline phosphatase in 3T3-L1 intracellular lipid accumulation

The first study has shown that an alkaline phosphatase isozyme that is inhibited by levamisole and histidine but not by Phe-Gly-Gly is expressed in the 3T3-L1 cell line. This is characteristic of the murine tissue nonspecific isozyme of alkaline phosphatase (Haneji et al., 1983; Ohkubo et al., 2000), which shares 90% amino acid-sequence homology with the human isozyme (Terao and Mintz, 1987). Furthermore, the activity of this enzyme is increased during adipogenesis. This is the first study to demonstrate this and is also the first to show that inhibition of alkaline phosphatase with levamisole or histidine reduces intracellular triglyceride deposition. Furthermore, the localisation of alkaline phosphatase to the triglyceride-storing lipid droplets is very suggestive of this enzyme playing a direct role in the intracellular accumulation of triglyceride. It is of interest to note that intestinal alkaline phosphatase has been indirectly linked to lipid transport in gut enterocytes (Zhang et al., 1996), and is found on the membrane surrounding lipid droplets in these cells.

Alkaline phosphatase has been the subject of intensive research due to its wide tissue distribution (McComb, 1979), clinical utility as a marker of liver and bone disorders (Wolf, 1986), and its early , controlled expression during defined stages of mammalian embryogenesis (McComb, 1979; Hahnel et al., 1990; McDougall et al., 1998). However this is the first study to demonstrate a possible role for alkaline phosphatase in the adipocyte differentiation pathway.

The results from all these experiments have shown increasing activity of alkaline phosphatase with adipogenesis. Inhibition of alkaline phosphatase using levamisole was paralleled with inhibition of adipogenesis. Levamisole has been used widely as an inhibitor for tissue nonspecific alkaline phosphatase.

Levamisole is known to have a number of other effects apart from inhibition of alkaline phosphatase. In particular, it is an agonist of both adrenergic and nicotinic receptors (Hsu, 1980). Also, levamisole has been demonstrated to increase the activity of both glycogen synthase in rat adipocytes (Basi et al., 1994) and pyruvate dehydrogenase in rat fat pads (Thomaskutty et al., 1993). Therefore, the inhibitory effect of levamisole on intracellular lipid accumulation in the 3T3-L1 cell line may conceivably be related to its non-alkaline phosphatase related activities. Another inhibitor of tissue nonspecific alkaline phosphatase that does not have these side effects i.e. histidine was therefore used. Results from these experiments were very similar to those obtained with levamisole. However, histidine also has other effects apart from inhibiting ALP, particularly inhibition of the amino acid System A transporter (SAT2) (Rae et al., 2003). SAT2 is known to be expressed in 3T3-L1 cells (Hyde et al., 2001) and its activity upregulated during adipogenesis (Su et al., 1998). Therefore, the ability of both levamisole and histidine to block ALP activity and reduce intracellular lipid accumulation in 3T3-L1 cells, although suggestive is not conclusive evidence that ALP is a prime modulator of this process.

In the present study, adipogenesis was measured using the triglyceride specific dye, oil red O. This method assesses intracellular triglyceride accumulation during the adipogenic process. However, as demonstrated by microarray (Burton et al., 2004) and proteome analysis (Welsh et al., 2004), adipogenesis in 3T3-L1 cells involves the up- and down-regulation of many other genes and enzyme systems besides those involved in lipid accumulation. Thus, it is possible that ALP may only be affecting lipid deposition and none of the other systems involved in adipogenesis. This can be tested by measuring the expression of other proteins that are known to be up-regulated during adipogenesis, such as PPARgamma, fatty acid binding protein-1 (FABP1) and glycerol 3-phosphate dehydrogenase (Gregoire et al., 1998).

The mechanism by which alkaline phosphatase may influence lipid accumulation in the 3T3-L1 cell line is not known. Its enzymatic activity not only includes an ability to hydrolyse organic phosphate at alkaline pH but also phosphotransferase, pyrophosphatase and adenosine-5'-triphosphatase (ATP'ase) activities (Granstrom, 1982; Pizauro et al., 1992). Thus, the stimulation of lipid accumulation by alkaline phosphatase must involve either an alteration in the phosphorylation state of surrounding proteins or the hydrolysis of ATP. The localisation of alkaline phosphatase to the membrane surrounding the lipid droplet suggests that it may modify biomolecules that are either anchored to or interact with this membrane. Studies have shown that the membrane surrounding the lipid droplets of adipocytes contain perilipin

(Greenberg et al., 1991), adipocyte differentiation-related protein (ADRP) (Brasaemie et al., 1997), caveolin (Brown, 2001) and vimentin (Blanchette-Mackie et al., 1995). Perilipin (Greenberg et al., 1991), caveolin (Mastick et al., 1995) and vimentin (Brandes et al., 1993) are all known to be phosphoproteins.

The murine preadipocyte cell line 3T3-F442A is similar to the 3T3-L1 cell line but unlike the latter requires the presence of growth hormone for the induction of adipogenesis (Sumantran et al., 1992). Studies have shown that the 3T3-F442A cell line can be stimulated to differentiate along the osteoblast pathway by treatment with bone morphogenetic protein (BMP)-2 and retinoic acid (Ji et al., 2000; Skillington et al., 2002). These factors induce expression of the early osteoblast differentiation marker, alkaline phosphatase as well as osteocalcin and collagen I (Skillington et al., 2002). Thus, preadipocytes can be induced to express alkaline phosphatase during differentiation into adipocytes or osteoblasts. Furthermore, inhibition of alkaline phosphatase activity in osteoblastic cell lines leads to decreased calcium uptake and reduced cellular mineralization (Fukayama and Tashjian, 1990; Iba et al., 1995). Transfection of alkaline phosphatase cDNA into alkaline phosphatase negative cells promotes both calcium and phosphate uptake (Yoon et al., 1989). Therefore, alkaline phosphatase does play an important role in the

differentiation pathway involved in osteoblast maturation and may also be involved in the accumulation of intracellular triglyceride during preadipocyte differentiation.

5.1.2. Changing constituents of 3T3-L1 transformation medium

In this study we tried to find out which factor in the transformation cocktail is the most important for the transformation of 3T3-L1 preadipocytes and for increasing ALP activity . As shown in the results (4.5A,B and ,4.6A,B) we found that removing IBMX from the cocktail did not allow the cells to differentiate, and significant decreases in both ALP activity and adipogenesis were observed. IBMX inhibits soluble cyclic nucleotide phosphodiesterase activity and results in increased intracellular cAMP levels. cAMP increases the synthesis of lipogenic enzymes, indicating that the presence of agents that increase cAMP levels are apparently important for full differentiation (Spiegelman and Green 1981).

At the nuclear level, treatment with IBMX results in activation of the related transcriptional factors C/EBP δ and/EBP β which induce transcription of C/EBP α and PPAR γ. cAMP may be a major factor involved in the transcriptional regulation during early preadipocyte differentiation. An increase in intracellular cAMP concentration during early differentiation is required for optimal adipose conversion in 3T3-L1 preadipocytes. It`s main function may be to activate cAMP response element binding protein (CREB) which is activated by cAMP through

protein kinase A, or calcium/calmodulin –dependent protein kinase to activate the expression of many genes (Roesler et al., 1988). Furthermore, treatment of confluent 3T3-L1 with low concentrations of cAMP potentiated the effect of insulin on differentiation .The duration of exposure to IBMX is also critical. Prolonged exposure to IBMX during 3T3-L1 differentiation can result in cell detachment and decreasing differentiation.

It has been demonstrated that cAMP causes a stimulation of ALP activity in cultured mouse L-cells. Induction of the enzyme was dependent upon de novo RNA and protein biosynthesis (Firestone and Heath,1981). Like many other studies Firestone and Heath (1981) have shown that ALP induced in L-cells is a glycoprotein and is associated with the plasma membrane. Tissue non-specific ALP activity in the murine L929 fibroblast cell line is induced by all trans-retinoic acid at concentrations between 10^{-6} and 10^{-5} M. At lower concentrations, retinoic acid is capable of inducing this enzymic activity on its own but also increases cyclic AMP mediated induction of ALP. This effect can be observed after incubation of retinoic acid with dibutyryl cAMP, 8-bromo cAMP or forskolin. This synergism is dependent on the order of addition of retinoic acid and the activator of the cAMP pathway. Addition of the two reagents together, or addition of cAMP before retinoic acid (but not addition of retinoic acid before cAMP) is necessary to produce this synergistic interaction (Gianni et al., 1993). It is therefore possible that one of the other mechanisms by which IBMX stimulates adipogenesis is via increasing cAMP levels which in turn induces ALP expression.

Removing dexamethasone from the transformation medium appeared to have a minimal effect on adipogenesis or ALP activity. Dexamethasone may effect differentiation negatively via affecting cAMP metabolism as well as other processes in the differentiated cells (Elks and Manganiello, 1985).

Removal of insulin from the media produced a minimal effect on adipogenesis and ALP activity . One study has shown that insulin is not required for adipogenesis in the 3T3-L1 cell line (Fong, 1990). However, insulin or insulin like growth factor-1 promote adipocyte differentiation in humans by activating phosphotidyl inositol 3-kinase (PI3-kinase) and increasing Akt activity. Modulation of the activity of the fork head transcriptional factor Fox 1, appears to be necessary for insulin to promote adipocyte differentiation.

The present study has shown that IBMX is an important regulator of both intracellular lipid accumulation and ALP activity in 3T3-L1 cells and that manipulation of the former also effects ALP activity. However, it should be noted that altered levels of lipid accumulation have only been studied using the manipulation of one factor i.e. IBMX and therefore it is not known whether manipulating other factors that effect adipogenesis, such as the thiazolidinediones, will have similar effects to IBMX.

5.2. Human studies

5.2.1. Human preadipocyte experiments

There are two important factors in the study of differentiation, the simplicity of the system, and the flexibility of the cells to differentiate under simple culture conditions. The majority of stromal cells present in human adipose tissue are able to undergo adipose differentiation, under serum-free chemically defined culture conditions. This cellular fraction appears to be by far more homogeneously composed of specific adipose precursor cells than originally believed. To ensure that ALP is presents in human preadipocytes and has the same relation with adipogenesis as in the 3T3-L1 cells, we repeated the same treatments with human preadipocytes.

5.2.1.1. Levamisole treatments

In the experiments with human preadipocytes we demonstrated for the first time that alkaline phosphatase is present in these cells and that increasing intracellular lipid accumulation in the absence of ALP inhibitors was associated with an increase of alkaline phosphatase enzyme activity paralleling, the data from the 3T3 –L1 preadipocytes. The method used in our studies to measure adipogenesis was the oil red O technique. This technique is based on well established protocols (Strutt et al., 1996). Quantitation of lipid accumulation by oil red O staining correlates well with the measurements of glycerol 3-phosphate dehydrogenase activity (Ramirez-Zacarias et al., 1992).

The results from our experiments with the human preadipocytes showed similar effects of the ALP inhibitors histidine and Phe.Gly.Gly to the results that we obtained from the 3T3-L1 experiments. In addition, when we removed the IBMX from the human transformation media (data not shown) neither the levamisole treated preadipocytes or the non-treated cells showed any sort of differentiation. This shows, that as in 3T3-L1 cells, cAMP is an important regulator of adipogenesis. Treatment with levamisole increased the activity of the ALP in the human preadipocytes. These results conflict with the results from the 3T3-L1 cells which showed that levamisole inhibits ALP activity but inhibits intracellular lipid accumulation in both cell types. However, treatment of the cell extract of human preadipocytes with levamisole caused a 50% inhibition of the enzyme. Thus, levamisole only inhibits ALP activity in cell extracts and not intact cells but is still able to inhibit intracellular triglyceride deposition. This suggests that levamisole may be blocking lipid accumulation via a mechanism that does not involve ALP. What this mechanism may be is not known. However, the fact that levamisole will inhibit ALP in cell extracts but not intact cells suggest that levamisole must be interacting with some factor in intact cells that blocks its ability to inhibit ALP. Levamisole is known to be an agonist for nicotinic and adrenergic receptors (Hsu, 1980) so one hypothesis may be that levamisole binds these receptors in intact cells reducing access to the lipid droplet-residing ALP. This theory must be explored with the use of antagonists and agonists of both receptor types. It is interesting to note that levamisole actually increases ALP activity in intact human preadipocytes and this has also been observed in rat

hepatocytes treated with levamisole (Engelmann and Richardson, 1986). Hepatocytes also accumulate intracellular lipid in membrane-bound compartments.

Levamisole treatment has shown that differences exist between 3T3-L1 cells and human preadipocytes in their response to this ALP inhibitor. The reason for this is not known and obviously warrants further investigation.

5.2.1.2. Phe. Gly. Gly

The tripeptide Phe Gly Gly did not show any effect on ALP activity or lipid accumulation in any of the preadipocyte preparations. These results parallel those from the 3T3-L1 experiments. This data suggests that the ALP isozyme present within the 3T3-L1 cells and the human preadipocytes is the tissue non-specific form. Tissue non-specific ALP is inhibited by levamisole (van Belle, 1976) and histidine (Fishman and Sie, 1971) but not by Phe.Gly.Gly, which is an inhibitor of the tissue specific ALP isozymes (Doellgadt and Fishman, 1977). This data must be confirmed by performing rtPCR analysis of both these cell types using primers specific for the tissue non-specific isozyme of ALP.

5.2.1.3. Histidine

Histidine been shown to suppress food intake through its conversion into neuronal histamine (Yoshimatsu, et al. 2002). Hypothalamic neural histamine suppresses food intake through H-1 receptors in the ventromedial hypothalamic

nucleus and the paraventricular nucleus .Increase in hypothalamic histamine concentration elevates peripheral glucose concentration, accelerates lipolysis in the adipose tissue, and decreases body temperature (Sakata and Yoshimatsu, 1977; Yoshaimatsu et al. 2002). In vivo treatment with histamine was able to reduce visceral fat predominantly leaving subcutaneous fat unaffected (Masaki, 2001). Studies have shown that the activation of histamine signalling promoted lipolysis through sympathetic nerves (cited in Masaki 2001). Our data suggests that histidine may also have direct effects on preadipocytes, not mediated by the central nervous system. Histidine blocks intracellular triglyceride accumulation and ALP activity in human preadipocytes, confirming the 3T3-L1 studies.

5.2.1.4. Ethnic differences

The prevalence of obesity in South Africa may be high due to urbanization of the population (Rossi-Espagnet, 1991). Sedentary life with increased energy intake and decreased energy expenditure has caused an increase in the prevalence of obesity in both white and black South African societies. It is known that black women tend to accumulate less visceral fat, compared with white women. A study has also shown that adipocytes collected from black women are more insulin resistant than those taken from white females (Buthelezi et al.,2001).

In our study we have seen significant differences between white and black women in both alkaline phosphatase activity and adipogenesis .These findings support a previous study showing that obesity is more prevalent in middle aged black women compared with white women (Puoane et al., 2002) .Increased

triglyceride deposition in preadipocytes isolated from the mammary tissue suggests that in black females there is a greater level of adipogenesis and this may partly explain the higher prevalence of obesity in this population. Obviously environmental factors must also play a role. Also it is possible that the low visceral fat depot size observed in black females is due to preferential storage in subcutaneous depots, where the adipogenesis rate is high. Obviously this must be confirmed via studies on adipogenesis rate in abdominal subcutaneous fat and visceral fat from white and black females. Indeed, researchers have found that a failure to differentiate new adipocytes compromises the capacity of lipid storage in adipose tissue which in turn leads to an increased fat accumulation in other tissues such as visceral fat, skeletal muscle, liver and perhaps pancreatic beta cells (Danforth, 2000).

5.2.1.5. The effect of body site on lipid accumulation and ALP activity

It has been shown that visceral preadipocytes differentiate more slowly compared with subcutaneous preadipocytes, indicating that the fat depot origin affects the adipogenesis level. Although adipogenesis is delayed in omental compared with abdominal subcutaneous preadipocytes, it does eventually proceed. Thiazolidinediones (TZD) cause more extensive differentiation of subcutaneous than omental cells. In vivo experiments found that TZDs decrease visceral but increases subcutaneous fat depot size by acting on adipogenesis (Arner, 2003). The new adipocytes resulting from TZD are smaller and more insulin sensitive (Arner, 2001). However, despite the differential effect of thiazolidinediones on

adipogenesis rate in different fat depots, it does not affect the level of PPAR γ expression in human subcutaneous and omental preadipocytes (Adams et al., 1997).

Fat cells isolated from different depots of rats and humans differ in size, response to insulin and lipolytic agents , lipoprotein lipase release, lipid synthetic capacity, fatty acid incorporation and other characteristics (Arner, 1997; Caaserta et al., 2001; Edens et al., 1993; Fried et al., 1993; Montague et al., 1997b; Prins and O`Rahilly, 1997).

Other differences in the characteristics of human preadipocytes from various depots have been described .It has been found that human visceral preadipocytes have a greater susceptibility to apoptosis induced by serum deprivation and tumour necrosis factor–alpha than abdominal subcutaneous preadipocytes (Niesler et al., 1998). Studies have shown more extensive transfer of fatty acids into differentiated human omental than abdominal subcutaneous preadipocytes from the same subjects, even after matching cells for lipid content , consistent with the greater fatty acid flux in visceral than in subcutaneous fat (Casserta et al., 2001).

Preadipocytes from different regions appear to be inherently distinct and studies performed with rat preadipocytes support this contention. Preadipocytes cultured from perirenal depots were shown to be capable of more extensive replication and differentiation in vitro and in vivo than preadipocytes from epididymal depots

(Djian et al., 1985; Kirkland et al., 1990; Kirkland et al. 1996; Wang et al., 1989). Preadipocytes cultured from various depots of rats retained distinct cell dynamic and biochemical responses despite exposure to similar hormonal manipulations in vivo such as estrogen administration, hypophysectomy, or castration (Kirkland et al., 1992; Lacasa et al., 1997). These depot-dependent differences in the innate characteristics of adipose cells could contribute to anatomic variation in function. Indeed, interdepot variation in cultured rat preadipocyte differentiation gene–dependent gene expression has been observed (Kirkland et al., 1996). Thus, rat preadipocyte studies are in accord with the hypothesis that inter-depot variation in fat cell function reflects differences in the innate characteristics of adipose cells themselves. Thus, our study showing differences in adipogenesis between human subcutaneous abdominal and mammary gland preadipocytes are supported by many other studies. The present study demonstrated that mammary gland preadipocytes contained more lipid before induction of differentiation than preadipocytes from the abdominal subcutaneous adipose site. After 12 days of differentiation however no differences existed in intracellular lipid content.\This data suggests that baseline lipid uptake is greater in mammary than subcutaneous abdominal preadipocytes, but that the lipid content in differentiated preadipocytes is not different. However, it should be noted that the subjects from whom mammary adipose tissue was obtained had a lower BMI, than subjects from whom subcutaneous abdominal adipose tissue was taken. Therefore, we cannot rule out the possibility that the differences observed in preadipocyte lipid content are due to differences in body mass. However, this is unlikely because

we were unable to demonstrate any correlation between BMI and the lipid content of the preadipocytes taken from either body site .

Subcellular localization of ALP

Alkaline phosphatase is normally located either on the plasma membrane (Harris, 1990; DePierre and Karnovsky, 1973) or on secretary vesicles (Detmers et al., 1995) and is anchored to cell membranes via glycosylphosphatidylinositol (GPI) (Low and Saltiel, 1988; Kihn et al., 1990). The present study describes the association of alkaline phosphatase with intracellular lipid droplets and in light of the large body of evidence demonstrating that alkaline phosphatase is a membrane-associated enzyme (Harris, 1990; DePierre and Karnovsky, 1973; Detmers et al., 1995; Low and Saltiel, 1988; Kihn et al., 1990) it is presumed that alkaline phosphatase is attached to the membrane of these organelles. This is the first study to demonstrate that alkaline phosphatase is not associated with only secretary vesicle membranes or the plasma membrane. Studies have shown that the membrane of lipid droplets is closely associated with that of the endoplasmic reticulum (Blanchette-Mackie et al., 1995; Targett-Adams et al., 2000), which, along with the Golgi system is also the source of secretory granules (Stephens and Pepperkok, 2001). The association of ALP with the cytoplasmic lipid droplets is also suggestive of ALP playing a role in intracellular triglyceride accumulation, as confirmed in the ALP inhibitors studies.

5.2.2. The effect of weight on ALP activity and other liver enzymes

The existence of ALP in human preadipocytes prompted an investigation of the possible link between serum ALP levels and BMI. Total ALP levels and levels of bone ALP were measured in 100 African subjects of varying BMI. Liver ALP levels were estimated by taking the difference between the values for total ALP and bone ALP. Serum levels of liver enzymes and products of hepatic synthetic pathways were also assessed to ensure no volunteers had any liver pathology, as this would contribute to elevated ALP levels in the blood. Univariate regression analysis showed that serum total ALP levels correlated with age and weakly with BMI.

Multivariate analysis demonstrated that the relationship between BMI and ALP levels were probably due to age acting as a confounding variable. However, when subjects were divided into lean, overweight and obese groups and ALP levels analysed using ANCOVA adjusted for age, sex and BMI there was a significantly higher ALP level in obese than lean subjects. No such relationship was found for the other liver function tests suggesting that adipose tissue rather than liver tissue may be contributing to this relationship. Furthermore, the estimated liver ALP in serum was also found to be higher in obese than lean subjects, whereas bone ALP was not. Another study has also found positive relationships between serum total ALP and BMI (Golik et al,1991). Liver enzymes, particularly ALT have also been found to increase with increasing BMI and WHR, and the hypothesis has been that this is due to fat deposition in the liver (steatohepatitis). Our data confirms the relationship between serum ALT

levels and WHR and also weaker relationships are noted for albumin, and total bilirubin with WHR. Thus, all liver function tests that have been shown to correlate with BMI and WHR in other studies have been shown to correlate with WHR in this study. This suggests that liver products found in the serum are released due to increased hepatic fat deposition, which in turn is related to abdominal obesity. ALP levels however, only show an association with increased BMI and not even a weak association with WHR.

Granted this relationship is only observed when subjects are grouped according to BMI, however it is suggestive of a relationship between total body fat mass and serum ALP levels, rather than of a relationship between liver derived ALP and abdominal obesity. The reason for the lack of a correlation between serum ALP and BMI in multiple regression analyses but the existence of a relationship in ANCOVA is difficult to explain. It may suggest a non-linear relationship between these 2 variables, and one possible explanation for this may be that ALP is derived from multiple sources within the body. The lack of a relationship between bone ALP and BMI suggests that the isoform in the serum is not the bone form. The inhibitor studies have shown that the isozyme in human preadipocytes is the tissue nonspecific form, which consists of isoforms from the liver, bone and kidney that differ by glycosylation pattern. ALP from the kidney is not found in serum and therefore the isoform in preadipocytes may be the liver isoform. This must be confirmed by gel electrophoretic analysis of ALP isolated from human preadipocytes.

Conclusions

Alkaline phosphatase has been the subject of intensive research due to its wide tissue distribution (McComb, 1979), clinical utility as a marker of liver and bone disorders (Wolf, 1986) and its early, controlled expression during defined stages of mammalian embryogenesis (McComb, 1979; Hahnel et al., 1990; McDougall et al., 1998). However, this is the first study to demonstrate a possible role for alkaline phosphatase in intracellular lipid accumulation during preadipocyte differentiation. This is important because studies have suggested that alkaline phosphatase may be involved in organogenesis (Hillman et al., 1975; McCulloch et al., 1990) and cell differentiation (Andracchi and Korte, 1991; Feldbush and Lafrenz, 1991) and the present study supports these hypotheses with strong *in vitro* evidence. The 3T3-L1 cell line may therefore represent a very good model for the study of the role of alkaline phosphatase in the control of cellular differentiation and may also be useful in identifying the endogenous ligands of the enzyme, which are as yet unknown (McComb et al., 1979; Harris, 1990).

The present study has also demonstrated that ethnic differences exist in the level of activity of ALP in preadipocytes and these differences are paralleled by similar differences in lipid deposition. The role of ALP in the control of intracellular triglyceride accumulation in human preadipocytes is however, unclear. Histidine did inhibit both ALP activity and lipid deposition whereas levamisole increased ALP activity but decreased lipid levels. In the 3T3-L1 cells levamisole inhibited ALP activity and blocked lipid build-up. These data

demonstrate that levamisole has different effects on ALP activity in these two cell types but similar effects on lipid accumulation. This demonstrates that the relationship between ALP activity, as modified by levamisole and intracellular lipid deposition, differs in the two cell types.

The immunocytochemical studies show that ALP is localised to the lipid droplet in both 3T3-L1 cells and human preadipocytes and this is suggestive of a role for ALP in controlling cellular triglyceride accumulation.

The presence of ALP activity in 3T3-L1 cells and preadipocytes from mammary gland tissue and subcutaneous abdominal fat depot suggest that ALP expression is a common feature of cells that accumulate triglyceride in cytoplasmic lipid droplets.

The higher levels of ALP activity in serum from obese than lean subjects suggests that adipose tissue as well as liver and bone may contribute to the ALP measured in human serum.

Future Studies

The results presented in this thesis represent preliminary studies on the possible involvement of alkaline phosphatase in intracellular lipid deposition within preadipocytes. The present study used inhibitors of ALP that are non-specific and have other side effects that may also induce inhibition of lipid accumulation. It is therefore important to use techniques that specifically knock out ALP gene expression to confirm that ALP does play a role in lipid deposition. Techniques such as the use of small inhibitory RNA (siRNA) would be useful in this regard. In

addition, the inhibitor studies suggest that the ALP isozyme present in 3T3-L1 and human preadipocytes is the tissue non-specific form. This must be confirmed using PCR analysis.

Levamisole was shown to inhibit lipid accumulation in human preadipocytes but this did not involve inhibition of ALP activity. This suggests that levamisole is affecting lipid deposition independently of any effect on ALP activity. Levamisole is known to have adrenergic and nicotinic effects (Hsu, 1980) and therefore it would be interesting to study the effect of levamisole on lipid accumulation in human preadipocytes in the presence of antagonists of adrenergic and nicotinic receptors. Furthermore, levamisole inhibited adipogenesis in the 3T3-L1 cell line in parallel with inhibition of ALP activity. This demonstrates differential effects of levamisole on ALP activity in human and 3T3-L1 preadipocytes and it would therefore be interesting to investigate the effects of levamisole on gene and /or protein expression in both cell types using microarray and proteomic technology.

These studies demonstrate that ALP inhibitors block intracellular lipid deposition within preadipocytes but they do not prove that adipogenesis has been inhibited. This must be studied by measuring the expression of molecules that are known to be upregulated during adipogenesis e.g. PPARgamma, fatty acid binding protein-1 (FABP1) and glycerol 3-phosphate dehydrogenase (Gregoire et al., 1998).

If we can confirm that ALP does play a direct role in the control of intracellular lipid deposition then it would be important to determine whether ALP inhibitors can block adipose tissue hypertrophy in rodent models of obesity. Furthermore, the present study has only analysed ALP activity in preadipocytes and has not investigated the level of this enzyme in mature adipocytes. Studies should therefore be performed on murine and human mature adipocytes to determine the activity of ALP and to relate this to the level of obesity.

We have shown that ALP activity is present in preadipocytes isolated from both mammary tissue and the subcutaneous abdominal fat depot. It has also been demonstrated that ethnic differences in the level of adipogenesis is mirrored by higher ALP activity in preadipocytes isolated from mammary tissue in black compared to white females. It would be interesting to investigate whether ethnic differences also exist for preadipocytes taken from the visceral and subcutaneous fat depots as studies have shown that obesity is more prevalent in South African black than white females (Puoane et al., 2002) and the former group tend to have lower levels of visceral fat compared to the latter, but greater gluteo-femoral fat depot size.

Adipocytes are not the only cells that accumulate lipid within membrane-bound intracellular compartments. Hepatocytes, adrenal cortical cells and the Leydig cells within the testes also contain lipid droplets (Greenberg et al., 1991). In addition, gut enterocytes possess membrane-bound lipid droplets that have

intestinal ALP bound to their surface (Zhang et al., 1996). Hepatocytes and adrenal cortical cells are also known to express ALP and the protein components of the membrane surrounding the lipid droplets in these different cell types share common features, notably the presence of perilipin (Greenberg et al., 1991). Future studies should therefore determine whether ALP activity is also associated with the lipid droplets found in these different cell lineages, and if so this would suggest that ALP is an essential component of all cells that accumulate intracellular lipids.

Drawbacks of present study

1. This study used pharmacological inhibition of ALP. The inhibitors used may also have non specific effect on adipogenesis independent of their effects on ALP. The specific inhibition of ALP, should therefore be investigated using molecular techniques such as small interfering RNA (siRNA).
2. The oil red O technique for assessing adipogenesis is a commonly used method, however, it is not the most sensitive technique available for measuring adipogenesis. The measurement of glycerol 3- phosphate dehydrogenase should be used in future studies.
3. ALP inhibitory studies, have shown that the isotype present in preadipocyte is tissue non-specific ALP and this must be confirmed using PCR analysis.
4. The measurement of ALP levels in serum is hindered by the fact that it is not possible to distinguish intestinal ALP from liver ALP using a simple ELISA. Future studies should be performed in which total body fat levels are measured

accurately and correlated with bone, liver and intestinal serum ALP levels. The three ALP forms found in serum may be measured using gel electrophoresis in conjunction with densitometric scanning of the gels.

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APPENDIX 1

Patient No	Ethnicity	Age	Weight (Kg)	Height (M)	BMI	ALP inhibitors used with preadipocytes
1	White	42	74	1.68	26.1	Leva
2	White	39	65	1.60	25.6	Leva.
3	White	43	59	1.63	22.2	Leva.
4	White	48	71	1.65	26.1	Leva.
5	White	35	70	1.65	22.7	Leva.
6	White	42	73	1.70	25.3	Leva.
7	White	----	----	----	----	Leva
8	White	----	----	----	----	Leva
9	White	47	74	1.70	25.6	Hist.
10	White	51	71	1.68	25.1	Hist.
11	White	42	63	1.65	23.1	Hist.
12	White	41	74	1.70	25.6	Hist + Phe Gly Gly.
13	White	51	71	1.68	25.1	Phe Gly Gly
14	White	45	68	1.71	23.2	Phe Gly Gly
15	White	39	67	1.68	23.9	Phe Gly Gly
16	Black	51	63	1.60	24.6	Leva.
17	Black	46	70	1.73	23.4	Leva
18	Black	55	68	1.61	26.2	Leva.
19	Black	53	65	1.58	26.0	Leva
20	Black	44	60	1.65	22.0	Leva
21	Black	39	70	1.62	26.7	Leva
22	Black	41	60	1.65	22.0	Leva
23	Black	46	74	1.68	26.2	Leva.
24	Black	----	----	-----	-----	Leva.
25	Black	41	67	1.60	26.2	Hist + Phe Gly Gly
26	Black	38	71	1.66	25.8	Hist + Phe Gly Gly
27	Black	46	68	1.63	25.6	Hist + Phe Gly Gly
28	Black	45	65	1.60	25.4	Hist + Phe Gly Gly

Table 1. Details of patients from whom mammary gland adipose tissue was obtained.

Leva = levamisole
Hist = histidine

Patient`s No	Ethnicity	Age	Weight	Height	BMI	ALP inhibitors used on preadipocytes
1	Black	54	95	1.65	34.9	Leva
2	Black	48	93	1.64	34.7	Leva
3	Black	42	75	1.60	29.3	Leva
4	Black	35	73	1.52	31.6	Leva
5	Black	47	100	1.67	36.0	Hist and Leva
6	Black	42	109	1.64	40.7	Hist
7	Black	42	78	1.48	35.6	Hist
8	Black	39	94	1.63	35.5	Hist
9	Black	47	130	1.56	53.5	Hist
10	Black	58	124	1.60	48.4	Hist
11	Black	54	100	1.76	32.5	Hist
12	Black	47	79	1.47	36.6	Hist
13	Black	49	121	1.57	49.2	Hist

Table 2. Details of the subjects from whom subcutaneous abdominal adipose tissue was obtained.

Leva = levamisole
Hist = histidine

APPENDIX 2

1. Data for ALP inhibitor studies in 3T3-L1 cells (means ± SEMs)

Units: ALP activity = mU/mg protein

Lipid accumulation = OD units

1.1 The effect of levamisole on ALP activity in 3T3-L1 cells (See figure 4.2A).

	Day 0	Day 3	Day 7	Day 11
With levamisole	1.90±0.62	13.77±8.50	11.30±3.60	10.44±3.90
Without levamisole	1.90±0.62	10.94±2.80	17.16±6.00	23.00±9.20

1.2 The effect of levamisole on lipid accumulation in 3T3-L1 cells (See figure 4.2B).

	Day0	Day 3	Day 7	Day 11
With Levamisole	0.29±0.05	0.35±0.07	0.47±0.11	0.59±.010
Without Levamisole	0.29±0.05	0.44±0.11	0.65±0.10	0.90±0.14

1.3 The effect of Phe Gly Gly on ALP activity in 3T3-L1 Cells (See figure 4.3A)

	Day 0	Day 7	Day 11
With Phe. Gly .Gly	1.60±0.50	30.6±1.6	56.7±9.8
Without Phe. Gly. Gly	1.60±0.50	24.9±3.3	71.7±4.9

1.4 The effect of Phe Gly Gly on lipid accumulation in 3T3-L1 cells (See figure 4 .3B).

	Day 0	Day 7	Day 11
With Phe.Gly. Gly	0.31±0.05	0.70±0.15	0.49±0.01
Without Phe.Gly.Gly	0.31±0.05	0.56±0.08	0.50 ±0.01

1.5 The effect of histidine on ALP activity in 3T3-L1 cells (See figure 4.4A).

	Day 0	Day 3	Day 7	Day 11
With Histidine	1.60±0.36	2.9±0.7	1.9±0.31	1.9±0.56
Without Histidine	1.60±0.36	9.2±1.6	22.8±1.6	39.1±9.6

1.6 The effect of histidine on lipid accumulation in 3T3-L1 cells (See figure 4.4B).

	Day 0	Day 3	Day 7	Day 11
With Histidine	.15±.011	.18±.005	.22±.014	.25±.020
Without Histdine	.15±.011	.3±.005	.47±.015	.62±.008

2. Data for transformation medium studies in 3T3-L1 cells (means ± SEMs)

2.1 The effect of removing IBMX or Insulin or DEXA on lipid accumulation in 3T3- L1 cells (See figure 4.5A).

	Day 0	Day 3	Day 6
IBMX 0	0.16±0.02	0.19±0.02	0.22±0.01
INSULIN 0	0.16±0.02	0.28±0.07	0.47±0.14
NORMAL MEDIA	0.16±0.02	0.36±0.04	0.55±0.08
DEXA 0	0.16±0.02	0.35±0.05	0.53±0.08

2.2 The effect of removing IBMX or Insulin or DEXA on ALP activity in 3T3-L1 cells (See figure 4.5B).

	Day 0	Day 3	Day 6
IBMX 0	1.39±0.38	1.75±0.34	2.75±1.70
INSULIN 0	1.39±0.38	11.97±4.00	16.9±7.2
NORMAL MEDIA	1.39±0.38	14.21±4.10	30.0±4.4
DEXA 0	1.39±0.38	13.07±3.00	31.0±7.0

2.3 The effect of level of concentration of DEXA and IBMX on lipid accumulation in 3T3 –L1 cells (See figure 4.6A).

	Day 0	Day 3	Day 6
0.22µM DEX:0.17µM IBMX	0.17±0.01	0.18±.002	0.21±0.01
0.22µM DEX:0.5µM IBMX	0.17±0.01	0.23±.010	0.27±0.03
0.22µM DEX:1.5µM IBMX	0.17±0.01	0.29±.037	0.31±0.05
0.66µM DEX:0.5µM IBMX	0.17±0.01	0.30±.033	0.33±0.05
0.07µM DEX:0.5µM IBMX	0.17±0.01	0.24±.025	0.27±0.04
0.023µM DEX:0.5µM IBMX	0.17±0.01	0.25±.026	0.29±.016

2.4 The effect of level of concentration of DEXA and IBMX on ALP activity in 3T3-L1 cells (See figure 4.6B).

	Day 0	Day 3	Day 6
0.22µM DEX:0.17µM IBMX	0.79±0.11	3.29±0.57	4.70±0.88
0.22µM DEX:0.5µM IBMX	0.79±0.11	10.40±2.85	15.50±1.86
0.22µM DEX:1.5µM IBMX	0.79±0.11	16.80±1.07	31.21±4.42
0.66µM DEX:0.5µM IBMX	0.79±0.11	13.73±1.02	20.21±2.41
0.07µM DEX:0.5µM IBMX	0.79±0.11	8.02±1.31	14.31±3.14
0.023µMDEX:0.5µM IBMX	0.79±0.11	10.60±2.36	16.55±1.41

3. Data for ALP inhibitor studies with preadipocytes taken from mammary gland tissue (means ± SEMs)

3.1 The effect of levamisole on lipid accumulation of mammary tissue preadipocytes taken from white women (See figure 4.7A).

	Day 0	Day 12
With Levamisole	0.26±0.01	0.35±0.01
Without Levamisole	0.26±0.01	0.50±0.02

3.2 The effect of levamisole on ALP activity of mammary tissue preadipocytes taken from white women (See figure 4.7B).

	Day 0	Day 12
Without Levamisole	37.9±8.0	133.8±20.9
With Levamisole	37.9±8.0	230.9±50.5

3.3 The effect of levamisole on lipid accumulation of mammary tissue preadipocytes taken from black women (See figure 4.8A).

	Day 0	Day 12
With Levamisole	0.31±0.01	0.40±0.01
Without Levamisole	0.31±0.01	0.70±0.01

3.4 The effect of levamisole on ALP activity of mammary tissue preadipocytes taken from black women (See figure 4.8B).

	Day 0	Day 12
Without Levamisole	135.0±16.5	269.9±.41.5
With Levamisole	135.0±16.5	444.4±86.4

3.5 The effect of Phe Gly Gly on lipid accumulation of mammary tissue preadipocytes taken from white women (See figure 4.9A).

	Day 0	Day 12
Without Phe.Gly.Gly	0.21±0.01	0.44±0.06
With Phe.Gly.Gly	0.21±0.01	0.43±0.07

3.6 The effect of Phe Gly Gly on ALP activity of mammary tissue taken from white women (See figure 4.9B).

	Day 0	Day 12
Without Phe.Gly.Gly	45.3±5.1	157.2±12.5
With Phe.Gly .Gly	45.3±5.1	143.3±16.6

3.7 The effect of Phe Gly Gly on lipid accumulation of mammary tissue preadipocytes taken from black women (See figure 4.10A).

	Day 0	Day 12
Without Phe.Gly.Gly	0.29±0.01	0.82±0.04
With Phe.Gly.Gly	0.29±0.01	0.81±0.04

3.8 The effect of Phe Gly Gly on ALP activity of mammary tissue preadipocytes taken from black women (See figure 4.10B).

	Day 0	Day 12
Without Phe.Gly.Gly	143.4±10.9	321.7±28.7
With Phe.Gly.Gly	143.4±10.9	320.9±25.4

3.9 The effect of histidine on lipid accumulation of mammary tissue preadipocytes taken from white women (See figure 4.11A).

	Day 0	Day 12
With Histidine	0.24±0.02	0.30±0.04
Without Histidine	0.24±0.02	0.51±0.04

3.10 The effect of histidine on ALP activity of mammary tissue preadipocytes taken from White women (See figure 4.11 B).

	Day 0	Day 12
With Histidine	31.5±11.2	9.1±2.0
Without Histidine	31.5±11.2	97.6±24.9

3.11 The effect of histidine on lipid accumulation of mammary tissue preadipocytes taken from black women (See figure 4.12A).

	Day 0	Day 12
With Histidine	0.29±0.005	0.38±0.02
Without Histidine	0.29±0.005	0.87±0.03

3.12 The effect of histidine on ALP activity of mammary tissue preadipocytes taken from black women (See figure 4.12B).

	Day 0	Day 12
With Histidine	143.4±10.8	12.55±2.81
Without Histidine	143.4±10.8	305.8±22.9

4. Data on lipid accumulation and ALP activity in mammary tissue preadipocytes isolated from white and black females (means±SEMs)

4.1 Ethnic differences in lipid accumulation during levamisole treatment (See figure 4.13A).

	Day 0	Day 12
With Levamisole White	0.26±0.01	0.35±0.01
Without Levamisole White	0.26±0.01	0.50±0.01
With Levamisole Black	0.31±0.01	0.40±0.01
Without Levamisole Black	0.31±0.01	0.70±0.01

4.2 Ethnic differences in ALP activity during levamisole treatment (See figure 4.13B).

	Day 0	Day 12
Without Levamisole White	37.89± 8.02	133.7± 21.0
With Levamisole White	37.89± 8.02	230.8±50.5
Without Levamisole Black	135.0±16.5	269.9±41.4
With Levamisole Black	135.0±16.5	444.4±86.3

4.3 Ethnic differences in lipid accumulation during Phe Gly Gly treatment (See figure 4.14A).

	Day 0	Day 12
Without Phe.Gly.Gly White	0.21±0.01	0.44±0.06
With Phe.Gly.Gly White	0.21±0.01	0.43±0.07
Without Phe.Gly.Gly Black	0.29±0.005	0.82±0.04
With Phe.Gly.Gly Black	0.29±0.005	0.81±0.04

4.4 Ethnic differences in ALP activity during Phe Gly Gly treatment (See figure 4.14B).

	Day 0	Day 12
Without Phe.Gly.Gly White	45.32±5.12	157.1±12.5
With Phe.Gly.Gly White	45.32±5.12	143.3±16.6
Without Phe.Gly.Gly Black	143.4±10.8	321.7±28.7
With Phe.Gly.Gly Black	143.4±10.8	320.9±25.4

4.5 Ethnic differences in lipid accumulation during histidine treatment (See figure 4.15A).

	Day 0	Day 12
With Histidine White	0.24±0.02	0.30±0.04
Without Histidine White	0.24±0.02	0.51±0.04
With Histidine Black	0.29±0.005	0.38±0.02
Without Histidine Black	0.29±0.005	0.87±0.03

4.6 Ethnic differences in ALP activity during histidine treatment (See figure 4.15B).

	Day 0	Day 12
With Histidine White	31.52±11.22	9.10±2.02
Without Histidine White	31.52±11.22	97.60±24.94
With Histidine Black	143.4±10.9	12.55±2.81
Without Histidine Black	143.4±10.9	305.8±22.9

5. Data for ALP inhibitor studies with preadipocytes taken from subcutaneous abdominal tissue of black females (means ± SEMs)

5.1 The effect of levamisole on lipid accumulation of abdominal tissue taken from black women (See figure 4.16A).

	Day 0	Day 12
With Levamisole	0.18±0.03	0.35±0.02
Without Levamisole	0.18±0.03	0.57±0.03

5.2 The effect of levamisole on ALP activity of abdominal tissue taken from black women (See figure 4.16B).

	Day 0	Day 12
Without Levamisole	108.8±12.6	380.8±6.8
With Levamisole	108.8±12.6	780.1±174.9

5.3 The effect of histidine on lipid accumulation of abdominal tissue taken from black women (See figure 4.17A).

	Day 0	Day 12
With Histidine	0.27±0.02	0.38±0.04
Without Histidine	0.27±0.02	0.71±0.06

5.4 The effect of histidine on ALP activity of abdominal tissue taken from black women (See figure 4.17B).

	Day 0	Day 12
With Histidine	140.3±8.3	44.0±21.6
Without Histidine	140.3±8.3	381.2±72.7

6. Data on lipid accumulation and ALP activity in mammary and abdominal tissue preadipocytes isolated from black females (means±SEMs)

6.1 The effect of site of origin of preadipocytes on lipid accumulation during levamisole treatment (See figure 4.18A).

	Day 0	Day 12
With Levamisole Mammary	0.31±0.01	0.40±0.01
Without Levamisole Mammary	0.31±0.01	0.70±0.01
With Levamisole Abdominal	0.18±0.03	0.35±0.02
Without Levamisole Abdominal	0.18±0.03	0.57±0.03

6.2 The effect of the site of origin of preadipocytes on ALP activity during levamisole treatment (See figure 4.18B).

	Day 0	Day 12
Without Levamisole Mammary	135.0±16.5	269.9±41.5
With Levamisole Mammary	135.0±16.5	444.4±86.4
Without Levamisole Abdominal	108.8±8.3	380.8±21.6
With Levamisole Abdominal	108.8±8.3	780.1±72.7

6.3 The effect of the site of origin of preadipocytes on lipid accumulation during histidine treatment (See figure 4.19A).

	Day 0	Day 12
With Histidine Mammary	0.29±0.005	0.38±0.02
Without Histidine Mammary	0.29±0.005	0.87±0.03
With Histidine Abdominal	0.27±0.02	0.38±0.04
Without Histidine Abdominal	0.27±0.02	0.71±0.06

6.4 The effect of the site of origin of preadipocytes on ALP activity during histidine treatment (See figure 4.19B).

	Day 0	Day 12
With Histidine Mammary	143.3±10.8	12.55±2.81
Without Histidine Mammary	143.3±10.8	305.7±22.9
With Histidine Abdominal	140.3±8.3	44.02±21.60
Without Histidine Abdoninal	140.3±8.3	381.2±72.7