RELATIONSHIP BETWEEN NUTRITIONAL STATUS AND IMMUNE FUNCTIONS IN ELDERLY PAKISTANI MEN

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ABSTRACT

BACKGROUND: The age-associated prevalence of malnutrition (both obesity and underweight) is increasing and becoming a global health problem, such that it has been recognized as a major contributor to causes of immune decline in the elderly.

OBJECTIVES: Our first objective was to investigate the prevalence of malnutrition in elderly men in a rural area of a poorly-investigated developing country. The second objective was to elucidate and identify differences in peripheral blood lymphocyte cell surface immune phenotypes and relate them to nutritional status in otherwise healthy obese (OB), overweight (OW) and underweight (UW) compared to normal weight (NW) elderly men. The third objective was to compare the immunological findings obtained here with those known for the elderly in industrialized countries.

METHODS: We randomly selected a convenience sample of 548 elderly men for our cross-sectional survey, conducted in the Peshawar Khyber Paktunkhwa area of Pakistan. Clinically healthy subjects were included when they had no history of disease and were not taking any drugs on a chronic basis. All subjects gave written informed consent to participate. Anthropometric measurements were carried out with the subject barefoot, wearing light clothing, and after an overnight fast. Body weight, height, and fat were measured. Body mass index (BMI) was calculated as body weight divided by the height squared (Kg/m^2). Percent body fat (%BF) was determined by Futrex 5000. Habitual dietary intake was assessed through 24-hr Dietary Recalls (24-hr DRs). For this, each item of food eaten during the previous 24 hrs was recalled by the subject and recorded in a questionnaire. Nutrient intakes were calculated from the reported dietary intake. In the second part of the study, we selected 100 subjects from the 548 (50 each young and the elderly, including subjects from all the four BMI groups) for blood sampling. Blood samples were collected for analysis of plasma concentrations of selected clinical chemistry values (albumin, total protein, triglycerides, CRP, ferritin; analyzed on a Modular Analytics SWA automated analyzer) and for assessment of T and B cell phenotypes by flow cytometry.

RESULTS: Based on BMI, the proportions of obese, overweight and underweight elderly were 13.1, 3.1 and 10.8%, respectively. Age was negatively correlated with BMI (p=0.028) and tended to be associated with energy intake (p=0.054) and protein intake (p=0.077), waist circumference (WC)(p=0.312) and waist to hip ratio (WHR)(p=0.122). %BF also significantly correlated with age (p=0.0001). Large variations existed in WC, WHR, %BF and nutrient intake within different BMI categories of both the young and elderly. The normal weight (NW) elderly had significantly (p<0.05) higher intake of all nutrients studied, except energy which was significantly (p<0.05) higher in obese and overweight elderly. Overall, however, the majority of subjects had lower than adequate nutrient intake for most of the other nutrients studied (67.3 – 100% of the recommended intake). The results of immune phenotyping showed that as a group, the elderly had a significantly lower CD4:CD8 ratio, a lower percentage of CD8+ naïve T cells and significantly higher percentage of late-differentiated CD8+ memory T cells. These results are broadly similar to those

seen in industrialized countries, but may be occurring at an earlier chronological age. Dividing the subjects into 4 BMI categories (normal weight, obese, overweight, and underweight) revealed an influence of nutritional status on immune parameters. This was greater within the young group, and unlike age, affected the CD4 subset more profoundly than the CD8 subset. No age- or nutrition-associated differences were seen in B or NK cells. CD8+ cells as a percentage of CD3+ T cells were positively associated with plasma CRP levels but not other factors (albumin, total protein, triglycerides, and ferritin).

CONCLUSIONS: Very few elderly people had adequate nutrient intake. Obese and overweight people had higher % BF as compared to normal weight elderly. Older age is associated with changes not only in anthropometrics and body composition but also in intake of key nutrients like energy and protein. In addition, age and malnutrition markedly affect cells of the immune system. In short, the results show that both nutritional status, but importantly age, have a cumulative effect on the immune signatures investigated in the current study.

ZUSAMMENFASSUNG

HINTERGRUND: Die mit dem Alter zunehmende Verbreitung von Fehlernährung (Fettleibigkeit und Untergewicht) steigt weiter an und wird zu einem globalen Gesundheitsproblem. Es wurde erkannt, dass sie wesentlich zu den Ursachen für die Abschwächung des Immunsystems bei älteren Menschen beiträgt.

ZIELE: Erstes Ziel war die Untersuchung der Verbreitung von Fehlernährung bei frei lebenden älteren Männern in einer ländlichen Gegend eines kaum untersuchten Entwicklungslands. Zweitens sollten die Unterschiede bei den Immunphänotypen auf der Zelloberfläche von Lymphozyten aus dem peripheren Blut geklärt, identifiziert und in Zusammenhang mit dem Ernährungszustand von ansonsten gesunden fettleibigen/adipösen (Obese, OB), übergewichtigen (Overweight, OW) und untergewichtigen (Underweight,UW) im Vergleich zu normalgewichtigen (Normal Weight, NW) älteren Männern gebracht werden. Zum dritten sollten die so gewonnenen immunologischen Daten mit denen verglichen werden, die vonälteren Menschen in industrialisierten Ländern bekannt sind.

METHODEN: Nach dem Zufallsprinzip trafen wir eine willkürliche Auswahl 548 älterer Männer für unsere Querschnittsstudie in der pakistanischen Region PeshawarKhyberPaktunkhwa. Als Teilnehmer aufgenommen wurden klinisch gesunde Versuchspersonen ohne Krankheitsgeschichte und ohne ständigen Medikamenten- oder Drogenkonsum. Sie gaben ihre schriftliche Einwilligung zur Teilnahme, nachdem sie über den Versuch informiert worden waren. Es wurden an den Probanden anthropometrische Messungen durchgeführt: Barfüßig, mit leichter Kleidung und nach nächtlichem Fasten wurden Körpergewicht, Größe und Körperfett ermittelt. Es wurde der Body-Mass-Index (BMI) als Körpergewicht dividiert durch Größe im Quadrat (Kg/m^2) errechnet. Mit Futrex 5000 wurde das Körperfett in Prozent (% Body Fat, %BF) bestimmt. Die gewöhnliche Nahrungsmenge wurde mit Hilfe von 24-Stunden-Recalls (24-hr DietaryRecalls, 24-hr DRs) ermittelt: Jedes Stück Nahrung, das in den vorangegangenen 24 Stunden aufgenommen wurde, mussten sich die Probanden ins Gedächtnis zurückrufen, damit es in einen Fragebogen eingetragen werden konnte. Aus der aufgenommenen Nahrung wurde die Nährstoffaufnahme errechnet. Im zweiten Teil der Studie wurden aus diesen 548 Personen 100 Männer (50 junge und 50 ältere, aus allen vier BMI-Gruppen) für Blutproben ausgewählt. Blutproben wurden auf Plasmakonzentrationen ausgewählter klinischer chemischer Werte analysiert (Albumin, Gesamtprotein, Triglyceride, CRP, Ferritin, analysiert mit einem automatisiertenModular Analytics SWA), und mittels Durchflusszytometrie wurden die T- und B-Zell-Phänotypen bestimmt.

ERGEBNISSE: Auf der Basis des BMIbetrug der Anteil an fettleibigen bzw. übergewichtigen bzw. untergewichtigen Älteren 13,1% bzw. 3,1% bzw.10,8%. Das Alter korrelierte negativ mit dem BMI (p=0,028) und war tendenziell assoziiert mit der Energiezufuhr(p=0,054) und der Eiweißaufnahme (p=0,077), dem Taillenumfang (WaistCircumference, WC) (p=0,312) und dem Verhältnis Taille zu Hüfte (Waistto Hip Ratio, WHR) (p=0,122). Auch der Faktor%BF korrelierte signifikant mit dem

Alter (p=0,0001). Innerhalb der unterschiedlichen BMI-Kategorien existierten sowohl bei jungen als auch bei alten Menschen erhebliche Variationen hinsichtlich WC, WHR, %BF und Nährstoffaufnahme. Ältere mit Normalgewicht (Normal Weight, NW) zeigten eine signifikanthöhere ($p<0,05$) Aufnahme aller untersuchten Nährstoffe, mit Ausnahme der Energiezufuhr, die bei fettleibigen und übergewichtigen Personen signifikant ($p<0,05$) höher lag.Ganz allgemein jedoch lag hinsichtlich aller anderen untersuchten Nährstoffe bei der Mehrheit der untersuchten Personen die Aufnahme unterhalb dessen, was als angemessen betrachtet wird (67,3 – 100% der empfohlenen Mengen). Die Ergebnisse der Immunphänotypisierung zeigten, dass die Älteren als Gruppe ein signifikant niedrigeres CD4:CD8-Verhältnis, ein niedrigeren Anteil an $CD8^+$naiven T-Zellen undeinen signifikant höheren Anteil an spät differenzierten $CD8^+$Gedächtnis-T-Zellen aufwiesen. Die Ergebnisse ähneln weitgehend denen, die man aus Industrieländern kennt, doch es ist möglich, dass sie in einem früheren chronologischen Alter auftreten. Die Aufteilung der Versuchspersonen in 4 BMI-Kategorien (Normalgewicht, Adipositas, Übergewicht und Untergewicht) zeigte einen Einfluss des Ernährungszustands auf Immunparameter. Dieser war in der Gruppe der Jüngeren größer, und anders als das Alter betraf er eher das CD4-Subset als das CD8-Subset. Bei B- und bei NK-Zellen waren keine alters- oder ernährungsassoziierten Unterschiede sichtbar. $CD8^+$Zellen als prozentualer Anteil von $CD3^+$ T-Zellen waren positiv assoziiert mit CRP-Plasma-Levels, nicht jedoch mit anderen Faktoren (Albumin, Gesamtprotein, Triglyceride oder Ferritin).

SCHLUSSFOLGERUNGEN: Nur sehr wenige ältere Menschen nehmen adäquat Nährstoffe zu sich. Fettleibige und übergewichtige Personen hatten einen höheren %BF-Wert als normalgewichtige ältere Personen. Es besteht nicht nur eine Assoziation zwischen dem Alter und anthropometrischen Werten und dem Körperbau, sondern auch zwischen dem Alter und der Aufnahme wesentlicher Nahrungskomponenten wie Energie und Protein. Hinzu kommt, dass Alter und Fehlernährung deutlich die Zellen des Immunsystems beeinflussen. Zusammengefasst zeigen die Ergebnisse, dass sowohl der Ernährungszustand als auch das Alter, das noch entscheidender ist, einen kumulativen Effekt auf die in der vorliegenden Studie untersuchten Immunsignaturen ausüben.

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LIST OF ABBREVIATIONS

APC	Allophycocyanin
APC	Antigen presenting cell
BD	Becton Dickinson
BMI	Body mass index
BSA	Bovine serum albumin
CCR7	C-C motif Receptor 7
CD	Cluster of differentiation
CMV	Cytomegalovirus
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
FACS	Fluorescence-activated cell sorting
FSC	Forward Scatter
FITC	Fluorescein
FoxP3	Forkhead box P3
H_2O	Water
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IRP	Immune risk phenotype
kg/m^2	Kilograms per metre-squared
KLRG1	Killer cell lectin-like receptor G1
M	Molar
mAb	Monoclonal antibody
Mg	Magnesium
mg/ml	Milligram per millilitre
MHC	Major histocompatibility complex
Ml	Millilitre
N	Number of subjects
NaCl	Sodium chloride
NaOH	Sodium Hydroxide
NK	Natural killer
Nm	Nanometers
NW	Normal Weight
PBMC	Peripheral blood mononuclear cells
OB	Obese
OW	Overweight
PBS	Phosphate buffered Saline
PE	Phycoerythrin
PE-Cy	Phycoerythrin cyanine
PHA	Phytohemagglutinin
SSC	Side Scatter
TCR	T cell receptor
Th	T helper

TNF	Tumour Necrosis Factor
Treg	Regulatory T cell
UW	Underweight
WC	Waist Circumference
WHR	Waist to Hip Ratio
%BF	Percent Body Fat
A	Alpha
B	Beta
Γ	Gamma
μg/ml	Microgram per milliliter

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CHAPTER 1

INTRODUCTION & BACKGROUND

1. INTRODUCTION&BACKGROUND

1.1. AGING DEMOGRAPHY

The number of elderly people is increasing globally. A large number of demographic studies show that improvement in living conditions and better health-care facilities has led to an increased average life expectancy, resulting in an increase in the numbers of the elderly everywhere (Cho et al., 2004; Gavazzi et al.,2004; CDC, 2003; Jitapunkul et al.,2003; Shahar et al., 2001). Worldwide, on average the older adult population was predicted to increase by 17%, reaching 39.4 million by 2010, with the number of older adults over age 85 increased by 56% as compared to a 13% increase in adults aged 65 to 84 (Anonymous, 1996). It is anticipated that the increasingly large elderly population will face various health and nutritional problems, which will require enhanced health-care and will levy social costs (Cho et al., 2004; Gavazzi et al., 2004; Jitapunkul et al., 2003; Suzana et al., 2002; Anonymous, 1996).

1.2. PHYSICAL AND PHYSIOLOGICAL CHANGES WITH AGING

Many physiological changes take place with aging. For example changes in overall body composition (Lee et al., 2010; Ding et al., 2007; Alibhai et al., 2005; Newman et al., 2005) and alterations in the number and functions of cells associated with immunity (Chernoff, 2009; Wintergerst et al., 2007; Campbell et al., 2002; ADA, 2000; Meydani, 1999; Mazari, 1998) have been reported to take place even with "healthy" and "normal" aging. These changes are closely interlinked and affect each other directly or indirectly and therefore, must be studied in combination (ADA, 2000; Meydani, 1999; Mazari, 1998).

Changes in body composition are considered to be the most significant in terms of health out-comes and well-being (reviewed by Han et al., 2011; Alley et al., 2008; Ding et al., 2007; Goodpaster et al., 2006; Newman et al., 2005; Sternfeld et al., 2002). Often a simple measure such as body weight can be a warning sign of more complex problems. For instance, the occurrence of age-related weight loss in the elderly is a phenomenon that often reflects poor health (reviewed by Han et al., 2011; Davison et al., 2002) and loss of lean body mass (reviewed by Han et al., 2011; Alley et al., 2008; Zoic et al., 2004). Weight loss in the elderly is typically unintentional and is associated with increased risk of functional impairment (reviewed by Han et al., 2011; Alley et al., 2008; Zoic et al., 2004; Despres et al., 1994) and mortality (Boyko et al., 1999). Due to these physiological changes, the elderly are the most vulnerable to poor health and malnutrition (Shatenstein et al., 2003; Keller et al., 1993), a fact that needs to be considered in preventive and/or corrective strategies.

There is now an overwhelming body of evidence from both cross-sectional (reviewed by Han et al., 2011; Aloia et al., 1996; Silver et al., 1993; Kuskowska & Rossner, 1990) and longitudinal (reviewed by Han et al., 2011; Chumlea et al., 1988; Steen et al., 1979) studies that there is a decrease in lean body mass (LBM) and an increase in percentage of body fat in the elderly. These changes can lead to reduced muscle mass and a decline in weight (mainly because of loss in LBM), physiological conditions

collectively called sarcopenia. There is also a redistribution of fat, i.e. intra-abdominal fat tends to increase and subcutaneous fat on the limbs tends to decrease. In cross-sectional studies, increases in subcutaneous fat have been observed with advancing age up to 60 years. Age-related differences in regional or segmented body composition are documented by higher waist diameters, higher waist-to-hip or waist-to-thigh ratios, and lower girths in the limbs in older than in younger subjects (Hughes et al., 2004). The relationships between age-related obesity and disease have been investigated extensively. It is beyond the scope of this thesis to describe these changes and their impact on health in detail, but more on age-related obesity and other diseases can be found in an excellent review by Han et al., (2011).

At the other extreme, weight loss in the elderly is very common; it is a phenomenon often reflecting poor health and compromised nutritional status. Weight loss is usually an indicator of loss of lean body mass (Wannamethee et al., 2005). Moreover, weight loss in the elderly is typically unintentional and is associated with increased risk of functional impairment and mortality (Hughes et al., 2004). With aging, elderly persons develop a low muscle mass coupled with high body fat, which have been shown to be a particularly strong predictor of poor physical function (Wannamethee et al., 2005; Hughes et al., 2004).

The prevalence of weight loss among elderly adults is not well-established as the literature on this topic is limited to a few small studies that utilize a variety of definitions for weight loss (reviewed by Han et al., 2011; Newman et al., 2001). Over the first 3 years of follow-up, more than 15% of the Cardiovascular Health Study cohort experienced ≥5% weight loss while an additional 5% had weight loss of ≥10% (Newman et al., 2001). In a separate study of male Veterans living in Seattle, one quarter of the sample experienced ≥4% weight loss over a 2 year period (Wallace et al., 1995). Thus, the weight loss prevalence ranges from 15% to 25% depending on the population under study and the definition of weight loss that is employed.

Weight loss may also be very variable in terms of the site of the body affected. However, most of the studies considered weight loss as a whole without any discrimination between the loss in lean body mass and the loss in adipose tissues. Physiological annual weight loss of less than 1% after the age of 60 years for both male and female subjects has been reported (Beck & Olevsen, 1998). Weight loss greater than this and overt malnutrition have been cited as common problems in older populations, and have been associated with adverse health outcomes such as infections, poor wound healing, and death (reviewed by Han et al., 2011; Wallace et al., 1995). Weight loss of 5 kg or more has been associated with a small increase in the risk of all-cause mortality (Yaari & Goldbourt, 1998). According to Davies & Knutson (1991), recent unintended weight loss can be considered as an important independent warning signal for malnutrition in the elderly. It assumes even greater urgency requiring various forms of practical preventive action, particularly, if weight loss it is associated with other risk factors.

Rumpel et al., (1993) and Losonczy et al., (1995) have shown that mortality is related to weight loss but not if the individuals have always been lean. Such people are

reported to be healthier than those of similar weight but who have lost weight due to conditions that cause unintentional weight loss, such as cancers, chronic heart and lung disease (Seidell & Visscher, 2000). This unintentional weight loss presents a problem in the interpretation of body weight in the elderly. Furthermore, persons who achieve weight loss intentionally, through lifestyle modification or pharmaceutical intervention, generally acquire a range of clinical benefits and quality of life, whereas unintentional weight loss is most commonly through illness and usually involves loss of lean tissue, which carries a poor prognosis, even if there is subsequent weight regain which may involve accumulation of fat tissue or edema (Seidell & Visscher, 2000).

1.3. AGING AND MALNUTRITION

Data from the third National Health and Nutrition Examination Survey (NHANES III: 1994) in the USA as well as other studies (Rolls et al., 1995; Subar et al., 1990; Wurtman et al., 1988) clearly demonstrate a linear decline in food intake from the age 20 to 80 years in both men and women. In general, the average decrease in food intake has been reported to be 1321 calories in males and 629 calories in females (Rolls & McDermott, 1991). This decrease in food intake was predominantly due to a decrease in carbohydrate and fat intake. In the study by Wurtman et al., (1988), older persons ate 55% less fat and 40% less carbohydrate than younger individuals. Protein consumption was equivalent in both age groups. In addition, older persons were also less likely to snack between meals. Rolls et al., (1991) found that older persons consumed less calories over a single meal compared to younger persons. However, when given a yogurt preload, older subjects were likely to overeat compared to younger subjects, suggesting a failure of normal energy-setting mechanisms that operate in younger persons. In another study by Rolls and McDermott (1995), it was found that in contrast to the young, older persons fail to develop sensory-specific satiety.

Age-associated malnutrition results mainly from insufficient intake of macro- and micronutrients (Brownie, 2006). On average, the global prevalence of malnutrition is as high as 5–10% of independently living older individuals, 30–60% of institutionalized patients and ≤35–65% of hospitalized patients. In nursing homes, even higher levels of prevalence have been observed; that is, ≤ 85% of nursing home patients shows significant nutritional deficits. The main nutrient deficiencies reported in the elderly include zinc (Prasad et al., 1993), vitamins A, B6, folate and B12 (Tucker, 2005; Lesourd & Mazari, 1999).

Energy is the key nutrient. Its requirements, however, diminish due to decrease in lean body mass with age. Average daily energy intake decreases by about 30% between 20 and 80 years (Chapman, 2008). Most of this decrease in energy intake is probably a response to the decline in energy expenditure that also occurs with aging. In addition, the loss of muscle mass can further contribute to a loss of mobility (Refai & Seidner, 1999), and decreased physical activity, which may lead to reduced energy requirement (Gariballa & Sinclair, 1998). However, despite the fact that elderly need relatively less energy per day, there is nonetheless a likelihood of deficit in energy intake in old age.

The age-associated energy intake deficit is, however, mainly attributed to lower than required food intake in old age, which in turn, is attributed to a number of other age-associated physiological changes. For example, both smell and taste decline with age and can decrease the appetite, which may affect the choice of food to a greater extent (Brownie, 2006; Refai & Seidner, 1999). The decline in smell and taste can be accompanied by early satiation in old age, which can also be explained by a diminished ability of the stomach to relax when filled (Asai, 2004) and the increased time required for emptying the stomach after a large volume of food (Gariballa & Sinclair, 2005). With increasing age, the ability to sense thirst can also be diminished (Asai, 2004), which in turn, can cause markedly lower food intake. The oral health and dental problems in old age have also been reported as the contributing factors for malnutrition in old age in Pakistani elderly (Alam & Bangash, 2010).

It is noteworthy that the rate of the aging process differs greatly between individuals, resulting in different subgroups within the elderly population (*e.g.* the healthy elderly versus the frail older group) (Hilmer, et al*.*, 2007), each of which may have their own issues with respect to nutritional requirements. All these changes are potential risk factors for causing malnutrition in the elderly. Risk factors for malnutrition are the underlying reasons explaining why people eat less or eat poorly. If the individuals have one or more risk factors, they are more likely to become malnourished. Different risk factors are often linked to each other and they may be more or less widespread in certain situations.

1.4. IMMUNE SYSTEM–AN OVERVIEW

Before we discuss in the forthcoming sections how immunity, aging and nutrition interact with one another, it will be useful to give here a brief overview of the immune system. The immune system is an integrated mechanism providing defense against invading pathogens, which may otherwise cause irreversible damage to the organism (Goldsby et al*.*, 2003; Kuby, 1997). The immune system is a network that protects against diseases, identifying and killing pathogens and possibly tumor cells, while not reacting excessively to symbionts in the gut. Functionally, the cells and molecules of the immune system are capable of mounting an immune response through two inter-related activities i.e., recognition and response (Kuby, 1997).

There are two broad components of immunity: innate immunity and specific or acquired immunity. Innate immunity is comprised of five types of defensive barriers: anatomic, physiologic, endocytic, phagocytic, and inflammatory. Anatomic sites such as the skin and the mucous membranes are the body's first line of defense and provide an effective barrier to the entry of many pathogens. Physiologic barriers include temperature, pH, oxygen tension, and various soluble factors. Temperature inhibits the growth of certain pathogens in many species, and gastric acidity is effective against microorganisms as very few survive low pH of the stomach. Soluble factors such as lysozyme (Alberts et al*.*, 2002; Boyton et al*.*, 2002; Moreau et al*.*, 2001; Hankiewicz, et al*.*, 1974), which is a hydrolytic enzyme capable of cleaving the bacterial cell wall, or complement (a group of serum proteins that participate in an enzyme cascade to destroy membranes of pathogens or facilitate their clearance), also contribute to innate

immunity (Kuby, 1997). The skin and respiratory tract also secrete certain antimicrobial peptides such as the β-defensins (Agerberth et al., 2006).

Besides the physical boundaries and factors to defeat the external environmental challenges, the innate immune system is composed of protective cells and molecules which rapidly become activated upon provocation. Some examples are natural killer cells (NK cells) (Goldsby et al., 2003; Kuby, 1997), scavenging macrophages (Kuby, 1997) and the complement system, as mentioned above (Salzet et al., 2006). To accommodate an immediate response, innate immunity is limited in terms of specificity and flexibility and can neither improve nor tailor its responses against a given antigen.

The other type of immunity is called adaptive immunity (Goldsby et al., 2003). In marked contrast to the innate immune response, adaptive immunity is highly antigen-specific and exhibits a capacity to adapt host responses to the nature of infectious agents. This type of immune system comprises certain types of lymphocytes, which can be divided into 'large lymphocytes' and 'small lymphocytes'. Large granular lymphocytes include the NK cells of the innate system, but the small lymphocytes consist of T cells and B cells of the adaptive system. They have highly polymorphic membrane receptors which enable them to recognize foreign antigens with fine specificity, and, in the case of B cells, to secrete them in the form of antibodies.

Lymphocytes account for 20-40% of the total white blood cells (WBCs) in peripheral blood. They are produced in the bone marrow by a complex biological process, called '*haematopoiesis*'. A detailed description of hematopoiesis can be found elsewhere (*e.g.* Williams, 2004). Briefly, hematopoiesis is the process of production, multiplication, and specialization of blood cells in the bone marrow. Hematopoiesis begins with the most basic blood cell, the stem cell or "*pluri-potent hematopoietic stem cell*" (PHSC). The end products of this process are mature white blood cells. Bone marrow is the primary organ where most of these processes are accomplished. On completion of their partial development, they leave the bone marrow, circulate in the blood and lymph, and reside in various organs of the lymphatic system (Alberts et al., 2002; Kuby, 1997). These cells provide specific recognition of an immense variety of environmental antigens by expressing a diverse repertoire of clonally distributed antigen-specific receptors. The most important immune cells are B cells producing antibodies and T cells that show attributes of specificity, diversity, and memory and self/non-self-recognition, after the crucial process of positive and negative selection in the thymus.

T and B lymphocytes are the two major cell types of adaptive immunity and constitute the majority of the lymphocyte population. Most of the lymphocytes in the blood are T cells; making up 22–30% of total nucleated white cells. Circulating B cells represent only 7–10% of white blood cells and consist of a number of different subsets that participate in immune responses. T and B cell differentiation stages can be distinguished by their expression or lack of expression of certain cell surface molecules. Thus, approximately 75% of circulating B cells in young people do not express a monomorphic TNF family co-stimulatory receptor called CD27, indicating that they have recently emerged from the bone marrow and have not yet encountered

antigen in the periphery (Schmidlin, 2009; Kuby, 1997). B cells mature in the bone marrow, while T cells migrate to and mature in a distinct organ, the thymus, where they go through further developmental stages, to produce mature CD4+ and CD8+ T cells. On completion of maturation, these naïve cells leave the thymus and migrate to the periphery. Näive T cells are long-lived cells and can circulate for years if not stimulated by their cognate antigen. They may, however, die before ultimately encountering their specific antigen, and this could happen in old age (Comans-Bitter et al., 1997). Otherwise, upon antigen encounter, näive T cells become activated, proliferate (clonal expansion) and differentiate into memory and effector T cells to exert their anti-pathogen functions. For example, the MHC class I-restricted CD8+ T (cytotoxic T cells) have direct cytotoxic reactivity once activated and can kill infected cells directly by production of cytotoxins such as perforins and granzymes (Shearer et al., 2002), whereas the MHC class II-restricted CD4+ T cells (helper T cells) 'help' macrophages and B cells during these responses by supplying cytokines which enable the maturation of these cells (Kuby, 1997).

CD4+ and CD8+ T cells are further divided and categorized into "sub-populations" based on the expression of additional cell surface molecules. There are at least three established models in use for the identification of T lymphocyte sub-populations, which are actually differentiation stages. The first utilizes the expression of CD27 in combination with the leukocyte common antigen CD45RA (Hamann et al., 1997; Sallusto et al., 2004). With this strategy one population of antigen inexperienced cells, the naïve subset, is defined (CD27+CD45RA+) together with three memory populations: central memory (CM; CD27+CD45RA−), effector memory (EM; CD27−CD45RA−), and 'revertant' memory cells, which have re-expressed the 'naïve' cell marker CD45RA (TEMRA; CD27−CD45RA+) (Hamann et al., 1997; Sallusto et al., 2004). The "classic" most-often applied model uses CD45RA and the chemokine/lymphoid homing receptor CCR7 to define naïve cells as CCR7+CD45RA+, central memory (CM) cells as CCR7+CD45RA-, effector memory (EM) cells as CCR7-CD45RA- and CCR7-CD45RA+ as late-stage "TEMRA" cells (Sallusto et al., 1999).In the third system, CD27 and the Ig family co-stimulatory molecule CD28 are used in combination with CD45RA (Appay et al., 2008; Sallusto et al., 2004; van Lier et al., 2003). Identifying cells on the basis of this model gives three distinct phenotypes, differing along a continuum of differentiation stage: early stage (CD27+CD28+), intermediate stage (CD27+CD28−), and late stage differentiated cells (CD27−CD28−) (Appay et al., 2002; Appay et al., 2008). These models were originally constructed to distinguish between naïve and different CD8+ memory T cell populations. Nevertheless, they do not allow drawing a clear distinction between naïve and memory cells (Appay et al., 2002; Appay et al., 2008). The reason for this limitation is that due to overlapping dynamic loss and re-expression of CD27, CD28, and CD45RA with cellular differentiation and antigenic stimulation, several of the sub-populations described share similar functional characteristics (Appay et al., 2008).

Naïve, CM and early-stage differentiated cells show a propensity to migrate to the secondary lymphoid organs. Here, these cells interact with antigen presenting cells (APC) and as a result their rapid proliferation occurs, which produces antigen-specific

effector cells. These antigen-specific effector cells then readily migrate to inflamed tissues (Sallusto et al., 2004; Sallusto et al., 1999). CM cells, which have previously responded to infection, have relatively greater capability to produce effector cells quicker than antigen inexperienced naïve cells (Sallusto et al., 2004; Sallusto et al., 1999). The EM and EMRA stages, strongly overlapping with the intermediate and late stage differentiated cells, migrate to peripheral tissue (*e.g.* the skin, mucous membrane etc). In addition, they express cytotoxic effector molecules (*e.g.* perforin and granzyme B) and readily produce inflammatory cytokines (*e.g. IFN*-γ) (Appay et al., 2002; Lanzavecchia and Sallusto, 2005; Takata and Takiguchi, 2006), which can help in providing immunity to the body in a number of ways.

What is the mechanism behind relatively long-lasting homeostasis of näive T-cells? It has now been well established that the homeostasis of näive T-cells remains relatively stable during life (Hazenberg et al., 2004). Production of large numbers of näive T cells in early life, when the thymus is a large and very active organ, is followed by their exposure to pathogens, clonal expansion of reactive cells, differentiation, performance of their function, clonal contraction and death of the majority with retention of some as memory cells. In humans, the number of näive T cells decreases with increasing age and exposure to pathogens, and is not compensated for because of thymic involution. Homeostatic proliferation of existing näive T cells in the periphery is then the main source of larger numbers of naïve cells in later life (Sakaguchi et al., 2004). A steady loss of näive T cells may occur by their death or by the differentiation of the näive T cells into memory/effector T cells in response to pathogens. Because thymic output declines significantly with age (Akbar et al., 2004), there is a decline in the contribution of the thymus to näive T-cell homeostasis over the life-span. This suggests that homeostasis of the näive T-cell compartment in adults relies mostly on peripheral T-cell proliferation and prolonged survival (longevity) of näive T cells. This contributes to the age-related changes in the immune system, particularly in T cells, as discussed in detail in the next section.

1.5. AGING AND IMMUNITY

Changes to the immune system with aging have been studied and reviewed extensively over the past few years (for example, Larbi et al., 2008; Pawelec, 2005; Sallusto et al., 2004; Pawelec et al., 2002; Campbell et al., 2002; Hamann et al., 1997; Douek & Koup, 2000; Sallusto et al., 1999; Lesourd & Mazari, 1999; Pawelec, et al., 1998; Myrvik et al., 1999). Most of these studies and reviews mainly concentrated on reporting changes in the immune system of so-called "WEIRD" (Western, educated, industrialized, rich and democratic) aged populations in the developed world. Thus, much of our knowledge in immunology may be relatively representative only of this minority of people worldwide.

It is usually difficult to investigate the changes in immune cell signatures in human in longitudinal studies due to time and resources constraints (Pawelec et al., 2004). It is, therefore, customary to study such changes in elderly compared to young in cross-sectional study settings. Such studies cannot document age-associated changes but only record differences between populations and make assumptions about changes.

However, there is limited number of longitudinal studies in human populations, eg. studies on individuals over 85 years of age in the Swedish OCTO/NONA longitudinal studies. These studies initially examined age-related changes in a few immune parameters but revealed an "immune risk profile" (IRP) characterized by an inverted CD4:CD8 ratio caused by an accumulation of late-stage differentiated CD8+CD28-negative T cells. As an example, the IRP was present in about 15% of the subjects at baseline, and was strongly associated with 2-, 4- and 6-year all-cause mortality at follow-up (Wikby et al., 2005). Most studies have noted marked differences between young and old subjects in the proportions of naïve and memory T cells present in the peripheral blood, and have assumed that these differences represent changes. In newborns, the ratio of naïve to memory T cells is quite high; in adults the ratio is reversed because most of the naïve T cells have been exposed to antigen, and hence converted to memory cells. The elderly have fewer or almost no naïve T cells at all, since as the thymus progressively involutes with age, fewer T cells are produced, and the naïve T cell subpopulation is not replenished (hence, naïve cells are not part of the IRP). Consequently, the stock of naïve T cells becomes depleted and the aged immune system cannot respond as well as a young person to a new antigen (reviewed by Pawelec et al., 2005)

T cell homeostasis ensures that as naive cells are lost, there is a compensatory increase in the numbers of memory cells and expansion of the remaining naïve cells. Consequently, the number and proportion of memory T cells with a late-differentiated phenotype (*e.g.* CD27−CD28−/CD45RA+) commonly increases (Pawelec, 2006; Akbar and Fletcher, 2005; Bosch et al., 2009). These phenotypic changes are particularly prominent in the cytotoxic T cell pool. The accumulation of CD8+CD27−CD28−/CD45RA+ cells is considered a hallmark of the aged immune system (Akbar & Fletcher, 2005; Bosch et al., 2009; Hadrup et al., 2006; Pawelec, 2006), which implies that young individuals exhibit marked alterations in their T cell repertoire as compared to the elderly (Chidrawar et al., 2009; Weinberger et al., 2007). This is reflected in the IRP, which suggests that these differences are indeed changes, which are, moreover, clinically relevant.

As mentioned above, the involution of the thymus is one of the most important features of normal anatomical as well as physiological development and has profound effects on aging in the context of the immune system. The shrinkage of the thymic lymphoid tissue starts right after or even before puberty, and a 4–5 fold reduction is observed by age 35–40. At 50 years of age, on average only 10% of the initial amount of lymphoid tissue is left. After this age, the amount of lymphoid tissue diminishes slowly or stays about the same (Kilpatrick et al., 2008; Consolini et al., 2000; Douek & Koup, 2000). Thus, the normal process of shrinkage of the thymus must have an active role as a weakening source of näive T lymphocytes and thymic hormones (Zhang et al., 1999). Consequently, the aging process results in immunity being greatly dependent on the existing pool of memory T cells and remaining unexposed naïve cells generated early in life (Woodland & Blackman, 2006) leading to some marked changes in the composition, function and competence of the human immune system, commonly termed "*immunosenescence*" (Gomez et al., 2008; Panda et al., 2009; Pawelec,

2005).This term was first coined by Dr. Roy Walford in his early book "*The immunologic theory of Aging*". (Walford, 1969). He proposed that normal aging in humans and animals is related to faulty immune processes, which may also be relevant in other contexts. For example, young adults who had been thymectomised in the first few years of life exhibit reduced numbers and proportions of naïve T lymphocytes, and sometimes increased numbers of cytotoxic T cells (Sauce et al., 2009; Torfadottir et al., 2006; Eysteinsdottir et al., 2004). These immune profiles of the young are quite similar to those of middle-aged (30-50 yr) and maybe to elderly adults, who also show little or no thymic output (Sauce et al., 2009; Torfadottir et al., 2006; Eysteinsdottir et al., 2004). However, it seems that reduced thymic output is only one cause of immunosenescence. For example, the investigation by Sauce et al., (2009) shows that young adults infected with Cytomegalovirus (CMV) and with no thymus, exhibited more severe alterations in the T cell repertoire compared to those who were free of CMV infection (Sauce et al., 2009). This combination of little or no thymic output and selective expansion/maintenance of cytotoxic T cell populations may lead to a gradual 'filling of immunological space' with CD8+ T cells (Brunner et al., 2010; Sauce et al., 2009; Akbar & Fletcher, 2005; van Lier et al., 2003). The over-riding impact of infection with the persistent herpes virus CMV in this respect is still little understood. Its importance is emphasized by its inclusion in the parameters making up the IRP.

Most research on "*immunosenescence*" has focused on adaptive immunity as it was once postulated that innate immunity is better preserved with aging (Franceschi et al., 2000). It is now appreciated, however, that age-related changes are discernible in nearly all cells of the innate immune system as well. For example, aging is associated with decreased natural killer cell function and altered neutrophil migration (Panda et al., 2009; Gomez et al., 2008), which starts as early as adolescence (Nikolich-Zugich, 2008; Akbar & Fletcher, 2005). Immunosenescence, by definition, is reflective of the erosion occurring in immune competence over the course of life and is at its maximum in old age (Larbi et al., 2008; Ostan et al., 2008; Pawelec & Larbi, 2008).

Alterations in the immune system with age are the likely reasons why older people experience increased morbidity and premature mortality from respiratory tract pathogens (Meyer, 2005), increased gastrointestinal infections (Shumaker et al., 2003), and diminished antigen-specific responses to vaccines (Hagiwara et al., 2003) amongst other things. Age-related phenotypic and functional changes to the T-cell component of adaptive immunity occur more drastically (*reviewed by* Pawelec, 2005; Pawelec et al., 2002; Pawelec et al., 1998), with adverse impact on overall immune functions. In the T cell compartment, a decline in naïve CD8 cytotoxic T cells has been associated with aging, while the CD4 subset seems to be preserved in most populations studied as long as nutritional status remains normal. However, while the CD4:CD8 ratio generally increases with age, and inverted CD4:CD8 ratio due to an increase in the number of at least partly dysfunctional late-differentiated CD8 cells predominantly specific for CMV epitopes is observed in a minority of very elderly people and is predictive of incipient mortality – this is the IRP mentioned above (Pawelec et al., 2004). This decline in CD4:CD8 with age has been related to the accumulation of antigenic pressure throughout life (Cakman et al., 1996).

Changes in B cells with age have also been studied (*reviewed by* Frasca et al., 2011; Curosos et al., 2009) and reported to be affected less than T cells (Goronzy et al., 2007; Castle, 2000; Bell & High 1997). Overall, numbers or proportions of B cells show little (Sansoni et al., 1993; Wikby et al., 1994; Cobleigh et al., 1980) or no change (Cakman et al., 1996; Oyeyinka et al., 1995) with age. However, lower numbers of B cells were also part of the IRP. If lymphocyte numbers are altered with age, then their functions as well as interactions with one another and other cells may be affected, leading to marked changes in immune response.

1.6. MALNUTRITION AND IMMUNITY IN THE ELDERLY

Malnutrition and a decline in the immune system go hand-in-hand as an individual ages. Malnutrition (both obesity/overweight and underweight) may affect the overall integrity of the immune system. Age-associated obesity, in particular, is closely associated with chronic inflammation (Dandona et al., 2004; Das, 2001), which results in increased plasma concentrations of C-reactive protein (CRP), IL-6, TNF and plasminogen activator inhibitor-1 (Dandona et al., 2004). On the other hand, undernutrition also exerts a strong negative effect on immune responses in the elderly (*reviewed by* Han et al., 2011). Protein–energy malnutrition (PEM) is the initial stage of malnutrition (undernutrition or underweight), followed by micronutrient deficiencies if left unaddressed. As a result, malnutrition or underweight is always associated with lower immune responses and this is observed for all types of immunity: cell-mediated immunity, antibody responses as well as innate immunity. In addition, the effects of micronutrient deficiencies are more often observed in aged than in young people (Lesourd, 2000) with adverse effects on the overall immune parameters. The evidence of such effects of micro- and/or macronutrient deficiencies on the immune system has mainly come from nutritional supplementation studies and their positive effects on the immune system. An appreciable number of previous research studies applying the cause and effect model have demonstrated that nutritional supplementation, whether macronutrient energy supplements in PEM or micronutrient supplements in micronutrient deficiency states, usually leads to increased immune responses (Lesourd et al., 1998). For example, although it is very unlikely that the elderly suffer from vitamin E deficiency, still cell-mediated immune responses are increased after vitamin E supplementation (Meydani et al., 1997), showing that vitamin E needs may be higher in aged individuals than the current recommendations and that such higher doses may be beneficial for the integrity of immune system.

It is, however, of particular note that the dysregulation of immune responses observed in the elderly might be due to cumulative antigenic pressure on the immune system throughout life (Albers et al., 2005), which results in driving T cell differentiation towards the accumulation of large numbers of partly-dysfunctional memory T cells with a limited available antigen repertoire. This effect is further coupled with decreased thymic output of näive T cells, as well as age-associated compromised function of näive cells produced earlier in life, resulting in increased susceptibility of the elderly to challenge by new pathogens. Undernutrition, whatever its type, may add

another detrimental factor to immune responses, the elderly being particularly susceptible to nutritional factors.

Epidemiological and clinical studies provide evidence that many nutrient deficiencies result in altered *immunocompetence,* which may cause an increased risk of developing infections (Gershwin et al., 2004; Selmi et al., 2004). There have been a few older studies which have investigated the effect of multi-micronutrient supplementation on resistance to infection in the elderly subjects (Graat et al., 1997; Chavance et al., 1993). While some have shown benefits of nutritional intervention in reducing the burden of infectious diseases in the elderly (Girodon et al., 1997), others have shown no significant effects (Graat et al., 2002; Chavance et al., 1993). These observed inconsistencies may be due to differences in nutritional status of the populations studied or to other methodological problems such as short duration of supplementation. This emphasizes the need for a better understanding of specific nutritional deficiencies of a particular population before an intervention strategy is proposed or carried out. A more detailed review of intervention studies with single nutrients or a mixture of nutrients in the elderly is provided in reviews (*e.g*. Meydani & Santos, 2000).

The effects of nutritional deficiencies on immunity and infections have also been studied extensively in intervention and supplementation studies. A multi-center nutritional trial (Bourdel-Machasson et al., 2000) demonstrated a slightly reduced risk of pressure ulcer infections in elderly patients who were given daily protein-calorie supplements. The purpose of the study was to assess the effect of nutritional supplementation on dietary intake and pressure ulcer development in critically ill older patients. The study involved 19 wards, stratified according to specialty and recruitment for critically ill older patients; 9 wards were randomly selected for nutritional intervention (nutritional intervention group), consisting of the daily distribution of two oral supplements, with each supplement containing 200 kcal, for 15 d. The study concluded that this energy protein intervention was associated with a decreased risk of pressure ulcer incidence. The study of institutionalized elderly persons that demonstrated clinical benefit (Girodon et al., 1997) also suggests that trace minerals, in particular, may be the key nutritional factors for preventing infection in older adults. Zinc (20 mg of elemental Zn++) plus selenium (100 μg) given daily, regardless of whether given with or without vitamins, decreased infection rates in this study and barely missed significance in a similarly designed second, larger trial (Girodon et al., 1999). The mean number of infections (respiratory and urinary tract) was reduced in both groups of subjects who took trace elements, as compared with those who took placebo or vitamins alone. Other studies of zinc supplementation in older adults have demonstrated enhanced DTH responses, and many have shown enhanced lymphocyte numbers and function of natural killer cells (Prasad et al., 1993; Bogden et al., 1990; Bogden et al., 1994; Fortes et al., 1998; Girodon et al., 1997). Some studies have examined the effects of vitamin C (ascorbic acid) supplementation as adjunctive therapy for respiratory tract infections. One such study (Hunt et al., 1994) recruited hospitalized elderly patients with bronchitis or pneumonia to compare vitamin C (200 mg/day) with placebo. The results of the study demonstrated that supplementation of vitamin C rapidly increased plasma and cellular vitamin C levels and might have

slightly improved functional status in elderly patients. Many other studies have shown the effects of nutritional supplementation in malnourished patients in restoring their reduced immune response to infections. The addition of the deficient nutrients back to the diet can restore immune function and resistance to infection (Calder & Kew, 2002). Taken altogether, states of malnutrition and infection can aggravate each other and lead to a vicious circle (*reviewed by* Katona & Katona-Apte, 2008).

Early work on nutrition and immune functions was primarily based on the findings from studies in nutritional deficiencies in young children from developing countries (Chandra & Chandra, 1986; *reviewed by* Albers et al*.,* 2005). Much evidence today points to nutrition as an important determinant of immune functions across all age groups worldwide. Immunological dysfunctions may occur because of single nutrient deficiencies, such as of vitamin A, iron or zinc, or because of multiple nutrient deficiencies in conjunction with general malnutrition (Harbige et al*.,* 2004; Beisel et al*.,* 1981). A large body of research has documented the effects of macro- and micronutrient deficiencies on immune structure and function (*reviewed by* Katona & Katona-Apte, 2008; Chapman, 2006; Ahluwalia et al*.,* 2001). Cell-mediated immunity is particularly sensitive to deficiencies in macronutrients (*reviewed by* Ames, 2006). Thymic volume declines dramatically; T lymphocytes circulate in reduced numbers; proliferative responsiveness is attenuated; and delayed-type hypersensitivity is suppressed (Ahluwalia et al*.,* 2001; Ryan et al*.,* 1992). In contrast, humoral-mediated immunity is relatively buffered: B lymphocytes remain within the normal range (except with severe under-nutrition), and immunoglobulin concentrations are in many cases elevated, possibly reflecting higher levels of pathogen exposure in malnourished individuals (Ryan et al*.,* 1992).

1.7. AGING, MALNUTRITION AND IMMUNITY IN DEVELOPING COUNTRIES: PAKISTAN AS AN EXAMPLE

The experience in survey research on the nutritional and health status of the elderly in developing countries is quite meager (Solomons, 2000). The first-ever survey was probably the one conducted from Perth, West Australia by Andrews et al*.,* (1986) (*cited by* Solomons, 2000). This cross-sectional survey covered almost all Pacific region nations, i.e. Fiji, Malaysia, the Philippines and the Republic of Korea, including samples of individuals 65 years of age and older. Other surveys in South Africa (Charlton & Ferreira, 1997) and Guatemala (Herman et al*.,* 1998) provided data on nutritional and health status in less economically developed populations. The most recent and relatively comprehensive survey of elderly nutritional status is that of the Cross-Cultural Research on Nutrition in Older Subjects (CRONOS); with an acronym named for the ancient god of time (Gross et al*.,* 1997). This survey was conceived and developed by the IUNS Committee on urbanization and nutrition. Many of the techniques, measures and interview questionnaires included in this survey seem to be purposely homologous to those used in SENECA (Survey in Europe on Nutrition and the Elderly: a Concerted Action; *reviewed by de* Groot et al*.,* 2004). The initial pilot testing was conducted in Asia (China, Indonesia, Malaysia, Philippines, and Thailand) and Latin America (Brazil, Guatemala, and Mexico).To date, only incomplete reports

are available in the form of graduate student thesis projects of Indonesia (Jakarta), Philippines (Metro Manila), and Vietnam (Hanoi) groups. Within the limited sites and data sets analyzed in non-industrialized but developing nations, the general impression is that undernutrition is not rampant and that in some instances overnutrition prevails.

On the other hand, more than 800 million people have been reported to suffer from undernutrition, mostly in the developing world (FAO, 2006). Clearly, more investigations of increasingly representative samples of elderly populations in low-income societies are needed before the true pattern of nutrition in older adults in developing societies can be specified in a robust fashion (Solomons, 2004). Currently, more information needed to calculate the cost-benefit ratio of nutritional interventions in the elderly of less-developed countries is essentially unavailable (Meydani et al*.,* 2005). Therefore, as indicated by Meydani et al*.,* (2005) more data on the type and prevalence of infectious diseases among this population is needed. Second, information related to their nutritional status needs to be completed. And finally, the degree of effectiveness of nutrient interventions in reducing the burden of infectious disease in this unique population needs to be determined through region-specific clinical trials.

Pakistan is a developing country with some recent appreciable trends towards substantially higher economic growth rate. With increasing life expectancy across the world, Pakistan is also experiencing a rise in its elderly population. With meager health care resources and a poor understanding of aging, Pakistan faces many challenges in caring for its elderly population (Itrat et al*.,* 2007). Pakistan's rapid transformation from an agricultural to a modern industrial state, coupled with sharp demographic changes towards lower fertility rate and greater urbanization has many implications for the health and social support of the elderly. Unfortunately, like the rest of the developing world not much information is available on the prevalence of malnutrition and diseases associated with malnutrition in the Pakistani elderly. Most of the surveys (*e.g.,* NNS, 2001-2002; NHS, 1990-94) conducted in the past report only the prevalence of diseases or disease burden by whole populations rather than by age-specific populations. Furthermore, as rightly pointed out by Meydani et al*.,* (2005), generally even if age-specific statistics are reported, they are presented in terms of the world population or populations of very large regions. As others have already indicated (*e.g.* Coyne & Hilsenrath, 2001; Navarro, 2002; Warren, 1997; Diczfalusy, 1996), in order to develop an efficient health care system, it is essential to have quantitative estimates of the age-specific disease burdens specifically in the population so as to develop strategies for preventing and treating disease with known effective and low-cost interventions.

Unfortunately, there are no universal standards for anthropometric status, body composition and related general and immunological health conditions for the elderly in the developing world. Equations on anthropometrics and bioelectrical impedance have been mostly developed in Western populations, which are unlikely to be transferable to groups of elderly of diverse backgrounds, for example, those in the developing countries (Tolonen et al*.,* 1988; *cited by* Katherine et al*.,* 2011), a fact which necessitates developing age-associated and area- or region-specific standards. In the case of Pakistan, some interest in geriatric health has recently been evinced in the

medical community. Articles citing the health problems of the elderly have been published (Itrat et al., 2007; Zafar et al., 2006; Baig et al., 2000). These studies have highlighted some medical and social problems faced by the elderly and emphasized further work in this area. Given these shifts in demographics resulting in an ever-increasing population of elderly people, nutrition science has to be integrated with other sciences. For this, we need a clear understanding of the relationship between aging, nutrition and immunity. We need tools and methods which may help in establishing these relations.

We, therefore, designed this study in an effort to elucidate and identify differences in lymphocyte cell surface immune phenotypes in otherwise healthy obese and underweight elderly male individuals. Our objective was to determine if obesity and underweight in this particular age group has any association with alterations in the frequency of peripheral blood lymphocyte subsets in order to provide supporting evidence for the presence of an immune diathesis of obesity and underweight and to identify specific lymphocyte subsets as targets for further studies. An important objective was to establish whether the immune alterations characterizing immunosenescence were the same in a population considered old at 50 in Pakistan asin the better-characterized populations of developed nations where

CHAPTER 2

METHODS

2. METHODS

2.1. PRESENT INVESTIGATIONS

The forthcoming sections of the thesis present a summary of the study results on anthropometric measurements and nutrient intake and their relationship with the selected plasma biochemicals and immunological parameters (T and B cells and their subsets). A short description of the methods is given in Chapter2. The results are compiled in the manuscripts attached, and are briefly discussed in Chapter3, while Chapter4 provides a short discussion of the results of the study. The strengths and weaknesses of the current study are discussed in Chapter5.

2.2. AIMS

The overall goal of the work reported in this thesis was to investigate the relationship between nutritional status and immune functions in elderly free-living subjects in a developing country.

1. To report the overall prevalence of malnutrition (*underweight and obesity*) in free-living elderly subjects.

2. To investigate the relationship between nutritional status and immune functions.

3. To study the effects of aging on their immune parameters.

4. To elucidate interactions between age and malnutrition influencing immune signatures

5. To compare age-associated immune signatures in elderly Pakistani men with WEIRD controls

2.3. BRIEF DESCRIPTION AND METHODOLOGIES

The details of methods of the study are described in papers **I, II, III, IV and V**. However, a brief overview is presented in the forthcoming section. Paper **VI** provides a detailed review of literature on the relationship between nutrition and immunity in the context of human aging. The study was conducted on subjects recruited in Peshawar. A convenience sampling strategy was adapted given the limitations of time and resources available (**Papers I and II**). In the first phase, a survey was conducted to assess the dietary intake and anthropometric measurements of the elderly (**Paper I**). In the second phase, a sub-sample from those interviewed previously was selected for collection of blood samples to be used for selected plasma biochemical and immunological analyses (**Papers II, IV,** and **V**). Methodologies for dealing with sampling in the field and freezing whole blood are dealt with in detail in **Paper III. Paper VI** provides a detailed overview of the literature review.

2.4. STUDY SITE & SAMPLE DESCRIPTION

Clinically healthy subjects were included when they had no history of disease and were not taking any drugs on a chronic basis (**Papers I and II**). In accordance with the Declaration of Helsinki (Benatar, 2004), after a clear explanation of the study protocol, all subjects gave written informed consent to participate. The study was approved by the Board of Studies, Department of Human Nutrition and the Research Ethics Committee of the Agriculture University, Peshawar.

Our first objective was to investigate the prevalence of malnutrition in free-living elderly men. We randomly selected a convenience sample of 548 elderly men for our cross-sectional survey. The selection procedure was as follows:

For recruitment of the subjects, city registration data and voter lists were obtained from the local NADRA (National Database and Registration Authorities) office in Peshawar. From these lists, addresses of subjects fulfilling the age criteria for the study were obtained. The subjects were contacted by phone or in person. The study purpose was explained to them. A final list of those who agreed to participate was developed. Initially, a total of 745 elderly were willing to participate in the study. However, collection of anthropometric data on only 548 and dietary data on 525 elderly could be completed. This drop-out from the study at various time points was mostly due to death of some of the elderly or their address change to other towns.

2.5. DATA COLLECTION

2.5.1. Anthropometry

Anthropometric measurements were carried out with subject barefoot, wearing light clothing, and after an overnight fast. Body weight, height, percent body fat (%BF) were measured and recorded in a questioner (**Appendix1**). Data on general and health characteristics were recorded **(Appendix2)**. Body mass index (BMI) was calculated as body weight divided by the height squared (Kg/m^2) (**Papers I and II**). We also divided the study subjects in various risk categories for developing obesity related diseases **(Paper II)**.

2.5.2. Dietary

Habitual dietary intake was assessed through 24-hr Dietary Recalls (24-hr DRs). Each item of food eaten during the previous 24 hrs was recalled by the subject and recorded in a questionnaire (**Appendix3**). The amount of food eaten was used for estimation of nutrient intake. Accepted food composition tables for Pakistan were used for this estimation (Hussain, 1985) (**Papers I and II**).

2.5.3. Blood Sampling and Analysis

In the second part of the study, 100 subjects (50 each young and the elderly) were selected. They were interviewed again for their dietary intake. Blood samples from these 100 subjects were collected, which were later used for analysis of plasma concentrations of selected clinical chemistry factors (albumin, total protein, triglycerides, CRP, ferritin) (**Paper II**) and for assessment of immune status (**Papers IV and V**). The number of CD4, CD8, B cells and their subsets were analyzed for the assessment of immune status. As we used frozen whole blood for these analyses, we developed a protocol to work on whole frozen blood before the actual analyses on these 100 blood samples (**Paper III**).

CHAPTER 3

RESULTS

3. RESULTS

3.1. PREVALENCE OF MALNUTRITION

Section 3.1 presents a summary of the results of **Paper I.** Briefly, the current study was conducted in Peshawar, Pakistan. Subjects for the current study were locals of the area and belonged to low-to-middle socioeconomic backgrounds. More than half (51%) were illiterate and relatively a high number (82%) depended on others for their livelihood and lived with their families. They had self-reported low to moderate physical activity.

Considering BMI as a standard, there were 13.1, 3.1, and 10.8% obese, overweight and underweight elderly, respectively. While based on WC and WHR, respectively, there were 7.7 and 1.7; and 43.5 and 9.5% obese and overweight elderly subjects. Overall, the data indicate a high prevalence of obese and overweight elderly subjects defined by any of the three criteria i.e. BMI, WC or WHR. Also, there were a high number (10.8%) of underweight elderly. All these results show relatively high prevalence of malnutrition in this less well-studied elderly population of Pakistan. These results also show that either BMI or WC alone may underestimate the prevalence of obesity in the elderly and therefore, WHR may be used as it is a more sensitive indicator for estimation of obesity and/or overweight In addition, these results also suggest that in the elderly, central or abdominal obesity (assessed by WC and/or WHR) may be more prevalent than general obesity (assessed by BMI).

The results show most of the overweight and/or obese elderly (defined either by BMI, WC, and WHR) were in the age group of 60.1–70 yr. Based on BMI, WC and WHR, 8.6, 4.9, and 29.2% elderly were either overweight or obese in this age category. The other age category with the second highest % prevalence of obesity and/or overweight was 70.1–80 yr. The prevalence of WHR-defined obesity was the highest (23.2%) in the age group 60.1–70 yr. These data suggest that elderly individuals in the age range of 60.1–70 were more likely to become obese or overweight. Onward this age limit, there might be a steady decrease in weight (primarily in lean body mass; LBM), which may mask the increasing fat mass associated with aging. Therefore, BMI only as a measure of obesity and/or overweight may underestimate body fatness and hence WC and WHR should also be used in conjunction with BMI.

Nutrient intake, in general, was reported to be very poor in the elderly subjects **(Paper I)**. The overall number of elderly individuals with adequate energy intake was 67.5%. In obese and overweight categories, respectively, 100 and 84% of the elderly had adequate energy intake, while very few in those two categories had adequate protein intake. Similarly, in the normal weight and underweight BMI categories, adequate energy and protein intake were reported for 64 and 22, and 47 and 17%, respectively. Similarly, for minerals and vitamins, even less than 45% of the elderly in obese, overweight and underweight categories had an adequate intake of Ca, Fe, Zn, vitamin A and vitamin C. As expected, the % of normal weight elderly with adequate intake for these nutrients was higher than either of the other BMI categories. These data suggest

that only a small number of elderly in the study area had adequate nutrient intake, which is truly a state of concern as far as the nutritional health and well-being of this segment of population is concerned.

There were great variations in the nutrient intake across various BMI categories. Only very few elderly had adequate protein intake in the four BMI categories: 25, 21, 47, and 17% of the obese, overweight, normal weight and underweight elderly, respectively, with an overall of 27.5% having adequate protein intake. This implies that a large proportion (72.5%) of the elderly had inadequate protein intake. The current study also documents a high prevalence of imbalance intake of important minerals and vitamins. The % number of elderly in the four BMI categories with adequate Ca, Fe, Zn, vitamin A and vitamin C intake ranged from 21–58% for Ca; 31–61% for Fe; 25–69% for Zn; 13 – 59% for vitamin A and 28 – 82 % for vitamin C. However, the overall percentages of elderly with adequate intake of these nutrients were only 37, 43, 41, 30, and 47%, respectively.

In short, these results suggest a high prevalence of malnutrition (either under- or over-nutrition) among the elderly subjects. The energy intake by the obese and overweight was higher than the recommended but they had proportionally very suboptimal intake of other important nutrients, including protein, minerals and vitamins. On the other hand, underweight elderly had lower than the recommended energy intake as well as very sub-optimal mineral and vitamin intake. Even those elderly with normal BMI had an unbalanced nutrient intake and we are tempted to suppose that these individuals may show clinical signs of malnutrition in the near future.

3.2. COMPARISON OF ANTHROPOMETRY, BIOCHEMICAL VARIABLES AND NUTRIENT INTAKE BETWEEN YOUNG AND ELDERLY

The results of our cross sectional survey (**Paper I:** presented in section 3.1) raised an important question: whether malnutrition is restricted to the elderly only or it is a general phenomenon in the study area? There is evidence that the health and social outcomes of the elderly are greatly influenced by their households in most Asian societies (Knodel & Debavalya, 2006; Alberts et al., 1990), implying the likelihood that young and elderly in the same household might have almost the same health and nutritional status. These similarities or differences in health and nutritional status have immense clinical as well as health-care policy implications. We therefore, selected 50 families of the elderly out of those already interviewed (**Paper I**). From each family, we selected one young (age 18-28 yrs) family member (**Paper II**). Young subjects were included in order to investigate and compare the nutritional status of young with the elderly living in the same household in order to get information on possible differences or similarities in nutrient intake, selected biochemicals (**Paper II**) and immunological parameters (**Papers IV and V**).

The results (**Paper II**) show that the young had higher mean BMI and WHR, while the elderly had higher weight, WC and %BF. However, except for %BF, neither of these differences had statistical significance. Regarding the nutrient intake, significant differences existed between young and the elderly; young had significantly higher

intake of energy, protein, fat, fiber, Ca, P, Fe, Zn, vitamin C, and thiamin. Concerning the concentrations of selected plasma biochemicals, only CRP level was significantly higher in the elderly than the young.

The study also reports large differences of anthropometrics, nutrient intake and plasma biochemicals in young and elderly individuals of the same BMI categories. Across all BMI matched pairs between young and the elderly, WC and %BF differed significantly but not WHR; energy intake differed significantly; protein intake differed significantly across the all BMI matched pairs except between NW young and NW elderly. Of particular note, significant differences were observed only for albumin (UW young *vs.* UW elderly), CRP (OB young *vs*. OB elderly) and ferritin (NW young *vs.* NW elderly). These large variations in anthropometric measurements, nutrient intake and to some extent concentrations of plasma biochemicals across the matched BMI pairs of young and the elderly may suggest differential aging effects on these parameters.

Some recent investigations have reported links between high levels of central adiposity in adults (as measured by waist circumference and/or waist-to-hip ratio), and higher risk of developing obesity-related disorders including type-2 diabetes, hypertension and heart disease, demonstrating that measures of central adiposity (*i.e.* WC and WHR) are independent predictors of future obesity-related diseases (Roger et al., 2008; Cozamanis, 2006). Therefore, we divided the study subjects in various risk categories relative to normal weight and waist circumference. For the purpose of the present study, for WC the risk categories were defined as: low risk (LR, WC; <89 cm), moderate risk (MR, WC; 90–99 cm) and high risk (HR, WC; ≥100 cm). For WHR, the risk categories were defined as low risk (LR, WHR; < 0.89), moderate risk (MR, WHR; 0.90–0.94) and high risk (HR, WHR; ≥ 0.95) (17–19) (Roger et al., 2008; Cozamanis, 2006; Welborn et al.,2003).The results (**Paper II**) show relatively higher number of elderly placed either in any of the high risk (HR) categories based on WC or WHR (HR-WC or HR-WHR). Furthermore, 24% of young obese and/or overweight were in the moderate risk (MR-WC) category; only 2% were in HR-WC category, while 44% of young obese and/or overweight were in HR-WHR category. In the elderly, 10% of obese and/or overweight were in HR-WC category. None of the normal weight young or elderly had a high WC and hence were at low risk (either LR-WC or LR-WHR) category.

With respect to body fat, 79.2% of the young obese and/or overweight had high body fat, while 100% of overweight and/or obese elderly had high body fat (HF-%BF of >25.1%). Also, 64.3% elderly with the normal BMI had high body fat (HF-%BF of >25.1%). All young subjects with normal BMI had healthy/normal body fat (10–20%). These results suggest that beyond the upper normal BMI threshold, an increase in BMI may be an indication of increased WC, WHR and %BF. These results further suggest that the elderly have increased chances of central obesity at higher than normal BMI and even at normal BMI.

The results **(Paper II)** on clinical chemistry show few differences in the young and the elderly. Obese elderly had relatively higher CRP levels as compared to other BMI

categories of the elderly and the young. This may suggest an increasing trend in CRP with aging and obesity. There were little differences of no statistical significance in the rest of bio-chemicals between the young and the elderly as well as across various BMI categories of the young and the elderly. There was a significant increase in %BF and CRP and a decrease in energy intake with increasing age. BMI also decreased with age but this decrease lacked any statistical significance.

From these results (**Paper II**), we conclude that the elderly had relatively poor nutritional status as compared to the young living in the same household. Great variations existed in WC, WHR, %BF and nutrient intake within different BMI categories of the young and the elderly.

3.3. FLOW CYTOMETRIC LYMPHOCYTE SUBSET ANALYSIS USING FROZEN WHOLE BLOOD

We collected blood samples from the young and the elderly for biochemical and flow cytometry analyses. These samples were frozen at –80^{o}C and were analyzed for biochemical factors (**Paper II**), and T and B cells and their subsets (**Papers IV and V**). Before analysis on these samples, however, a small pilot study (**Paper III**) was conducted in order to establish a protocol for T cell phenotyping using whole blood (WB) frozen at –80^{o}C stored for a short period (10 days) or relatively long period (120 days) to assess potential deterioration over time, systematically compared with the standard method of carrying out all such analysis on cryopreserved PBMC.

The results showed no significant differences in the frequencies of CD4+ and CD8+ T cells in the WB samples compared to PBMC (stored 10 days). The frequencies of CD4+ and CD8+ T cells within the recovered CD3+ T cells were in perfect agreement with values obtained using isolated frozen PBMC. This indicates that frozen whole blood can be used to identify parameters such as the CD4/CD8 ratio. We also tested whether not only CD4+ and CD8+ frequencies were maintained within CD3+ T cells but other subsets could be identified in frozen WB as with PBMC. Naïve and memory cells were distinguished using a range of surface markers including CD27, CCR7, CD57, CD28, and PD-1. The *p-values* for comparisons between conventionally cryopreserved PBMC and frozen whole blood were in the range of 0.074–0.998 for CD4+ T cells and their subsets, indicating that the differences between CD4+ subsets analyzed in these samples were not significant. Similarly for the comparison of CD8+ T cells and subsets analyzed, none of the slight differences observed were statistically significant (*p-value,* 0.093– 0.993). Nonetheless, although the percentages of the subsets were very similar no matter how the samples had been processed and frozen, the density of expression of some surface molecules did vary.

The results (**Paper III**) suggest that frozen WB may be used to evaluate T cell subset frequencies when logistic matters do not allow PBMC separation following good laboratory practice. Further, the results show that red blood cell lysis of the thawed WB has no effect (positive or negative) on the percentage of T cells analyzed, so including this steps useful for minimizing the time required for flow cytometric measurement. While some markers may be affected by freezing conditions, most

appeared to be very reliable (including CD28 and CD27) and are thus recommended for use on frozen WB samples.

3.4. IMMUNE CELL NUMBER AND NUTRITIONAL STATUS

We elucidated and identified differences in phenotypes in otherwise healthy obese (OB), overweight (OW) and underweight (UW) young and elderly men (**Paper IV**) and compared them with those of normal weight (NW). Obese and UW individuals may present with a difference in their immune profile as compared to the NW individuals. Our objectives were to determine if the elderly compared to the young and malnourished (OB and UW) compared to well-nourished (NW) individuals differ regarding in the frequency of peripheral blood lymphocyte subsets, in order to provide supporting evidence for the presence of an immune diathesis of obesity and underweight and to identify specific lymphocyte subsets to focus on in further studies.

The four BMI categories of young were compared for immune signatures within CD4+ and CD8+ T cell compartments. The results show significant (p<0.05) differences in percentages of CD4+ T cells between NW and OB young; in CD45RA+CD27+ cells between NW and OB between NW and OW and between NW and UW young; in CD45RA-CD27- cells between NW and OW and NW and UW young; in CD28-CD27-KLRG1+CD57+ cells between NW and UW young. In the CD8+ compartment, significant differences (p<0.05) were found only in the percentage of CD8+CD45RO-CD27+ cells between NW and UW young; in CD8+CD45RO+CD28- cells between NW and OW young; and in CD28+CD27+KLRG1-CD57- cells between NW and OW young. Differences in the percentage of other subsets of CD4+ and CD8+ cells among the three BMI categories (OB, OW, and UW) *vs.* NW were not significant (p for all trends ≥0.05). In the elderly, the frequencies of all other phenotypes within the CD4+ subset between the three BMI categories vs. NW did not differ significantly (p for all trends ≥0.05) except CD45RA+CD27+ (p=0.0303), and CD45RO-CD28+ (p=0.0093) cells in OB vs. NW. In the elderly, the percentages of CD8+ cells in UW were significantly lower than in the NW (p=0.0443), while the differences in the frequencies of subsets within CD8+ among the three BMI categories vs. NW of the elderly were not significant (p for all trends ≥0.05). The elderly tended to have more B cells, more IgD-CD27+ B cells, and NKT cells than the young (p, for all trends ≥0.05). Comparing CD4+, CD8+ and B cells of young and the elderly men in the same BMI category suggests some differences. CD4+ cells differed significantly only between UW young *vs.* UW elderly (p=0.0003); the UW young had more CD8+ cells than the UW elderly (mean: 22.9 vs. 37.6 %). The differences in CD8+ cells were significantly (p=0.002) more CD8+ in OB elderly than OB young (Means: 25.9 vs. 16.0 %); and significantly more in the NW elderly than the NW young (mean: 27.8 vs. 21.2; p=0.0005). There was only a tendency for altered percentages of B cells between any BMI matched category of young and the elderly (p for all trends≥0.05).

While BMI and %BF had no effect on the percentage CD8+ cells (p for all trends ≥ 0.05), these were inversely correlated with CD4+ cells (p= 0.0223; and 0.021, respectively). None of the nutrients correlated significantly with CD8+, CD4+ or their subsets (p for all trends ≥ 0.05). There was a significant increase in both CD8+ (p=

0.0001) and CD4+ (p=0.001) cells with an increase in plasma CRP. Other plasma factors measured (albumin, total protein, triglycerides, and ferritin) had no significant effect on CD8+ and CD4+ counts (p, for all trends ≥ 0.05).

According to the results (**Paper V**), the young had a higher percentage of CD4+ T cells, while the elderly had significantly ($p<0.0001$) more CD8+ cells. The present study also found that the elderly in general had significantly ($p<0.05$) lower CD4:CD8 ratios; fewer CD8+CD28+CD27+,CD8+CD45RA+CD27+, CD8+CD45RA+CD28+, and CD4+CD28+CD27+ cells, but significantly ($p<0.05$) more CD8+CD45RA-CD27-,CD8+CD45RO-CD28+,CD8+CD45RA-CD28-, CD4+CD45RO+CD27-, CD4+CD45RA-CD28- cells. Similarly, the elderly had significantly ($p<0.05$) more CD8+CD28-CD27-KLRG1+CD57+ and CD4+CD28-CD27-KLRG1+CD57+ cells compared to young.

These results are broadly similar to those seen in industrialized countries. We conclude that there are differences in the immune signatures of the young and the elderly that immune profiles in younger and older men in a developing country seem broadly similar to the more extensively-documented state reported in industrialized countries, despite the marked societal, nutritional and many other differences. Hence, from these results (**Papers IV and V**), it may be concluded that the elderly and young have different immune signatures in rural Pakistan, as in other localities. Furthermore, comparisons between the three BMI categories of malnourished (i.e. OB, OW, and UW) versus the well-nourished (*i.e.,* NW) in the young and the elderly regarding their immune phenotypes indicate significant differences, particularly in the young and in the CD4+ compartment. The results of this study support the common notion that malnutrition (both obesity and underweight) markedly affects cells of the immune system. The risk of having serious health complications due to impaired immunity may be relatively higher in malnourished subjects at old age than those with healthy weight.

Chapter 4

DISCUSSION

4. DISCUSSION

4.1. PARTICIPATION TURN-OUT

This study was carried out in Peshawar, NWFP Pakistan. The study aimed to find the predictors of relationships between nutritional status, nutrient intake and immune functions of free-living elderly in a developing country where "old" is 10-20 years younger than in the West. The indicators for nutritional status used in this study were weight, BMI, WC, WHR, %BF and some selected plasma biochemicals (*albumin, total protein, triglycerides, CRP, ferritin*). The percentages and types of T and B cells were used as indicators of immune status of the subjects.

Initially, a total of 745 elderly provided their consent to participate in the study. However, collection of anthropometric data on only 548 and dietary data on 525 elderly could be completed. For blood collection, 100 subjects (50 each young and elderly) were selected. The young group was included for comparison with the elderly with respect to their nutritional status, body composition and immune status. Since subjects in poor health are often not able and also not willing to participate, selectivity in favor of subjects in better health can hardly be avoided in studies with elderly people (Scroll et al., 1991). The same holds true for poorly-educated persons (Gunzelmann et al., 1997). The participation turnover of elderly in our study was, however, very satisfactory; relatively a small number (25.4%) of elderly dropped out from the study; mostly due to their death or change of address during the study period (**Paper I**).

4.2. ANTHROPOMETRY

In general, the anthropometric measurements showed differences in various anthropometric parameters between the BMI-matched young and the elderly, thus documenting the age-related changes in anthropometric measurements independent of BMI **(Paper II)**. Based on BMI, we identified a substantial number of elderly as obese (3.1%) and overweight (10.8%). Also, there was relatively higher number (13.1%) of underweight elderly (**Paper I**). These are alarmingly higher numbers of prevalence of malnutrition in these elderly in otherwise medically healthy subjects and particularly with more relaxed BMI cut-off criteria. The current recommendations for the Asia-Pacific region (2000) define adult overweight at BMI ≥23 and obesity at BMI ≥25. There is growing support for the use of lower BMI cutoffs for Asians, especially given their propensity to abdominal obesity (Seidell et al., 2000; Singh et al., 1996). If BMI values ≥25 and ≥23 had been used to define obesity and overweight, respectively, then there would have been more elderly classified as obese or overweight. However, we used the WHO BMI classification as there are still a large body of controversies of using the cut offs proposed for Asians (Seidell et al., 2000).

Data on malnutrition of the elderly is very fragmentary in Pakistan and other studies documenting the prevalence of obesity and overweight in the elderly seem essentially absent. There has been no nationwide study to document the prevalence of obesity in

other population groups either. Some small-scale local studies, however, reported very high to low rates of overweight and obesity in Pakistan. The National Health Survey (NHS) 1990-1994 revealed that 1% of the population in Pakistan was obese and 5% overweight (Sheikh & Hatcher, 2007; NHS, 1990-94). However, the DAP-WHO (1994-98) study found that 16% had a BMI <18.5, (underweight), 36.8% had a BMI between 18.5-24.9 (normal), 44.6% had a BMI 25-26.9 (overweight) and 17% were obese with a BMI of > 27 (Iqbal & Naz, 2005). Another study by Afridi et al., (2004) reported the prevalence of obesity as 7-8%. Similarly, the age-adjusted prevalence of BMI > or = 25 (overweight/obesity) was 13.5% for men and 14.1% for women (Shah et al., 2004). The prevalence of obesity and overweight in relatively educated segment of population in Pakistan has been reported to be 8 and 29.6%, respectively (Khan et al., 2003). Pappas et al., (2001) reported that the prevalence of obesity for adults aged 25 to 64 years, moving from low to middle to high socioeconomic status (and rounding to the nearest whole number): for rural areas, 9%, 15%, and 27%; for urban areas, 21%, 27%, and 42%. Obesity in terms of waist circumference has been found in 46–68% of the Pakistani population (Basit & Shera, 2008). The results of our study are comparable with these studies previously conducted in Pakistan. The differences in the rate of prevalence may be due to the size, age and locality of the samples used in these and our studies.

In the current study, there was a trend of increasing body fat with age (**Paper I)**. The change in prevalence of obesity and/or overweight with age in Pakistan has also been reported previously. Nanan (2002) reported that in Pakistan obesity and overweight increase with advancing age; peaking at 45 - 64 years for both men and women and then decreasing after 65 years. Another study by Shah et al.,(2004) reported that overweight/obesity increased with age (Shahet al., 2004). Our results (**Paper I)** also demonstrated that most of the overweight and/or obese elderly (defined either by BMI, WC, and WHR) were in the age group of 60.1–70 yr. This implies that it is likely to become malnourished (particularly obese and/or overweight) around at this point of age.

Malnutrition (both under-and overweight) is a general phenomenon; reports even from developed countries document high rate of malnutrition prevalence (*reviewed by* Chapman, 2006; Semba, 2004; Thurnham & Northrop-Clewes, 2004). In general, the incidence of malnutrition ranges from 12-50% among the hospitalized elderly population and from 23-60% among institutionalized older adults (Endoy, 2005). In some of the developed countries, the rate of malnutrition in hospitalized elderly has been reported to be up to 86.2 % (Table 1). However, we cannot compare our results with these findings as most of these studies report the prevalence of malnutrition in a mixed population of elderly (*i.e.,* free living, institutionalized, hospitalized etc.).

Table 1: Prevalence of Malnutrition in the elderly in different countries

Country	Malnutrition prevalence	Malnutrition Risk	References
Germany	20 %	65%	Hengstermann et al., (2008)
UK	17 %	17%	Harris et al., (2008)
Netherlands	-	30 %	Essed et al., (2007)
Finland	56.7 %	40.7 %	Suominen et al.,., (2007)
Brazil	21.7 %	21.7 %	Cabrera et al., (2007)
Mexico	18.6 %	50.5 %	Reyes et al.,(2007)
Sweden	58 – 64 %	-	Olofsson et al., (2007)
Switzerland	10.4 %	20.1 %	Venzin et al.,(2009)
Sweden	21.7 %	50 %	Elkan et al.,(2007)
Korea	21.9 %	59.2 %	Tsai et al.,(2007)
Germany	39.5 %	58 %	Hengstermann et al.,(2007)
France	17.9 %	41.2 %	Dion et al.,(2007)
S. Africa	53 %	50.4 %	Charlton et al.,(2007)
Germany	8.9 %	81.4 %	Norman et al.,(2007)
Italy	56 %	56 %	Ghisla et al.,(2007)
Israel	18.5 %	-	Feldblum et al.,(2007)
Finland	86.2 %	-	Pitkälä et al.,(2006)
Netherlands	17% - 11 %	-	Nijs et al.,(2006)
Turkey	9.5 %	56.2 %	Afsar et al.,(2006)
Japan	8.9 %	51.2 %	Izawa et al.,(2006)

4.3. DIET AND NUTRIENT INTAKE

As reported in the results (**Paper I**), fewer elderly had adequate nutrient intake. Energy intake seemed to be adequate (66.7-100% of the recommended intake) in 100, 84 and 64%, respectively of obese, overweight and normal weight elderly, but only in 22% of the underweight elderly. The overall number of elderly individuals with adequate energy intake was 67.5%, which means more than 33% were energy-deficient and had inadequate (<66.7% of the recommended intake) energy intake. Similarly, in almost all BMI categories, the elderly had lower intake of most of the nutrients. In a heterogeneous population with respect to different economic backgrounds, the dietary patterns and hence the nutrient intake differs even from individual to individual (Rickertsen, 1998).

In our study (**Paper II**), in almost all cases, the mean nutrient intake was greater for young than the BMI-matched elderly subjects. So far, a limited number of studies have been undertaken to document the nutritional status of the elderly living in developing countries. The Western Pacific studies (Andrews et al., 1986) described socio-cultural factors, but not nutritional factors of free-living elderly people living in Fiji, the Republic of Korea, Malaysia and the Philippines. Low BMI values in the Asian elderly population have been reported in the IUNS Study (Roche 1995). Wahlqvist et al., (1995) reported the food habits, lifestyles and health status among the aged in

developed and developing countries. They concluded that elderly people living in developing countries have an inadequate intake of macro- and micronutrients (*e.g.,* vitamin A, thiamin, riboflavin and vitamin C) and most of them are malnourished.

The prevalence of energy deficiency in Pakistan is not unexpected. Some studies conducted in the general populations in the recent past have reported higher rates of energy deficiency in almost all segments of population (Khattak et al*.,* 2008; Khattak & Ullah, 2006; Afridi et al*.,* 2004; NNS of Pakistan 2001-2002). The FAO (Food and Agriculture Organization) reported a general deficiency of energy intake in Pakistani population ranging from 34-67% of the masses (FAO, 1998). In our study **(Paper I)**, the mean energy intake (1593±296.3) was well below the reference value for the elderly. Energy intake often decreases in old age. This "*anorexia of aging*" has been discussed in several reviews (Morley, 2001; Elahi et al*.,* 1983). According to Elahi et al*.,* (1983), the decline in energy intake appears to be a pure effect of age, with no confounding cohort or time effects. Elderly individuals also seem to have a diminished capacity to compensate for temporary alterations of energy intake. While young men regain their initial weight after over- or underfeeding, the elderly remain at a higher or lower body weight (Morley, 1997). Moreover, the elderly seem to be less able to compensate for a caloric pre-load at a subsequent meal (Rolls et al*.,* 1995), and experience less hunger during dieting than young individuals (Moriguti et al*.,* 2000). The nutritional risk increases in the community-dwelling elderly who are sick, poor, homebound, and have limited access to medical care.

The current study reported very suboptimal protein intake by the elderly. Only a small number of elderly had an intake which reached the level of adequate intake (**Paper I**). Although requirements for protein in the elderly are still under debate (Morse et al*.,* 2001), it is quite safe to say that there was a high risk of protein deficiency in our study group of elderly. Similar trends in protein intake have been reported from studies of other groups of elderly people (McGandy et al*.,* 1986; Nicolas et al*.,* 2001). In addition, most of the recent studies suggest the current RDA for protein might not be adequate for older people to maintain skeletal muscle (Campbell et al.,2002). For the mature adult and the elderly, protein needs may be higher. In a 14 week study, 12 men and 17 women (age range 54-78 years) consumed the RDA for protein (0.8 g/kg/day) but were divided into one of three different groups; (1) sedentary (SED); (2) lower body resistance training; or (3) whole body resistance training. While the study suggested that the RDA of 0.8 g/kg/day was adequate for all subjects, there was a loss of fat-free mass in all three groups and a decrease in mid-thigh muscle area in the SED group, which led the researchers to question whether the RDA for protein was adequate for the older population (Campbell et al*.,* 2002).

The decrease in skeletal tissue mass that comes with aging means the protein stores ordinarily provided by skeletal muscle may not be adequate to meet the needs for protein synthesis, especially under conditions of stress. This makes dietary protein intake critically important in order to meet the body's essential needs (Mahan & Escott-Stump, 2004). Some studies suggest it is better to err on the side of excess protein in elderly individuals rather than too little (*reviewed by* Morse et al*.,* 2001; Campbell et al.,2001). Even though physical growth has ended, adults need to

consume enough protein to replace body nitrogen losses in order to maintain lean mass. Sources of protein loss include cells shed by the gastrointestinal tract and skin, body secretions and nitrogenous end products of body metabolism (Schlenker, 1998). Protein needs increase in the elderly due to a higher incidence of chronic diseases. Because of physiological stress and various metabolic changes, dietary nitrogen utilization is less efficient and there is an increase in nitrogen excretion in older individuals (Mahan & Escott-Stump, 2004). Due to decreased energy needs with age, it may be advisable for the mature adult to increase the proportion of total energy from protein to maintain nitrogen status without creating energy excess (Prothro, 1989).

Inadequate consumption of protein by the elderly may compromise the immune system and slow recovery from illness or surgery (Campbell et al., 1995; Castaneda et al., 1995). In a 9-week study, marginal protein intake (0.45 g/kg/day) led to impairment of immune function in the study subjects with losses in functional capacity (Castaneda et al., 1995). Protein energy malnutrition (PEM) is also considered a major prognostic factor for mortality in older adults (Sullivan et al., 1990).

The mean intake of selected minerals (Ca, Fe and Zn) in our study (**Paper I**) and those reported earlier for general Pakistani adults are not comparable. The mean intake of Ca, Fe and Zn by Pakistani adults have been recently reported, which are 325 mg/day, 31.9 mg/day and 13.6 mg/day, respectively (Akhtar, 2005). Our sample of elderly is at even higher risk of deficiency for these nutrients as compared to the general adult population in Pakistan as reported by Akhtar et al., (2005). The mean intake of these minerals have been reported for some other countries of the region *e.g.,* Bangladesh (Miah, 2000), India (Dang et al., 2001), Iran (IAE, 1992) and some developed countries (USA, UK, Canada, and Germany) (IAE, 1992). The mean Ca intake of the elderly in our study was higher than that in Bangladesh (294 mg/day) and India (360 mg/day) but lower than that in Iran (679 mg/day), USA (852 mg/day), UK (827 mg/day), Canada (875 mg/day) and Germany (822 mg/day). In general, although mean calcium, iron, and zinc intake in the present study seemed well within the intake range of most countries (477 to 895 mg), however, there were only fewer subjects with adequate intake of these nutrients was very low (**Paper I**). The deficiencies of minerals in the elderly diet can result in a number of physical and physiological problems. Minerals are essential micronutrients required for the proper functioning of the body. Lifetime calcium intake appears to be a factor in the incidence of osteoporosis in the elderly (Blumberg, 1997). In the presence of adequate vitamin D nutrition, it appears that calcium intake in the range of 800-1200 mg/day will have beneficial effects on bone mineral density. Other potential benefits of high calcium intakes, such as reduced blood pressure and decreased risk of colon cancer, may also be important health effects (Russell & Suter, 1999). The adequate intake reference value for Ca suggested by the Food and Nutrition Board is 1200 mg/day for those aged 51 years and above. This is an increase of 400 mg/day above the 1989 RDA. The iron deficiency seen in the elderly is due mainly to inadequate iron intake (Mueller & Burke, 1996). Adequate zinc intake is necessary to support many bodily functions, such as wound healing, growth, proper immune function, storage and release of insulin and proper development of sexual organs and bone (Wardlawa & Insel, 1993). Some studies

indicate that supplementation with RDA levels of zinc in older people is associated with improvements in immune responsiveness (Bogden et al., 1994).

The mean intake of selected vitamins (A and C) was well below the reference values (**Paper I**). The deficiency of vitamins in the daily diets of the elderly has great health impacts. Vitamins are vital for a number of physiological functions in human body. Best understood of the actions of vitamin A is its role in night vision. Although vitamin A is not distributed widely in foods, excess daily amounts can be stored in the body (Groff et al.,1995). Adequate vitamin C is thought to be effective in helping prevent certain cancers, as well as cataracts in the eye. It is also vital for the function of the immune system and for iron absorption (Wardlaw & Insel, 1993). The current RDA for vitamin C is 60 mg/day. Although vitamin C is abundant in many foods, intake in the elderly varies widely. Factors such as smoking, medication, and emotional and environmental stress all adversely affect vitamin C nutrition (Groff et al., 1995).

4.4. CORRELATION & CLINICAL CHEMISTRY ANALYSES

Our results (**Papers I and II**) show that age was positively and significantly correlated with %BF and plasma CRP but negatively and significantly correlated with energy intake. BMI decreased with age but this did not approach statistical significance (**PaperII**). These results are in agreement with previous studies on relationships between age and body fat (Kuk et al., 2009), age and CRP (Wong et al., 2001) and age and energy intake (Wong et al., 2001). A decrease in BMI with advancing age has also been reported (Endoy, 2005). There are changes in body fat with advancing age. Age-related increases in body weight and fat mass and a decrease in lean body mass have been reported previously (Perissinotto et al., 2002). A number of studies have quantified the gain in adiposity; approximately doubling of body fat between 20 and 50 years of age. The Fels Longitudinal Study found that total body fat increases with age by 0.37 kg/year in men and by 0.41 kg/year in women. Thus, the percentage of body fat in the Fels Longitudinal Study was 23.6 % in men and 33.4 % in women at 40 years of age, reaching 29.3% and 37.8 %, respectively, at 60 years of age (Guo et al., 1999). Other studies have determined that fat increases at a rate ≥7.5 % per decade (Hughes et al., 2002), and that older subjects have a mean of fat tissue 7 kg higher than young (Piers et al., 1998).

4.5. IMMUNOLOGICAL STATUS

The effects of nutrition and aging on immune functions have been extensively investigated, but almost exclusively in the so-called “WEIRD” subjects (Western, educated, industrialized, rich, and democratic) and rarely in concert. It is not very well established whether immune alterations found in these populations are representative of the majority of the world’s peoples. The possible detrimental effects of aging on the immune system are further aggravated by numerous physiological and physical conditions, which in many cases can be associated with malnutrition.

In the present study (**Paper IV**), we investigated the effects of nutritional status on immune signatures in a group of healthy young and elderly individuals from a rural area of Pakistan; a developing country where nutritional issues tend to be even more extreme (both in terms of over-as well as under-weight) than in developed countries. The primary findings of this study (**Paper IV**) were that there were some significant differences in the subsets of CD4+ and CD8+ cells but only tendential differences in the percentages of B cells and NK cells between the young and the elderly men based on their nutritional status. While the current study observed changes in both CD4+ and CD8+ compartments, the impact of nutritional status on CD4+ cells and their subsets seemed to be more profound than on CD8+ cells and subsets. Some previous studies suggested that fat has a more direct effect on CD4+ T cell count, total lymphocyte count, and WBC count than on CD8+ T cells (Janeway et al., 2005). It has been reported that CD4+ subtypes are directly stimulated by various cytokines including tumor necrosis factor-α, which contributes to the differentiation of CD4+ T cells into the TH1 subset (Rudin & Barzilai, 2005) and leptin. In contrast, cytokines produced by fat tissue are not central to CD8+ activation. Therefore, whereas fat directly influences CD4+ cell counts via the action of various adipokines, it may influence CD8+ counts only indirectly via its ability to activate CD4+ T cells.

In contrast to our initial expectations, the current study did not identify many differences among the three BMI categories versus normal. We are not aware of concrete evidence from previous studies to support the present observations. Some conflicting results, however, demonstrated an increase in CD4+ cells and a decrease in CD8+ cells in obese people (based on BMI) (Cottam et al., 2004). Others have attempted similar studies of lymphocyte subset frequency in obesity, with conflicting results. Total circulating lymphocytes and monocytes were reported as increased (Nieman et al., 1999) or the same (Merritt et al., 1980) as in the lean controls. Lymphocyte subsets have also been studied, and some investigators have found no differences in numbers of circulating T-cells, B-cells, and NK cells (Kawashima & Watanabe, 1998), while others have shown increased or decreased lymphocytes and T-cells in obese people, and correlated the magnitude of these differences with increasing BMI (Caruso et al., 2009). Still other investigators have demonstrated a relationship between morbid obesity and CD8+ count only, but not mere overweight and/or obesity when compared with normal weight (Nieman et al., 1999). Despite these conflicting results, a preponderance of evidence suggests alterations in the immune system of both underweight and obese individuals. For example, monocyte function has been shown to alter in obese humans resulting in increased oxidative burst and phagocytic activity (Nieman et al., 1999). Such alterations in monocyte function could contribute to the state of systemic inflammation associated with obesity. T-cell phenotypes are likewise altered in obesity as reported previously (Nieman et al., 1999).

Nevertheless, our findings on the comparison between OB and NW subjects are in agreement with Lynch et al. (2009), who reported significantly more CD8+ and NK cells in lean controls compared to obese individuals. They further reported no differences in CD4+ levels between obese and lean individuals. In addition, they observed that the phenotypes of immune cells were also different between obese and

lean individuals with regard to expression of activation markers and that obese individuals expressed significantly less CD45RA on their T cells. However, when obese individuals were further divided into metabolically healthy (MH) and unhealthy (UH) groups, it was found that circulating NK cells and CD8+ T cell levels were significantly reduced only in the UH obese group. In our study, all the overweight and obese individuals seem more likely to be metabolically healthy as our inclusion criteria excluded all those who had a present or recent past history of diseases including diabetes, hypertension, CVD etc. We, therefore, need further studies in elderly Pakistani subjects including both metabolically healthy and unhealthy obese elderly individuals to investigate the differences as reported previously (Brochu et al., 2001). Others have suggested that the unique metabolically healthy subgroup of obese individuals appear to be protected or more resistant to the development of comorbidities associated with obesity (Brochu et al., 2001). Despite having excessive body fat, these individuals display a favorable metabolic profile characterized by high levels of insulin sensitivity, no hypertension, normal lipid, inflammation, and hormonal profiles and, importantly in the context of the present study, a favorable immune profile (Lynch et al., 2009).

In the present study (**Paper IV**), we divided young and the elderly subjects into high, medium and low risk categories on the basis of their WC and WHR values. The comparisons between the low risk (LR) and the other risk groups (high risk, HR; medium risk, MR) show that HR-WC young subjects had significantly (p=0.0145) lower percentages of CD8+ cells (p=0.014) and B cells (p=0.0201) compared to the LR-WC category. In the elderly subjects, there were also differences between these categories but none of them was significant (p for all trends $\geq$0.05). In the present study, the high body fat (HF-% BF) category in the young had significantly (p=0.0201) lower percentages of CD4+ cells compared to the normal fat (NF) category. In general, it has been suggested that a decrease in the lean body mass is related to the decrease in body cell mass (Niyongabo et al., 1997). Furthermore, these results may suggest that compared to the elderly, in young people, WC, WHR and %BF are more sensitive anthropometric measurements influencing the percentages of circulating CD4+, CD8+ and B cells.

No significant differences were found in the four BMI categories of young and the elderly regarding the number of B cells (**Paper IV**). In the IgD+CD27-, IgD-CD27+ and NK cells (CD16/CD56) significant differences were noted, respectively, between UW vs. NW; UW vs. NW and OB vs. NW elderly. Previous research reporting differences in these cellular phenotypes in the well- and malnourished elderly is scarce. Some studies on the relationship between weight and NK cell number in other age groups, however, have shown a close but conflicting link between body weight and NK cells. As an example, Kelley et al., (1994) reported that individuals who reported losing weight had fewer and less effective NK cells than those who had never lost weight. Likewise, Scanga & co-workers (1998) pointed out that obese women consuming a restricted diet had apparent decrements in NK cell cytotoxicity. However, another experimental trial indicates that obesity is related to lower T and B-cell mitogen-induced lymphocyte proliferation but normal numbers and function of NK

cells (Nieman et al., 1999). Again Lynch et al., have shown decreased NK cell levels only in metabolically unhealthy obese compared to a similarly obese but metabolically healthy group (Lynch et al.,2009). This study had also shown that the levels and phenotypes of NK cells in metabolically healthy obese were similar to lean healthy controls. This decrease in number of NK cells in the unhealthy obese suggests that the immune system is altered in obese people who are at risk from obesity-related comorbidities. In contrast, some other researchers have reported no differences between the percentages of B lymphocytes in malnourished versus well-nourished subjects (Bhaskaram, 1992). If obesity is considered an inflammatory disease (Das, 2001), then one might expect that NK cell number, or activity, should be increased. In contrast, results on adults suggest that NK cell number and activity may not be changed by obesity (Nieman et al., 1999). Thus, our results (**Paper IV**) showing no significant difference in NK cells between OB and NW young are in agreement with these previous findings. Taken together, all these studies confirm that malnutrition (whether obesity or underweight) has an effect on the circulating B and NK cells, particularly in the elderly.

Similarly, like malnutrition, the consequences of aging on immune functions have been extensively investigated, but almost exclusively in "WEIRD" subjects (Western, educated, industrialized, rich, and democratic). While it is very difficult to conclude whether age-associated immune changes found in these populations are the true representative of the majority of the world´s peoples or not, nevertheless, the conclusion of the previous studies is that even with healthy aging, the proportion of memory cells versus naïve cells increases. During aging, more memory cells are generated; the body has only a limited capacity of cells it can sustain and the immune system favors memory over naïve cells, which is the basis of adaptive immunity. These age-associated alterations and their consequences have been reviewed elsewhere by ourselves (Derhovanessian et al., 2008; Larbi et al., 2008, Pawelec et al., 1999) and many others.

The present study (**Paper V**) found a tendency towards the presence of lower percentages of CD4+ cells and reciprocally, highly significantly more CD8+ ($p<0.0001$) cells in the elderly compared to the young. There was also a significantly ($p=0.02$) lower CD4:CD8 ratio in the elderly than the young. In analogy to older Western (Swedish) populations, these values would put many Pakistani elderly in the high IRP group, which is found in a minority of people over 65 in Sweden, and which is known to become predictive of incipient mortality from 85 years of age (Wikby et al., 2005). The definition of "aged" in Pakistan is considerably lower than 65 yr. (Alam et al., 2011), and in parallel with this, possibly the IRP also occurs at an earlier age. Whether this is indeed predictive of mortality in this Pakistani population remains to be established in longitudinal follow-up studies.

Consistent with many previous studies, there was a reduction in CD28 expression by CD8+ T cells with ageing. This might be because of increased antigen exposures over time and lack of expression of CD28 on CD8+ memory cells. In a more extreme manner than usually reported in Western populations, however, there was also a decrease in CD4+ naïve T cells. This may be due to exposure to different

constellations of more pathogens, and is similar to what we have observed in Western Alzheimer patients, where Amyloid-β may be driving naïve CD4+ T cells to differentiate to memory cells (Larbi et al., 2009).

Consistent with this scenario established in Western populations, in the present study (**Paper V**), compared to the young, elderly people had more CD8+CD27-CD28-KLRG1+CD57+ ($p<0.001$) and CD4+CD27-CD28-KLRG1+CD57+ cells ($p<0.001$). It has previously been shown that older individuals have relatively higher frequencies of KLRG1+, CD57+ and CD28- cells in the peripheral blood in CD4+ and/or CD8+ T lymphocyte subsets compared to young individuals (Brzezinska, 2005; Koch et al., 2008). Cells with these phenotypes have been reported to be incapable of proliferation in response to antigenic stimulation (Ibegbu et al., 2005). A larger proportion of CD45RO+ cells has also been noted in this population in older individuals (Gabriel et al., 1993). It has previously been suggested that the KLRG1+CD57+ population is a senescent phenotype and the KLRG1+CD57- subset is a population of effector or central memory cells destined to become senescent (Ibegbu et al., 2005).

Although B and NK cells seem to be the least affected by aging as also demonstrated in the current study (non-significant differences in percent B cells, IgD-CD27+ and NK cells (Paper V), changes in the peripheral B cell number with aging have been reported in Western populations (e.g. Caruso et al., 2009) with reductions in naïve B cells and increases in memory cells (Weksler & Szabo, 2000; Gibson et al., 2009). However, discrepant results have been reported for memory B cells (Agematsu et al., 2000; Colonna-Romano et al., 2009).

In general, it seems that most or all of the differences between young and old Pakistani men that we have established here appear to be very similar to results from studies conducted in industrialized countries, although the impact on CD4+ as well as CD8+ T cells seems more notable (Derhovanessian et al., 2009) and there are more people in the IRP at a younger age. The data are consistent with chronologically earlier onset of immunosenescence in Pakistani men than in Western populations. They may thus represent true reflections of the impact of ageing on immunity, independently of a plethora of differences between the different populations tested, including nutritional and socioeconomic, as well as potentially genetic and psychological factors, but with different kinetics in different populations.

CHAPTER 5

STRENGTHS & LIMITATIONS

5. STRENGTHS & LIMITATIONS

There are several areas of potential strengths and weakness in our study design:

i. The major strength is the use of validated tools for anthropometric measurements and nutrient intake (**Papers I and II**) through thorough interview sessions and careful evaluation. Also, a method (**Paper III**) was especially developed to perform the immunological analyses on whole frozen blood which was all that could be collected under field conditions in this study.

ii. One of the major limitations includes the possibility that BMI cut-off points (**Paper I**) used in this study may understate health risk. The cut-off points are those recommended by the WHO. Although these have been proven to be fairly robust for classifying obesity across elderly populations, they are based primarily on the association between BMI and mortality in European and North American populations. As a small cross-sectional study, the present analysis is limited in its ability to elucidate causal relationships between risk factors and overweight. BMI can overestimate body fat in individuals who are very muscular and underestimate body fat in individuals who have lost muscle mass, such as many elderly. However, estimates from these potentially misclassified groups are expected to be reduced as we used other phenotypes for classification of obesity i.e. WC and WHR. Our previous experience with other parts of similar datasets gives us some confidence that data quality is sufficient for this type of study and that our results are deemed to provide useful additional evidence on the relationship between nutritional status and immune parameters of the elderly subjects.

iii. The sample (**Papers I, II, IV and V**) that we are using reflects the population of Peshawar, the central city of *Khyber Pakhtunkhwa* (KPK) province, and as such is largely of *Pashtune* ethnicity and middle-class. Thus we will not have sufficient power to look separately at race, ethnicity, cultural, or socio-economic effects. However, this is a population with which we have considerable experience in our previous studies, and can anticipate relatively trouble-free recruitment and follow-up (if needed in future).

iv. Our study (**Papers I, II, IV and V**) is restricted to the participants living in one relatively restricted geographic area of the country (Peshawar, *Khyber Pakhtunkhwa*). While this is appropriate in an initial study of this type, future studies should include sites with different geographical conditions and social backgrounds. The study cohort for the immunological assays was very small, and is more in the nature of a pilot study.

v. The present study (Papers I, II, IV and V) reports nutritional and immunological characteristics of men only. No women participated in the study because of some reasons of cultural constraints on the participation of female observed in the area. While gender differences in nutritional and immunological characteristics are important to be established, future studies should consider including female subjects as well.

vi. We could only report (Papers IV and V) on the number of T and B cells and their subsets in the young and the elderly but not on functional characteristics of these cells (e.g. intracellular cytokines, cell proliferation assays etc) due to our inability of conducting such analyses on whole frozen blood. These functional assays provide some mechanistic insight into the differences in immunological characteristics between the young and the elderly and must be considered in future studies.

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APPENDICES

APPENDIX1

ANTHROPOMETRY DATA RECORD®

RECORD FORM

Department of Human Nutrition
Faculty of Nutrition Sciences
NWFP University of Agriculture, Peshawar
NWFP-Pakistan

(Anthropometry)

Relationship Between Nutritional Status and Immune Functions in Elderly Pakistani Subjects.

Notice: The information on this for will be used only for research purpose and the identity of the subjects will be kept in strict confidence, will be used only for purposes stated for this study, and will not b disclosed or released to others.

Age	Sex	Examiner Name	Recorder Name
-------- Yrs ------- mons	-------M --------F	----------	---------- -- Date----------

1. Bioacromia breadth (cm) ---------- 2. Iliac Crest (cm)----------

Right Side ○ Left Side ○

3. Elbow Breadth(cm)----------
4. Triceps skinfold (cm)----------
5. Subscapular Skinfold (mm)----------
6. Illiac crest skinfold (mm)----------
7. Medial Calf Circumference(cm)------
8. Medial Calf skinfold (mm)----------

9. Chest Circumference (cm) ---------- 10. Abdominal Circumference (cm)----------
11. Waist (cm)..... 12. Hip (cm).....

13. Examinee right/left handed R------ L------ Both------- Not sure---

14. Weight (Kg) ----------

15. Sitting Height (cm)----------

16. Standing Height----------13. Knee Height.....

17. % Body Fat: BIA Reading.....

APPENDIX2

Health & Activity Form®

GENERAL QUESTIONAIR

Date .....

Name of Investigator.....

- ID_____
- Name_____
- Address _____ (permanent yes___No__)
- Age (yrs) _____ (according to NIC)
- No. of family members_____
- Sons_____daughters_____grandsons_____granddaughters_____others_____
- No. of family dependent on you_____
- How many brothers do you have?_____
- How many sisters do you have? _____
- Are you an ex-service man? Yes ---- No----

MARITAL STATUS

- Unmarried
- Married
- Divorced
- Widowed

LIVING SITUATION

- Alone
- With family

EDUCATION

- No schooling at all
- Basic/primary school
- Middle
- Matriculation
- SSC
- University
- Madrassa Basic

PROFESSION & INCOME

- Farming
- Shop-keeping
- Driving
- Masson

- Government servant
- Labor
- Do you have currently your own income? Yes_____ No_____
- If yes, how much per month_____ (Rs)
- Present income sources_____

HEALTH STATUS

- Dentition....Poor____good____

REMARKS:

- Using artificial teeth yes___ No___
- Teeth hygiene condition Good___Poor___

EYE-SIGHT

- Good___ poor___ REMARKS:
- Glasses yes____No___ REMARKS:

HEARING

- Good____poor____ REMARKS:

GI TRACT & DIGESTION

- Are you satisfied with your digestion? yes____No____
- Do you suffer very often from constipation yes_____No____
- How is your appetite? Good____poor____
- Chewing problems always_____with hard foods____
- Swallowing problems always_____sometimes____
- Problems cutting foods always_____ sometimes_____

CHRONIC DISEASES

- Have you suffered from any chronic disease in recent past Yes____ No ____
- If yes, which one_____ REMARKS:

MOBILITY

- Can easily walk for _____hrs daily.
- Can offer his prayers 5 times a day in the mosque. yes____ No.___
- Can join people in HUJRA yes_____ No_____

LIFE STYLE

- Sports yes____No____ REMARKS:
- Exercise yes____No___ REMARKS:
- Smoking yes____No___ REMARKS:

APPENDIX3

24-hr Dietary Recall (24-hr DR) Questionnaire (Summarized Version)

Instruction for 24-hour diet recall

What is it?

Data on household food consumption can be gathered by applying the 24-hour diet recall method during the monthly visits of the research student to the selected households' residences. Basically, it is a listing of all foods consumed by the household during the last 24 hours. This is done with the respondent in the household who is responsible for food preparation, or if that person is unavailable, another adult who was present in the household the previous day. The registration of food intake refers **to the household as a whole**, not to a single member of the household. The respondent should be instructed to also include the food prepared in the home for consumption by household members outside the home (e.g. at lunchtime in the fields). Likewise, foods consumed outside the home that were not prepared in the home should also be included.

How to do it?

To facilitate data registry, the research student can make use of a questionnaire format, as it is very important to ask all households the same questions. In the example section a possible questionnaire format is given. The format can be filled in following the next steps:

Step 1	After filling in the necessary ID-numbers of the interviewer, the interviewee plus the number of the week you are recording in, ask the interviewee the question 'Has any person in your household, in the past 24 hours, been eating any …', repeating this question one by one for each of the categories of food which are mentioned on the questionnaire format.
Step 2	Fill in the interviewee's response: yes (Y) or no (N), by marking the Y or the N.
Step 3	If the interviewee responds yes, ask what product(s) exactly was consumed, how much and whether it was bought, home-grown, bartered for or consumed outside of the household. Please be aware that a household could have consumed more than one different product within the same food group. The column 'Amount' has two sub-columns: one in which the amount in local measurements can be filled in, and the other in which the local measurements can be converted into standard volume measurements after the interview, such as for example kilograms. The conversion rates can be agreed upon during a focus group meeting during the preparation stage, if not yet available.

Questionnaire (summarized version)

Researcher ID # ☐

Household ID # ☐

Week # ☐

	Can you remember what you ate yesterday in breakfast, lunch, dinner, snacks? (Y/N).		If yes, indicate …			
				AMOUNT (TOTAL FOR WHOLE HOUSEHOLD)		
			WHAT	LOCA	STANDAR	SOURCE *)
A	bread, rice noodles, biscuits, or other foods made from millet, sorghum, maize, rice, wheat	Y / N	1. 2. 3.	1. 2. 3.	1. 2. 3.	1. 2. 3.
B	potatoes, yams, manioc, cassava or any other foods made from roots or tubers?	Y / N	1. 2. 3.	1. 2. 3.	1. 2. 3.	1. 2. 3.
C	vegetables?	Y / N	1. 2. 3.	1. 2. 3.	1. 2. 3.	1. 2. 3.
D	fruits?	Y / N	1. 2. 3.	1. 2. 3.	1. 2. 3.	1. 2. 3.
E	beef, pork, lamb, goat, rabbit wild game, chicken, duck, or other birds, liver, kidney, heart, or other organ meats?	Y / N	1. 2. 3.	1. 2. 3.	1. 2. 3.	1. 2. 3.
F	eggs?	Y / N	1. 2. 3.	1. 2. 3.	1. 2. 3.	1. 2. 3.
G	Fresh or dried meat, liver fish or shellfish?	Y / N	1. 2. 3.	1. 2. 3.	1. 2. 3.	1. 2. 3.
H	Foods made from beans, peas, lentils, or nuts?	Y / N	1. 2. 3.	1. 2. 3.	1. 2. 3.	1. 2. 3.
I	cheese, yogurt, milk or other milk products?	Y / N	1. 2. 3.	1. 2. 3.	1. 2. 3.	1. 2. 3.
J	foods made with oil, fat or butter?	Y / N	1. 2. 3.	1. 2. 3.	1. 2. 3.	1. 2. 3.
K	sugar or honey?	Y / N	1. 2. 3.	1. 2. 3.	1. 2. 3.	1. 2. 3.
L	other foods, such as condiments, coffee or tea?	Y / N	1. 2. 3.	1. 2. 3.	1. 2. 3.	1. 2. 3.

Remarks:

Curriculum Vitae

Iftikhar Alam

Assistant Professor, Agriculture (HUMAN NUTRITION

Bacha Khan University (BKU), Charsadda, KPK, Pakistan 25000

PERSONAL

- Date of Birth: 10-03-1974
- Place of Birth: Charsadda
- Nationality: Pakistani
- Marital Status: Married (Safia Beum (wife) and three daughters, Adiba , Zeba and Mihrun Nisa

ACADEMICS

- Ph.D. (scholar) at the Institute of Medical Research, Tuebingen University, Germany
- M.Sc (Hons.) Human Nutrition (A grade CGPA: 3.69; 83%)
- B.Sc. (Hons) Human Nutrition (A grade CGPA: 3.29; 75%)
- HSSC, FSc (Pre-Medical) - (71.2%) A-grade Ist Div
- Intermediate (Science) – (74%) A-grade, Ist Div

DISTINCTION

- Best Academic Performance Certificate of the year, **Edwards College Peshawar**
- Won **DAAD Fellowship Award** for PhD 2007-2010

SHORT-TERM COURSES TRAINING AND INTERNSHIPS

- **Child Care** (AIOU, Islamabad)
- **Food and Nutrition** (AIOU, Islamabad)
- **Applied Food and Nutrition** (AIOU, Islamabad)
- **Family Health and Care**(AIOU, Islamabad)
- **Awareness of Public About Special Needs of children**(AIOU, Islamabad)
- **Common Health Problems, Their prevention and Nutritional Therapy** (AIOU, Islamabad)

TRAININGS

- **Experts Training in Fortification**, Dubai UAE
- **Laboratory Bio-safety and Bio-security**, Bangkok, Thailand
- **Total Nutritional Therapy (TNT)** by Abbott, Pakistan
- **PCM & Logical Framework**, Asia Link Program, EU Karachi
- **Clinical Nutrition**, Paracha Hospital Peshawar
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CURRENTPOSITION & RESPONSIBILITIES

- Presently working as ***Lecturer and Junior Research Specialist (JRS)*** in the Department of Human Nutrition, teaching Immunology, Human Physiology, Laboratory Techniques and other nutrition subjects. Also responsible for the supervision of M.Sc. (Hons) research thesis and B.Sc. (Hons) internship students.

- Working on Relationship between **"Nutritional Status and immune functions of elderly Pakistani men"** for PhD with Tübingen Aging and Tumor Immunology group Sektion für Transplantationsimmunologie und Immunohämatologie at the University of Tübingen, Zentrum für Medizinische Forschung, Waldhörnlestraße 22, 72072Tübingen, Germany

RESEARCH AREAS & INTERESTS

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- Child & Maternal Nutrition
- Diabetes and Nutrition
- Laboratory Techniques & research Methodologies
- Enteral, Parenteral and Clinical Nutrition
- Nutrient to nutrient interaction
- Ethics in Nutrition and Food Composition

JOURNAL ARTICLES

RECENT NATIONAL & INTERNATIONAL PUBLICATIONS

- **Alam I,** Larbi A, Pawelec G**.** Nutritional status influences peripheral immune cell phenotypes in healthy men in rural Pakistan. *9:16 doi:10.1186/1742-4933-9-16***.**

- **Alam I,** Goldeck D, Larbi A, Pawelec G**.** Aging affects the number of T and B cells in a group of elderly in developing countries – a pilot study from Pakistan. *Age (Dordr.)* 2012 Jul 19. [Epub ahead of print].

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- **Iftikhar Alam** and Parvez Iqbal Paracha. 2009. **Caring for the Muslim patients-some religious issues**. Int. Med J. Vol. 8 No 1

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BOOK / PERIODICALS / VIDEOS

- **Iftikhar Alam** and Parvez Iqbal Paracha. 2009. Breastfeeding During Crises and Emergencies. Inc. Breastfeeding: Methods, Benefits to the infant and mother and difficulties. Nova Publishers, NY USA

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- **Iftikhar Alam**. 2007. Reaching the unreachable. (A 30 minutes video documentary on malnutrition: written, ^& produced by Alam I).

PAPERS CITED BY OTHER AUTHORS

- Four papers cited, a total of fifteen times in other Papers/Thesis/periodicals. Recent citation by Anderson et al (2010): Breast-feeding in a complex emergency: four linked cross-sectional Studies during the Bosnian conflict. *Public Health Nutrition: page 1 of 8*

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- **American Society of Nutrition**, USA
- **Feline Nutrition Education Society** (FNES), USA
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REFERENCES

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MANUSCRIPTS

All the manuscripts are given in chronological order.

NUTRITION JOURNAL

RESEARCH Open Access

Relationship between anthropometric variables and nutrient intake in apparently healthy male elderly individuals: A study from Pakistan

Iftikhar Alam[1,2*], Anis Larbi[3], Graham Pawelec[1] and Parvez I Paracha[4]

Abstract

Background: The elderly population is increasing worldwide, which warrants their nutritional status assessment more important. The present study was undertaken to establish the nutritional status of the least-studied elderly population in Pakistan.

Methods: This was a cross-sectional study with a sample of 526 generally healthy free-living elderly men (mean age: 68.9 yr; range: 50-98 yr) from Peshawar, Pakistan. Anthropometric measurements (weight, height, WC) were measured and BMI and WHR were calculated from these measurements following WHO standard procedures. Dietary intake was assessed by 24-hr dietary recall. Nutrients were calculated from the information on food intake. Nutrients in terms of % of RNI were calculated using WHO data on recommended intakes.

Results: Based on BMI, the numbers of obese, overweight and underweight elderly were 13.1, 3.1 and 10.8%, respectively. Age was negatively and significantly correlated with BMI ($p = 0.0028$). Energy ($p = 0.0564$) and protein intake ($p = 0.0776$) tended to decrease with age. There was a significant increase in % BF with age ($p = <0.0001$). The normal weight elderly had significantly ($p < 0.05$) higher intake of all nutrients studied, except energy which was significantly ($p < 0.05$) higher in obese and overweight elderly. Overall, however, the majority of subjects had lower than adequate nutrient intake (67.3 - 100% of recommendation).

Conclusions: Malnutrition is common in apparently healthy elderly Pakistani men. Very few elderly have adequate nutrient intake. Obese and overweight had higher % BF as compared to normal weight elderly. Older age is associated with changes not only in anthropometrics and body composition but also in intake of key nutrients like energy and protein.

Background

There has been a rapid increase in the number of elderly people in Pakistan [1] hence maintaining health and well-being of this age group is becoming even more important. Beside so many other health risks associated with old age, this population is potentially the most vulnerable group for malnutrition [2]. Poor dentition, neuropsychological problems and immobility in older age directly affect their nutritional status [3].

The prevalence of overweight and obesity is increasing [4], particularly in the elderly [5], where it is associated with increased mortality and a number of metabolic and cardiac disorders [6]. Overweight and obesity also contributes to functional decline and disability in the elderly [7]. At the same time, quite significant numbers of old individuals are reported to suffer from underweight and are at higher risk for acute illness and death [8]. They also have significantly higher risk of dying within the first year of hospitalization than those with adequate nutrition [9]. Weight loss has been shown to be associated with a higher risk of disability [10]. Decreased body Mass Index (BMI) is an indicator of chronic energy deficiency and malnutrition, and is associated with compromised immune function, increased

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susceptibility to infectious illnesses, and reduced survival in the elderly [6].

Similar to other developing countries, Pakistan can be expected to experience the impact of an increasingly ageing population over the next few decades [1], with a steady rise in the average life expectancy from 59.1 years in 1991 to 65 years in 2002. This quite sudden demographic shift can be very challenging in terms of health and nutritional care. Essential information about individuals' food intake and habits, activity, cultural influences, and the economic and social situation provide a database for nutritional assessment. Developed countries have established dedicated health care systems in order to meet the special needs of the elderly. However, such programs are lacking in developing countries like Pakistan. To the best of our knowledge, so far no separate study has been undertaken to document the nutritional status of the elderly in Pakistan and this type of important information thus remains fragmentary or absent. Those nutritional surveys that have been conducted in the past, however, do show very marginal nutritional status and high nutrient deficiencies in the general population (not specifically the aged) [1]. In this context of higher prevalence of malnutrition in general population in Pakistan, it can be assumed that the elderly might have an even more impaired nutritional status. The present study, therefore, aimed to investigate the nutritional status and nutrient intake of Pakistani elderly. The results are expected to help in designing policies and making plans regarding health care provision for the elderly in Pakistan. Nutritional status is particularly worrisome in the context of the ageing population, which is becoming a serious demographic problem. Hence, elucidating the nutritional status of the elderly is of prime importance for formulating preventive strategies to lower morbidity rates, improve quality of life and reduce health care costs.

Methods

Study site and sample selection

The current study is a cross-sectional survey using focused interviews, conducted during 2008-09 in Peshawar, Pakistan. Participants of the study were elderly men from Peshawar in the province of *Khyber Pakhtunkhwa* (previously, the North West Frontier Province: NWFP) of Pakistan. In order to increase representation of the elderly, subjects were selected randomly from eight different sites in Peshawar. Women were not included mainly due to cultural constraints of the area. Taking into account the limited resources and time available, the convenience sampling method was adopted; recruiting a final total of 526 elderly men defined as ≥50 years of age. For our present work, we defined elderly as individuals ≥50 years of age partially based on the arguments of Glascock and Feinman (1980) [11], which provide a basis for definition of old age in developing countries. It is recommended to use change in social role (*i.e.* change in work patterns, adult status of children and menopause) as a criterion for definition of old age. We adopted this criterion as we observed that in Pakistan (and particularly in our study area) this social change in the life span starts at the age of around 50 years. For recruitment of the elderly subjects, city registration data were obtained from the local office of NADRA (National Database and Registration Authorities) in Peshawar. Addresses of the elderly subjects, who fulfilled the age and health criteria for the study, were obtained from the lists provided by NADRA.

Data Collection

Data were collected by the first author assisted by trained graduate students of the Department of Human Nutrition, Agricultural University, Peshawar.

Age and Anthropometric Data

Age was assessed using official documents (the National Identity Card, NIC). Weight and height were measured and BMI was calculated as weight/height^2 (kg/m^2). Waist circumference (WC) and waist-to-hip ratio (WHR) are simple anthropometric indices for assessing the amount and distribution of body fat that can help in risk assessment for many health problems [12]. WC and HC (Hip Circumference) were measured according to the standard procedures reported in details elsewhere [13]. Briefly, WC was measured at the part of the trunk located midway between the lower costal margin (bottom of lower rib) and the iliac crest (top of pelvic bone) while the subject was standing with feet apart and weight equally distributed on each leg. The measurer (the first author) stood beside the individual and fitted a non-flexible tape snugly, without compressing any underlying soft tissues. The circumference was measured to the nearest 0.5 cm, at the end of a normal expiration. HC was measured with the same tape, placed around the point with the maximum circumference over the buttocks. The subject stood with feet fairly close together and weight equally distributed on each leg. The subject was asked to breathe normally and the reading of the measurement was taken at the end of normal expiration. The measuring tape was held firmly, ensuring its horizontal position. Due care was taken that the tape should be loose enough to allow the observer to place one finger between the tape and the subject's body.

Subjects were categorized into four groups as obese, overweight, normal weight and underweight based on their BMI values [2,4]. For assessment of central obesity, we used cut-off values of WC and WHR. Subjects with

WC of <94, 94-101.9 and ≥ 102.0 cm were classified as normal weight, overweight and obese, respectively [2,4]. WHR (waist to hip ratio) was calculated as: WC/HC and subjects with WHR values of <0.90, 0.90-0.99 and ≥1.0 were classified as normal weight, overweight and obese, respectively. WC and WHR are not used to define underweight [2,4].

Percent body fat (%BF) of each subject was measured by Futrex-5000 according to the procedures recommended by the manufacturer (Futrex®, Hagerstown MD, USA). The device emits near-infrared light into the body at very precise frequencies (938 nm and 948 nm) at which body fat absorbs the light and lean body mass reflects it. From the amount of light absorbed and emitted the device calculates % BF. The measurements were taken at the midpoint of each participant's dominant bicep.

Dietary Data

The dietary data were collected using 24-hr dietary recalls (24-hr DR) through face-to-face interviews conducted primarily in *Pashto*, the local language. These 24-hr DRs were repeated three times over the three alternative days of a week. No data, however, for Sunday (a weekly holiday in the study area) was collected. Because we observed in our pilot trial for validation of the 24-hr DR questionnaire that most of the subjects were away from homes for social reasons on Sunday and it was difficult for them to recall exactly what they had eaten when they were away. Nevertheless, this exclusion did not bias the results as our other analyses (data not shown) suggest that differences in nutrient intake over the weekend and weekdays were not significant in our study area, although some studies in other countries, for example the USA, have reported differences in nutrient intake over the weekdays and weekends [14]. During the 24-hr DR interviews, the intake reported by the subject was verified by someone in the household to avoid over- or under estimation of dietary intake because elderly might easily forget what they had eaten during the previous 24 hrs.

Household measures such as cups, bowls, and spoons were used to help estimate quantities of foods consumed. Quantities were recorded according to the amount of a particular bowl, for instance, 1/2 of the small brown bowl. When interviewees gave answers like, "I used a little or a lot of milk in tea", they were asked to show this with the cup they used, and the cup volume was later measured to estimate the amount. Nutrient intakes were computed using an in-house nutrient calculator (Microsoft Office Excel 2003, USA). This calculator is based on the data from food composition tables for Pakistan [15]. Mean and standard deviation (SD) of energy, protein, selected minerals (Ca, Fe, Zn) and vitamins (A and C) were determined from dietary intake data. The vitamins and minerals selected are those known to be important, particularly for the older population [16]. Reference Nutrient Intakes (RNI) of the World Health Organization/Food and Agriculture Organization (WHO/FAO) [17] were used because Pakistan has no nutrient recommendations of its own. The percentage of elderly with adequate nutrient intake was ascertained. Nutritional adequacy for each nutrient was calculated by comparing the actual intake with the recommended values for a nutrient. For most of the nutrients, recommendations are usually set about 30% above the average requirement in order to cover the need of almost all healthy people of the respective sex and age group [18]. For this reason, it has been customary to use a cut-off value of two-thirds (66.7%) of the recommended intake to estimate the proportion of a population with adequate intakes [18]. Therefore, adequate consumption was considered to be 66.7-100% of the RNI for a particular nutrient.

Statistical Analysis

All anthropometric measurements were made in duplicate and the means of paired values were used in the analyses. The data were statistically analyzed using JMP (Version 7.0. SAS, USA). As the current study involved four BMI categories, the means of nutrient intake in these four BMI categories (*i.e.* obese, overweight, normal weight and underweight) were taken for one-way analysis of variance (ANOVA), and post-hoc comparisons with Dennett's test taking the normal weight group as reference. BMI-adjusted partial correlation coefficients were calculated to establish associations between anthropometric measurements and nutrient intake. The resulting *p*-values demonstrate significance or lack thereof. The cut-off points used were: $p \geq 0.05$ is a non-significant difference and $p < 0.05$, a significant difference.

The current study was approved by the Board of Studies, Department of Human Nutrition, Agricultural University Peshawar. Written informed consents were obtained from all the participants before the start of study.

Results and Discussion

The present study included only apparently healthy individuals with no recent past or present smoking or any other drug addiction history. Table 1 shows general and socio-demographic characteristics of the study subjects. Table 1 also shows % number of elderly in four BMI categories and mean (SD) % BF of elderly in these BMI categories. As evident, more than half (51%) of study subjects were illiterate and relatively a high number (82%) were living with their families. Based on BMI,

Table 1 General and anthropometric characteristics of the study subjects

Mean age (yrs)	68.9 (8.80); Range: 50 - 98 yr
Education (% number of subjects)	
Primary	24
High	8
Others (non-conventional)[1]	17
Illiterate	*51*
% number of economically active[2]	41
% number living with families	82
% number whose wives had died	48
% number in four BMI groups [3]	
≥ 30	13.1%
24.9 - 29.9	3.1%
18 - 24.9	73.0%
<18	10.8%
Mean (SD) % BF in four BMI groups	
Obese	38.4 (7.21)
Overweight	32.2 (5.18)
Normal Weight	25.6 (5.52)
Underweight	15.1 (6.41)

[1]Non-conventional refers to the particular education system imparted in local *Madrassas* (the religious education system in Pakistan).[2]Economically active refers here to an engagement in a job or service for earning purpose.[3]BMI categories as per WHO (2003)

there were 13.1, 3.1, and 10.8% obese, overweight and underweight elderly, respectively. The mean (SD) % BF ranged from 15.5 (6.41) to 38.4(7.21), respectively in the underweight and obese elderly.

Table 2 shows % number of overweight and obese elderly defined by BMI, WC and WHR. Most of the overweight and/or obese elderly defined by any of these three criteria were in the age group of 60.1 - 70 yr. Based on BMI, WC and WHR, 8.6, 4.9, and 29.2% elderly were either overweight or obese in this age category; the highest as compared to other age categories. The other age category with the second highest percent prevalence of obesity and/or overweight was 70.1-80 yr. The prevalence of WHR-defined obesity was the highest

Table 2 Percent of overweight (OW) and obesity (OB) by body mass index (BMI), waist circumference (WC) and waist-hip ratio (WHR) cut-offs

Age (yrs)	N	BMI		WC		WHR	
		OW	OB	OW	OB	OW	OB
50-60	59	0.7	0	1.3	0.2	4.7	1.1
60.1-70	260	6.2	2.4	3.8	1.1	23.2	6
70.1-80	154	3.1	0.9	1.5	0.4	9.3	1.5
80.1-90	65	0.4	0	0.7	0	4.7	0.7
>90	7	0.2	0	0.4	0	1.6	0.2
Overall	526	10.6	3.3	7.7	1.7	43.5	9.5

BMI = Body Mass Index; WC = Waist Circumference; WHR = Waist to hip ratio; OW = Overweight; OB = Obese

(23.2%) in the age group 60.1 - 70 yr. Furthermore, in all age groups WHR gave the highest prevalence of obesity followed by BMI- and WC-defined obesity. These results show that either BMI or WC alone may underestimate the prevalence of obesity in elderly and, therefore, WHR may be a stronger and more sensitive indicator for estimation of obesity and/or overweight in epidemiological studies. These results further show that in elderly central or abdominal obesity (assessed by WC or WHR) may be more prevalent than general obesity (assessed by BMI).

Table 3 presents the mean daily intake of selected nutrients by elderly stratified by BMI groups. There were large differences in nutrient intake comparing all the three groups (*i.e.* obese, overweight and underweight) to the normal weight group. Obese and overweight elderly seemed to be consuming significantly ($p < 0.0001$) more energy than people of normal weight but significantly less protein, calcium, iron, vitamins A and C. Further, the results show that underweight elderly had significantly lower mean intake of all nutrients studied as compared to the normal weight elderly (p value ranged from 0.0001 - 0.0006).

The % number of elderly with adequate nutrient intake in each BMI category is depicted in Figure 1. Overall, very few elderly had adequate energy and protein intake. In obese and overweight categories, 100 and 84% of the elderly had adequate energy intake, while very few people in those two categories had adequate protein intake. Similarly, in the normal weight and underweight BMI categories, adequate energy and protein intake were reported for 64 and 22, and 47 and 17%, respectively. Similarly, for minerals and vitamins, even lesser than 45% of the elderly in obese, overweight and underweight categories had an adequate intake of Ca, Fe, Zn, vitamin A and vitamin C. As expected, the percentage of normal weight elderly with adequate intake for these nutrients was higher than either of the other BMI categories.

One encouraging fact was that the participation rate in this study was fairly high (73.6%). Because subjects in poor health are often not able and also not willing to participate, selectivity in favor of subjects in better health can hardly be avoided in studies involving the elderly. The same holds true for poorly-educated persons [19].

The nutritional assessment of free-living elderly in Pakistan in the present study has demonstrated the need to promote a healthy lifestyle in this population. BMI, WC, WHR, and % BF measurements showed that most of the elderly people had abnormal nutritional status with very high energy intake in the obese category and inadequately lower energy intake in the rest of the BMI categories. The need for the elderly to improve their nutritional status and balance their dietary intake has

Table 3 Mean (SD) of nutrient intake in four BMI categories

Nutrients	Obese (OB)	Over-weight (OW)	Normal weight (NW)	Under-weight (UW)	p-value[1]		
					OB-NW	*OW-NW*	*UW-NW*
Energy (Kcal)	2266 (312.2)	2058 (219.5)	1651 (311)	817 (312)	<0.0001	<0.0001	<0.0001
Protein (g)	41.8 (6.68)	42.3 (6.79)	43.4 (6.41)	27.0 (7.06)	0.002	0.0421	<0.0001
Fiber (g)	6.8 (1.62)	7.6 (2.06)	9.4 (1.60)	3.5 (1.14)	0.0481	0.0041	<0.0001
Calcium (mg)	342.4 (79.1)	392.2 (91.6)	451.4 (111.1)	270 (83.1)	<0.0001	0.0052	<0.0001
Iron (mg)	11.2 (2.48)	12.7 (3.5)	13.1 (2.81)	7.2 (2.90)	0.0139	0.0139	<0.0001
Zinc (mg)	7.3 (1.31)	7.2 (1.7)	7.5 (1.58)	4.4 (1.18)	0.1421	0.0411	<0.0001
Vit A (RE)	283.6 (97.2)	298.3 (113.1)	314.9 (194)	219 (106.5)	0.0439	0.0501	0.0006
Vit C (mg)	32.3 (17.3)	25.9 (13.7)	44.4 (12.3)	14.2 (8.16)	0.0431	0.0411	<0.0001

[1]. p-values were calculated using Dennett's test in JMP. The normal weight castigatory was considered as reference. Alpha value for significance was 0.05

been a long-standing topic of discussion among nutritionists. Many studies have associated higher energy intake with obesity and overweight and lower energy intake with body decomposition, which may result in a decreased DNA repair capability, lower plasma glucose levels, diminished insulin sensitivity and overall unhealthy lifespan [6,19].

In current study, all the anthropometric variables were included on the basis of their association with food habits, health and well-being in the elderly [20]. Weight reflects the recent and present balance between energy utilization [21]. Height/stature reflects genetic potential and nutritional status during growth and is also related to fat-free or lean body mass, which is a good index of

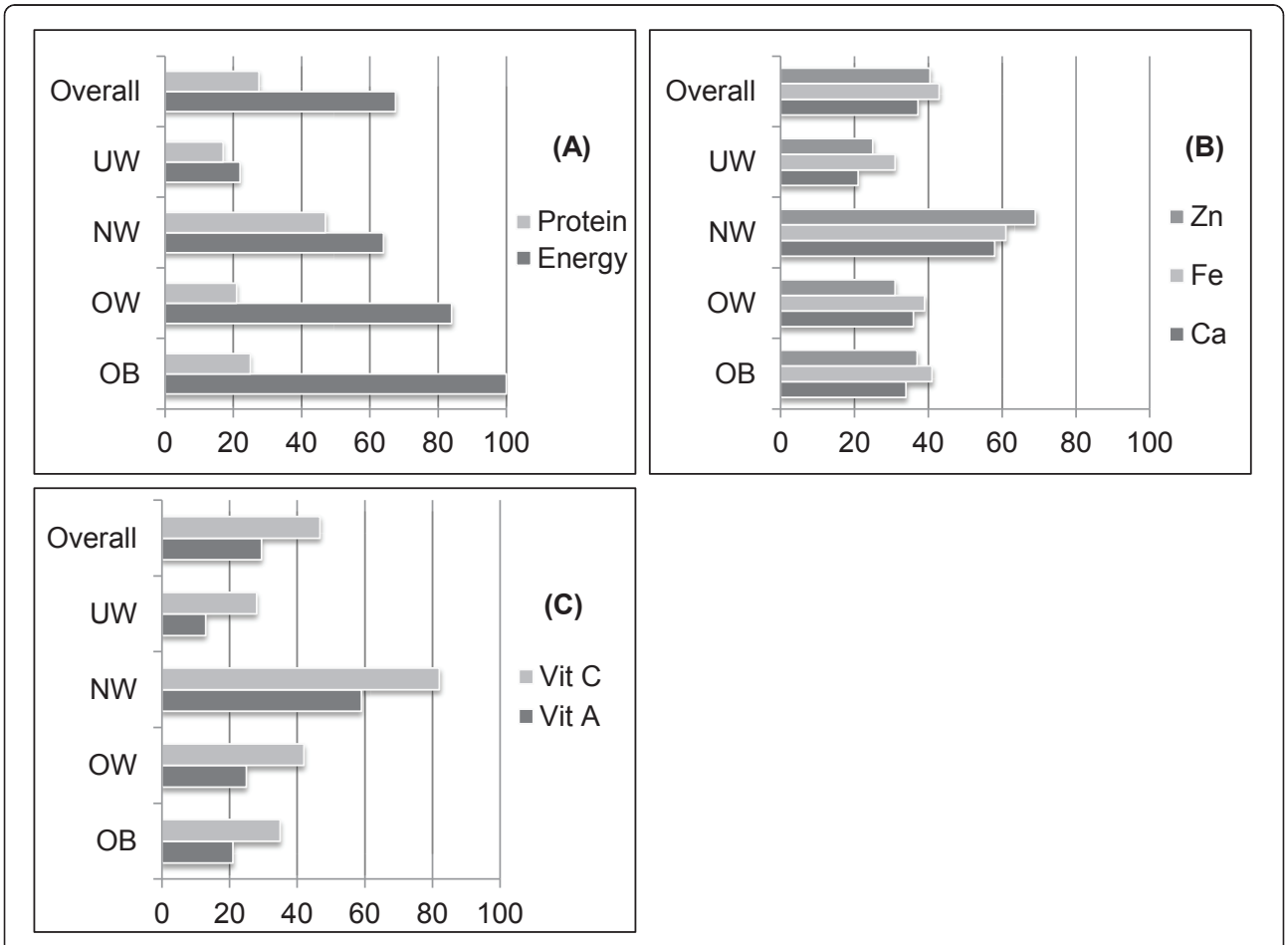

Figure 1 Percent (%) Number of elderly in four BMI categories with adequate intake of nutrients. The adequate intake is defined as intake 67.3 - 100% of the recommended intake

protein stores [22]. BMI calculated from weight and height [23] is related to percentage of body fat and to fat-free mass, while WC and HC are useful indices of adipose tissue and central obesity [24].

The present study highlights an alarmingly high prevalence of overweight, obese and underweight even in relatively healthy and wealthy Pakistani elderly men, measured either by BMI, WC or WHR. In particular, very high numbers (43.6%) of elderly were found to be either overweight or obese assessed by WHR (Table 2), which is especially important in view of the fact that Asian adults have higher cardiovascular risk factors already at lower BMI and WC than Western populations [16]. These arguments may support the fact that alone BMI is not enough to determine the risk of developing obesity-related conditions. Excess abdominal fat, regardless of overall body fat, will predispose to obesity-related disease. This highlights the importance of measuring WHR. It is possible that two persons with very similar BMI may vary substantially in the proportion of abdominal fat. Accordingly, a person with a BMI in the "normal" weight range may exceed the safe range of abdominal fat. In aged individuals with a decline in lean muscle mass, their BMI may not change or may even decrease, but fat levels could increase with the accompanying redistribution of body fat. WHR and WC are useful and reliable measures of abdominal obesity but both of them have their individual strengths and weaknesses and both are usually measured in a clinical evaluation.

In addition, BMI has also been criticized for its poor discrimination between fat and muscle mass. Thus, those individuals who are overweight not because of an increased amount of body fat, may have a high BMI value, but should not be considered obese. There are data indicating that even though BMI is a reliable measure of fatness in children and young individuals [25], an adolescent's percentage of fat can change by as much as -3 to +7% without any difference in BMI. For an individual adult, the same BMI can correspond to changes in fat of ±5% [26]. Additionally, BMI seems to have a reduced applicability to the elderly [27]. For this very reason, WC and WHR are used for better discrimination of obesity, particularly the central or abdominal obesity [24,26,28]. However, all these anthropometric measurements have certain limitations [29] and therefore, cannot be used in isolation to predict results.

Data on nutritional status of elderly is also very fragmentary in Pakistan. Other studies documenting the prevalence of obesity and overweight in the elderly seem essentially absent. There has been no nationwide study to document the prevalence of obesity in the other population groups either. Some small-scale local studies, however, reported variable rates of overweight and obesity in Pakistan [30]. Higher prevalence of obesity and/or overweight in Pakistani population with increasing age has also been reported previously [30,31]. The results of these studies are in close agreement with ours, finding the highest mean measurements of BMI, WC and WHR in the elderly age group of 60.1-70 yr. The difference in prevalence as reported by the current and the previous studies might be mainly due to difference of age of the sample, sample size and sample characteristics.

In current study, we found fewer elderly had adequate nutrient intakes (Figure 1). Energy intake seemed to be adequate (66.7-100% of the recommended intake) in 100, 84 and 64%, respectively of obese, overweight and normal weight elderly, but only in 22% of the underweight elderly. The overall number of elderly individuals with adequate energy intake was 67.5%, which means more than 33% were energy-deficient and had inadequate (<66.7% of the recommended intake) energy intake.

The prevalence of energy deficiency in Pakistan is not unexpected [32], particularly in the elderly [33]. If BMI < 18.5 kg/m^2 is used as an indicator of chronic energy deficiency in the elderly [34], prevalence of chronic energy deficiency as high as 13.1% is reported in the current study. Low BMI values in relation to low energy intake in Asian elderly populations have also been reported in the IUNS Study [35]. Even in developed countries, data show a high prevalence of energy deficiency in the elderly [36]. Lower energy intake causes body decomposition [18]. On the other hand, due to problems with mastication and poor dentition [33,37], elderly prefer caloric-dense foods with proportionally limited amounts of other necessary nutrients, which might be a contributing factor to age-related obesity and deficient intake of other important nutrients.

In current study, protein intake in all four BMI categories seemed to be inadequate (Table 2). Only very few elderly had adequate (66.7-100% of the recommendation) protein intake in the four BMI categories (Figure 1A): 25, 21, 47, and 17% of the obese, overweight, normal weight and underweight elderly, respectively, with an overall of 27.5%, had adequate intake. This implies that a large proportion (72.5%) of the elderly had inadequate (<66.7% of the recommendation) protein intake. Requirements for protein in the elderly are still under debate [31]; but it is quite safe to say that there was a high risk of protein deficiency in our study group of the elderly.

The % number of elderly in the four BMI categories with adequate Ca, Fe, Zn (Figure 1B) and vitamin A and vitamin C (Figure 1C) intake ranged from 21 - 58% for Ca; 31 - 61% for Fe; 25 - 69% for Zn; 13 - 59% for vitamin A and 28 - 82% for vitamin C. However, the overall numbers of elderly with adequate intake of these

nutrients were only 37, 43, 41, 30, and 47%, respectively. To the best of our knowledge, there have been no separate data on the intake of these nutrients by Pakistani elderly. However it has been reported that mean intake of Ca, Fe and Zn by adults in the general Pakistani population is much lower than the recommendations [38]. Mean calcium, iron, and zinc intake in the present study seemed well within the intake range of most countries [39]. However, the % number of subjects with adequate intake of these nutrients was very low.

It is also noteworthy that most nutrients consumed by the elderly in the present study were derived from plant sources (data not shown). This intake pattern is similar to that in many other developing countries [40], which may be one of the reasons for deficiencies in certain nutrients in this age group. For example, phytates present in whole-grain breads, cereals, legumes and other plant foods bind zinc and inhibit its absorption [41]. Factors found mainly in plant foods including phosphorus, flavonoids, oxalates and soy protein can also inhibit iron absorption and decrease its bioavailability [42].

The correlation analyses (Figure 2) show that with increasing age there was a significant decrease in BMI (p = 0.0028; r = -0.1304). Energy (p = 0.0564; r = -0.1236) and protein intake (p = 0.0776; r = -0.0771) tended to decrease with age but not significantly, while a non-significant increase in WC (p = 0.3124; r = 0.0422) and significant increase in % BF (p = <0.0001; r = 0.3655) with age were noted. Unlike WC, WHR decreased with age. However, this decrease was not

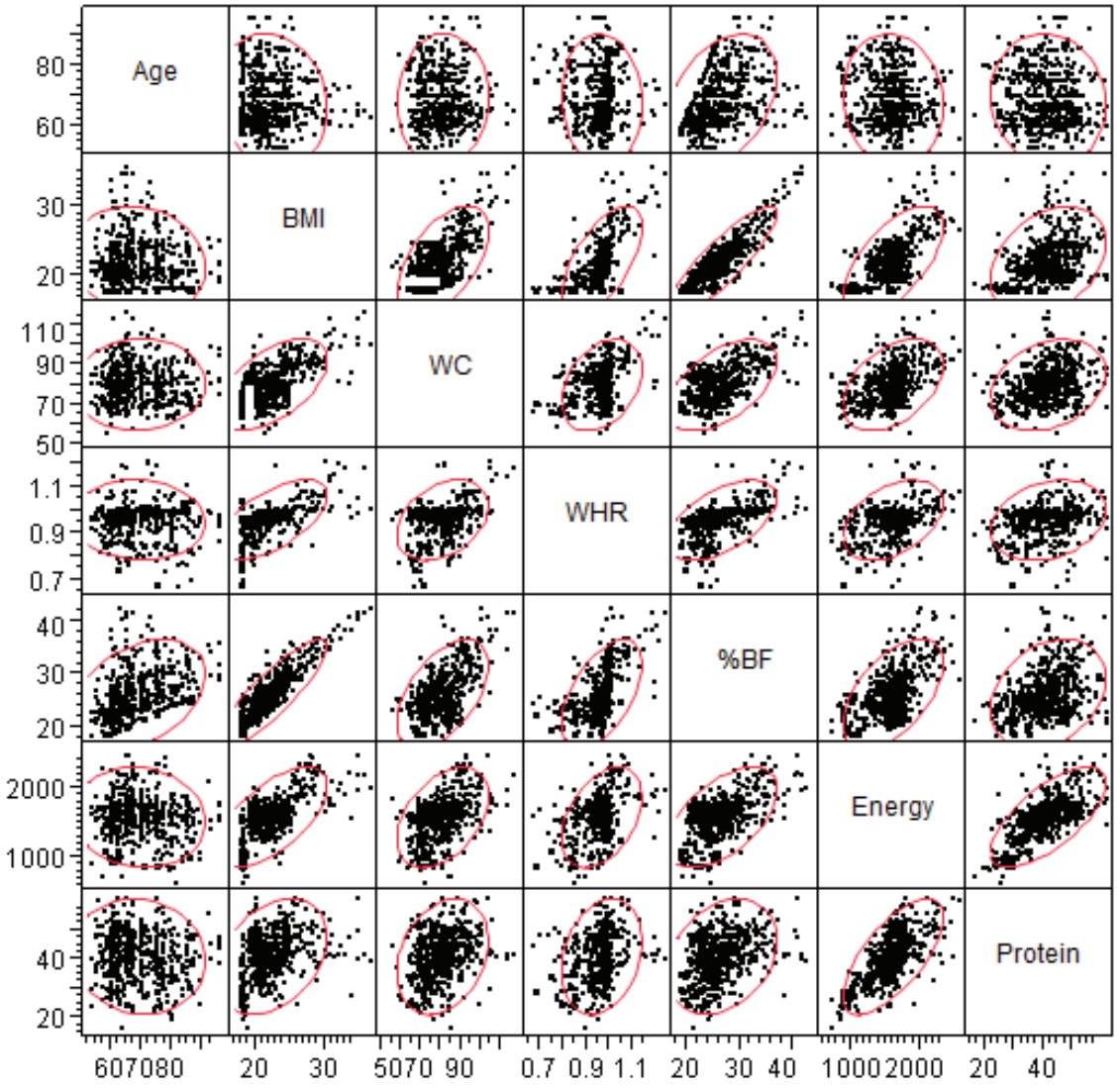

Figure 2 Correlation Matrix. The correlation analysis was performed for age, anthropometric measurements (BMI, WC, WHR,), %BF, energy and protein. The alpha level of significance is 0.05.

significant statistically (p = 0.1220; r = -0.0675). Studies show a decrease in BMI with age, particularly after 60 yr [43,44], an increase in fat mass [45] and a decrease in energy intake [36]. However, these changes are very variable [43-45]. Nevertheless, all these associations of selected anthropometric measurements and nutrients with age are important from the aging and nutrition point of view as an understanding of the underlying factors affecting body composition may facilitate correction by simple nutritional interventions. An increase in body fat with aging may be partly attributed to a loss in muscle mass, even in independently-living healthy subjects [27]. Furthermore, skeletal muscle mass loss in men is masked by weight stability, resulting from a corresponding increase in total body fat mass. Progression of sarcopenia, particularly in men, may therefore be clinically silent and comparable to the loss of bone mineral density in osteoporosis [27].

In conclusion, there is a high prevalence of underweight, overweight and obesity in elderly Pakistani men. We report a limitation of prediction made either by BMI, WC or WHR alone as a measure of overweight and obesity, based on our results and the published literature. The nutritional data demonstrated that majority of subjects had a suboptimal nutrient intake. We propose that the current BMI-based categories be reviewed for the Pakistani population, particularly for the elderly. Furthermore, we suggest that BMI, WC and WHR should be used in combination to define nutritional status. In addition, we suggest that attention should also be paid to the problem of underweight in old age.

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Authors' contributions

IA and GP designed research; IA, and PIP conducted research and collected the data; IA and AL analyzed the data; IA wrote the manuscript; Critical revision of the manuscript for important intellectual content was the responsibility of IA, AL and GP. IA had full access to all the data in the study and takes full responsibility for the integrity of the data and the accuracy of the analysis. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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A COMPARISON OF ANTHROPOMETRICS, BIOCHEMICAL VARIABLES AND NUTRIENT INTAKE BETWEEN YOUNG AND ELDERLY RURAL PAKISTANI MEN

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Abstract: Aging health is associated with nutritional changes which are not well understood or investigated in developing countries, and were therefore evaluated in this study by comparing the nutritional status of elderly with young subjects in Peshawar, Pakistan. *Subjects:* The participants in this study were young and elderly men (n=50 each), represented by each of the four BMI categories (obese, overweight, normal weight, underweight). *Methods:* Anthropometrics (height, weight, body mass index (BMI), percent body fat (%BF) were measured; nutrient intake was assessed by 24 hr Dietary Recall (24-hr DR); clinical chemistry variables (albumin, total protein, triglycerides, CRP, ferritin) in plasma were analyzed on a Modular Analytics SWA automated analyzer. *Results:* Our results show no significant differences in mean weight, waist circumference (WC) and waist to hip ratio (WHR) between young and elderly ($p \geq 0.005$). Mean %BF of elderly was significantly ($p=0.02$) higher than young. Of the sample, 10% and 34%, respectively, of the elderly fall either in high risk categories of WC (HR-WC) or WHR (HR-WHR). Intake of almost all nutrients studied was significantly higher in young compared to elderly ($p < 0.005$). There were differences in plasma factors but only the mean plasma CRP level was significantly higher in the elderly ($p=0.0376$). With increasing age, there was a significant increase in % BF and CRP ($p=0.0160$ and 0.0222, respectively) but decrease in energy intake ($p= 0.0001$). BMI decreased with age but not significantly ($p=0.5821$). *Conclusions:* The elderly had relatively poor nutritional status as compared to the young. Great variations existed in WC, WHR, %BF and nutrient intake within different BMI categories of young and elderly. These results suggest almost the same poor nutritional status of elderly as reported in most developed and developing countries.

Key words: Nutritional status, elderly, anthropometry, body fat.

Introduction

The numbers of elderly people, potentially the most vulnerable group for malnutrition (1), are increasing in developing countries like Pakistan (2, 3). Poor dentition (2), neuropsychological problems and decreased mobility at older age directly affect nutritional status (4). Poor health and disability (5) are linked to nutritional risk indicators, which often lead to poor nutritional status in old age.

The prevalence of malnutrition (both under- and overnutrition) is increasing in many countries in the elderly population (6, 7). Obesity is associated with increased mortality, metabolic and cardiac disorders (8) and contributes to functional decline and disability in the elderly (9). At the same time, a significant number of older individuals are reported to suffer from underweight (10) and to be at higher risk for acute illness and death.

Anthropometry and clinical chemistry variables are good indicators of nutritional status (11). Body mass index (BMI), waist circumference (WC) and waist-to-hip ratio (WHR) are simple anthropometric indices for assessing the amount and distribution of body fat (12-14), which additionally can help in risk assessment for many health problems. Low BMI is indicative of chronic energy deficiency and malnutrition, and is associated with compromised immune function, increased susceptibility to infections, and reduced survival among the elderly (8). In addition, there are well-documented links between adiposity, measured by WC and/or WHR, and the risks

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of obesity-related conditions including type-II diabetes, hypertension and coronary heart disease (CVD). These links may remain intact even once BMI is adjusted for age and other variables, demonstrating that measures of central adiposity are independent predictors of future obesity-related diseases (15-18). It is customary to categorize individuals in various risk categories for these diseases based on WC as: low risk (WC<89 cm), moderate risk (WC=90–99 cm) and high risk (WC≥100 cm). For WHR, these risk categories are usually defined as: low risk (WHR< 0.89), moderate risk (WHR= 0.90 – 0.94) and high risk (WHR ≥ 0.95) (17, 18).

Similar to other developing countries, Pakistan can be expected to experience the impact of an increasingly aged population over the next few decades. Essential information about peoples' food intake and habits, activity, cultural influences, and the socioeconomic situation provide a fundamental base for nutritional assessment. Our main objective in the present study was to investigate the overall nutritional status of elderly people in rural Pakistan. We were also interested to compare nutritional status of these elderly with young from the same family in order to ascertain whether malnutrition runs in families within the same socioeconomic background.

Materials and methods

Study Site and Sample Selection

For the current study we used a sub-sample from our previous study conducted during 2008-09 in Peshawar, Pakistan (15). We selected a convenient sample of 50 families and from each family we selected one young and one elderly subject fulfilling the inclusion criteria. Clinically healthy subjects were included when they had no history of disease and were not regularly taking any drugs. Based on their BMI values (1), young and elderly subjects fell into one of four BMI categories i.e., obese (OB; BMI ≥ 30; N=12), overweight (OW; BMI=25-30; N=12), normal weight (NW; BMI = 18.5 – 24.9; N=14) and underweight (UW; <18.5; N=12). In the present study, in most cases young and elderly were close relatives like son and father or grandson and grandfather. Selection of one young and one elderly person from the same family was deliberately done in order to minimize the effects of possible confounding factors like genetic and socio-demographic variations.

Anthropometric Data

Detailed procedures for collection of data on anthropometric measurements, body composition and nutrient intake are reported elsewhere (15). Briefly, age was assessed from the official records of the subjects (the National Identity Card, NIC). Weight and height were measured and BMI (Body Mass Index) was calculated as: weight/height^2 (kg/m^2) (1). WC and HC (waist and hip circumferences) were measured in accordance to the standard procedures (16). Percent body fat (%BF) was assessed using Futrex-5000 according to the procedures recommended by the manufacturer (Futrex®, Hagerstown, MD) (15). Based on their WC and WHR values, subjects were grouped into one of three risk categories. For WC, the risk categories were defined as: low risk (LR, WC; <89 cm), moderate risk (MR, WC; 90 – 99 cm) and high risk (HR, WC; ≥100 cm). For WHR, the risk categories were defined as low risk (LR, WHR; < 0.89), moderate risk (MR, WHR; 0.90 – 0.94) and high risk (HR, WHR; ≥ 0.95) (17-19). Similarly, on the basis of their body fat, subjects were divided into any of the three categories i.e., low fat (LF, % BF; <10%), normal fat (NF, % BF; 10-25%) and high fat (HF, % BF; >25%) (14).

The dietary data were collected using 24-hr dietary recalls (24-hr DRs) through face-to-face interviews. These 24-hr DRs were repeated three times over the three alternative days of a week (15). From information of 24-hr DR, nutrient intakes were computed using an in-house nutrient calculator (using Microsoft Office Excel 2003, USA) based on the data of food composition tables for Pakistan (20).

Clinical Chemistry Analysis

Blood samples were collected by a trained medical technician; plasma was separated by centrifugation at 1200 g and stored in a -80°C freezer in the Department of Human Nutrition, Agriculture University Peshawar. These samples were shipped on dry ice to the Center for Medical Research (ZMF), Tübingen University, Germany, where they were stored at -80°C until further analysis. Albumin, ferritin, C-reactive protein (CRP), triglycerides and total protein concentrations were measured on a Modular Analytics SWA automated analyzer system according to the manufacturer's recommendations (Roche Diagnostics, Mannheim, Germany). All the clinical chemistry analyses were performed in the facilities of Department of Clinical Chemistry, University Medical Center Göttingen, Germany.

Statistical Analysis

All anthropometric measurements were made in duplicate and the means of paired values were used in the analyses. The data were statistically analyzed using JMP (Version 7.0. SAS, USA) and GraphPad (5.0). As the current study involved four BMI categories, mean values of nutrient intake in these categories were taken for one-way analysis of variance (ANOVA), and post-hoc comparisons with Dunnett's test taking the normal weight group as reference. BMI-adjusted partial

correlation coefficients were calculated to establish associations between anthropometric measurements, clinical chemistry and nutrient intake.

The study was approved by the Board of Studies, Department of Human Nutrition, Agriculture University Peshawar. Written informed consent was obtained from all the participants before the start of the study.

Results

Age, anthropometric measurements, nutrient intake and selected plasma factors of young and the elderly subjects are shown in Table 1. Young men had higher BMI and WHR while elderly men had higher body weight, WC and % BF. However, the only statistically significant difference between young and elderly was in % BF (p=0.02). There were significant differences in nutrient intake between young and elderly (p<0.05, Table 1); the former had significantly higher intake of almost all nutrients.

Young and elderly subjects were stratified into four BMI groups. Table 2 and Table 3 show mean (±SD) age, anthropometrics, nutrient intake and selected plasma factors of the four BMI groups of young and elderly, respectively. For both age groups, there were no significant differences in mean age of the subjects in any of the four BMI categories (p, for all trends ≥0.05). Both in young and elderly, weight, WC, WHR and % BF of the three BMI categories differed significantly (p<0.05) compared to their respective NW BMI categories. Comparison of energy and protein intake between the three BMI categories (OB, OW, UW) of young vs. NW young were as follows: for energy, OB young had significantly higher intake (p= 0.007); OW young tended to have higher intake (p=0.295); UW young had significantly lower intake (p=0.001); for protein, OB and OW young tended to have higher intake (p=0.591 and 0.075, respectively), while UW young had significantly lower intake compared to NW young (p=0.001). In the elderly, however, only protein intake of people in the OB group did not differ significantly compared to NW (p=0.0566), while energy intake differed significantly between the three BMI vs. the NW BMI categories (p<0.05).

Young and elderly in the matched BMI categories were compared for their anthropometrics, nutrient intake and plasma chemistry and the results are shown in Figures 1 and 2 and Table 4. Great variations of significant differences in these parameters of BMI matched young and elderly were observed as summarized in Table 4.

Table 1 and Table 2, respectively, also show mean (±SD) values of selected plasma factors of young and elderly men in the four BMI categories. Within young, no significant differences were noted in the plasma chemistry between the three BMI vs. NW BMI (p, for all trends≥0.05), whereas in UW elderly both albumin and ferritin levels were significantly lower than the NW elderly (p=0.0005 and 0.0501, respectively).

For both young and elderly, the WCs and WHRs were divided into three groups. Figures 3A and 3B depict percent frequencies of young and elderly subjects with low, moderate and high risk based, respectively, on their

Table 1

Mean (SD) age, anthropometric measurements, nutrient intake and plasma biochemicals

	Young		Elderly		
	Mean	Range	Mean	Range	p-value[1]
Anthropometry					
Age (years)	24.2 (3.43)	18.0 - 29.2	67.3 (8.77)	50.1 – 85.5	-
Weight (Kg)	67.6 (14.02)	45.3 - 92.4	68.7 (14.57)	46.0 – 97.0	0.7329
BMI (Kg/m^2)	25.0 (5.37)	16.3 - 33.4	24.2 (5.47)	15.4 – 33.8	0.5056
WC	82.1 (11.20)	64.1-102.2	86.7 (12.39)	62.1- 113.2	0.0735
WHR	1.0 (0.11)	0.70 – 1.17	0.9 (0.12)	0.67 – 1.21	0.3676
%BF	17.7 (8.48)	5.5 – 33.1	21.3 (7.99)	9.0 – 32.6	0.0200
Nutrient intake					
Energy (Kcal)	2344 (498.8)	1262 - 3280	1778 (479.9)	659 -2487	<0.0001
Protein (g)	48.8 (11.39)	29.5 – 77.2	37.6 (12.19)	14.6 – 68.6	<0.0001
Fat (g)	111.5 (38.97)	53 – 222	61.5 (25.07)	14.5 -149.2	<0.0001
Fiber (g)	6.3 (2.46)	1.0 – 12.0	5.0 (1.95)	1.0 – 10.0	0.0155
Calcium (mg)	485.8 (155.89)	227 – 819	344.6 (102.54)	133.0 – 664	<0.0001
Phosphorus (mg)	753.5 (148.23)	490 – 1147	550.6 (179.39	215.0 – 1037.0	<0.0001
Iron (mg)	15.2 (4.38)	7.0 – 27.0	12.1 (5.42)	3.0 – 25.0	0.0021
Zinc (mg)	9.8 (4.97)	0.5 – 23.5	7.6 (3.95)	0.5 -18.5	0.0181
Vitamin A (RE.μg)	234.6 (70.88)	51.0 – 354	227.5 (83.13)	92.0 – 469.1	0.0688
Vitamin C (mg)	32.4 (7.78)	21.0 – 52.0	25.2 (6.40)	13.0 – 41.1	<0.0001
Thiamin (mg)	0.9 (0.26)	0.50 – 1.40	0.7 (0.15)	0.5 – 1.1	0.0221
Riboflavin (mg)	0.7 (0.32)	0.20 – 1.40	0.5 (0.28)	0.10 – 1.40	0.0761
Cholesterol (mg)	156.0 (78.42)	35.0 – 352.0	172.3 (109.21)	22.0 – 488.1	0.0811
Blood Chemistry					
Albumin (g/dL)	3.7 (0.48)	1.93 – 4.7	3.6 (0.64)	1.91 – 4.65	0.3537
Total protein (mg/dL)	5.9 (0.56)	3.1 – 7.3	5.7 (0.76)	3.11- 7.33	0.2131
Triglycerides (mg/dL)	117.7 (67.71)	26.0 – 344.0	109.1 (58.33)	26.0 – 296.1	0.6171
C-Reactive Protein (mg/L)	2.0 (2.38)	0.1 – 17.2	2.4 (1.41)	0.10 – 5.7	0.0376
Ferritin (mg/dL)	72.6 (52.86)	40.2 – 206.6	79.8 (43.68)	9.29 – 196.8	0.2962

1. Significant at p <0.05

Table 2

Mean (SD) of anthropometrics, nutrient intake and blood biochemicals in the four BMI categories of the young

	OB	OW	NW	UW	P-value[1] OB-NW	OW-NW	UW-NW
Age & Anthropometry							
Age (years)							
Weight (Kg)	81.6 (4.1)	76.6 (5.5)	60.7 (6.6)	52.3 (7.5)	0.0002	0.0005	0.0035
BMI (Kg/m^2)	31.5 (0.8)	28.5 (2.0)	22.9 (2.6)	18.5 (2.0)	<.0001	<.0001	<.0001
WC (Cm)	96.2 (1.9)	87.1 (6.5)	77.2 (8.0)	70.9 (5.4)	<.0001	0.0040	0.0028
WHR	1.0 (0.02)	1.0 (0.1)	0.9 (0.1)	0.9 (0.1)	<.0017	0.0023	0.0027
% BF	29.6 (1.8)	21.7 (3.0)	14.2 (3.1)	7.8 (2.8)	0.0021	0.0201	<.0001
Nutrient Intake							
Energy (Kcal/day)	2847 (383.6)	2512 (364.3)	2305 (283.1)	1804 (326.1)	0.007	0.295	0.001
Protein (g)	52.9 (8.8)	48.8 (8.0)	56.6 (10.2)	37.1 (7.8)	0.591	0.075	0.001
Blood Chemistry							
Albumin (g/dL)	3.7 (0.4)	3.6 (0.6)	3.8 (0.5)	3.7 (0.6)	0.6040	0.3272	0.545
Total protein (mg/dL)	5.5 (0.5)	5.5 (0.8)	5.9 (0.8)	5.9 (0.9)	0.4712	0.4588	0.9951
Triglycerides (mg/dL)	112.0 (36.8)	117.8 (86.5)	117.8 (84.1)	122.3 (54.9)	0.3514	0.5326	0.8798
C-Reactive Protein (mg/L)	2.1 (0.4)	1.5 (0.5)	1.8 (0.8)	2.3 (0.4)	0.8886	0.9997	0.4731
Ferritin (mg/dL)	81.7 (43.5)	61.3 (45.3)	64.1 16.8)	60.6 (14.6)	0.5025	0.4008	0.3501

1. Significant at p <0.05

Table 3

Mean (SD) of anthropometrics, nutrient intake and blood biochemicals in the four BMI categories of the elderly

	OB	OW	NW	UW	P-value OB-NW	OW-NW	UW-NW
Anthropometry							
Age (years)							
Weight (Kg)	88.1 (6.63)	72.1 (6.54)	62.0 (9.59)	53.6 (4.63)	<.0001	0.0025	0.0135
BMI (Kg/m^2)	31.7 (1.28)	26.4 (1.59)	21.2 (2.22)	17.7 (0.78)	<.0001	<.0001	<.0001
WC (Cm)	100.2 (7.81)	92.2 (3.89)	83.4 (4.88)	71.5 (9.23)	<.0001	0.0056	0.0001
WHR	1.08 (0.08)	0.99 (0.06)	0.89 (0.04)	0.79 (0.72)	<.0001	0.0008	0.0027
% BF	31.1 (1.04)	24.7 (2.54)	19.6 (4.13)	10.2 (0.81)	<.0001	<.0001	<.0001
Nutrient Intake							
Energy (Kcal/day)	2202.2 (133.9)	1935.3 (235.3)	1655.2 (128.4)	1172.3 (393.7)	<.0001	0.0148	<.0001
Protein (g)	37.10 (5.96)	36.2 (7.63)	45.6 (11.91)	25.9 (8.66)	0.0566	0.0305	<.0001
Blood Chemistry							
Albumin (g/dL)	3.70 (0.34)	3.60 (0.45)	3.93 (0.36)	3.03 (0.91)	0.6040	0.3272	0.0005
Total protein (mg/dL)	5.53 (0.44)	5.52 (0.77)	5.90 (0.89)	5.96 (0.81)	0.4712	0.4588	0.9951
Triglycerides (mg/dL)	124.7 (47.7)	117.8 (86.5)	91.4 (34.6)	105.4 (56.7)	0.3514	0.5326	0.8798
C-Reactive Protein (mg/L)	3.39 (1.51)	2.23 (1.37)	2.27 (0.52)	1.63 (0.43)	0.0386	0.9997	0.4731
Ferritin (mg/dL)	99.8 (53.6)	79.8 (41.5)	77.1 (36.6)	59.2 (32.5)	0.5025	0.4008	0.0501

1. Significant at p <0.05

Table 4

Summary of p-value statistics for comparison of anthropometrics, nutrient intake and plasma factors in the same BMI categories of young versus elderly

	OB young – OB elderly	OW young – OW elderly	NW young – NW elderly	UW young – UW elderly
Anthropometrics				
WC (cm)	0.074	0.0257	0.0168	0.7280
WHR	0.1927	0.2971	0.5625	0.0921
%BF	0.0501	0.0035	0.0018	0.0005
Nutrients				
Energy (Kcal)	0.0072	0.0067	<0.0001	0.0032
Protein (g)	0.0027	0.0012	0.1081	0.0281
Fat (g)	0.0022	0.0004	0.0660	0.0002
Fiber (g)	0.6799	0.0308	0.0067	0.0280
Calcium (mg)	0.0018	0.0211	0.0051	0.0781
Phosphorus (mg)	0.0041	0.0003	0.0431	0.0022
Iron (mg)	0.0319	0.0432	0.1595	0.0114
Zinc (mg)	0.0979	0.5046	0.3210	0.0219
Vitamin A (RE.μg)	0.3864	0.0367	0.0731	0.0833
Vitamin C (mg)	0.0559	0.0782	0.0021	0.0002
Thiamin (mg)	0.0004	0.1382	0.1299	0.2112
Riboflavin (mg)	0.0167	0.0051	0.8121	0.7233
Biochemicals				
Albumin (g/dL)	0.7068	0.0842	0.6290	0.0496
Total Protein (mg/dL)	0.7942	0.3266	0.7130	0.7431
Triglycerides (mg/dL)	0.2134	0.1660	0.2123	0.4555
C-Reactive Protein (mg/L)	0.0014	0.2122	0.0812	0.3104
Ferritin (mg/dL)	0.0855	0.3398	0.0432	0.5253

WC, Waist Circumference; WHR, Waist to Hip Ratio; % BF, % Body Fat. Significant at p< 0.05.

WC and WHR values. Similarly, the percent number of young and elderly subjects with low fat, normal fat and high fat are depicted in Figure 3C.

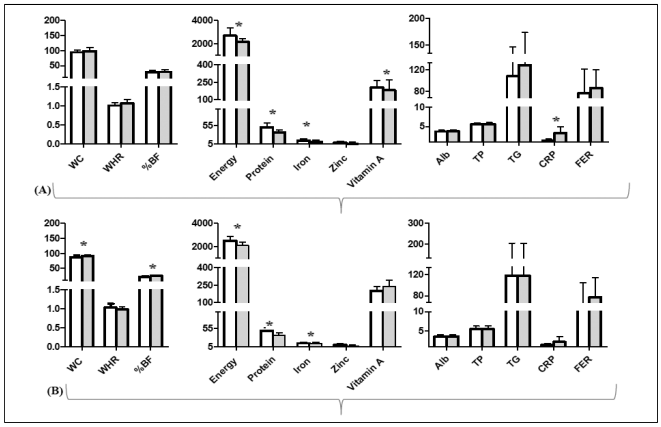

Figure 1. Percentages of young (white bar) and elderly (grey bar) subjects in each of three WC (A), WHR (B) and % BF (C) categories. HR, High Risk; LR, Low Risk; MR, Moderate Risk, HF, High Fat; LF, Low Fat; NF, Normal Fat. For WC, the risk categories were defined as: low risk (LR-WC), moderate risk (MR-WC) and high risk (HR-WC). For WHR, the risk categories were defined as low risk (LR-WHR), moderate risk (MR-WHR) and high risk (HR-, WHR) similarly, on the basis of body fat, subjects were divided into three categories i.e., low fat (LF, % BF), normal fat (NF, % BF) and high fat (HF, % BF).

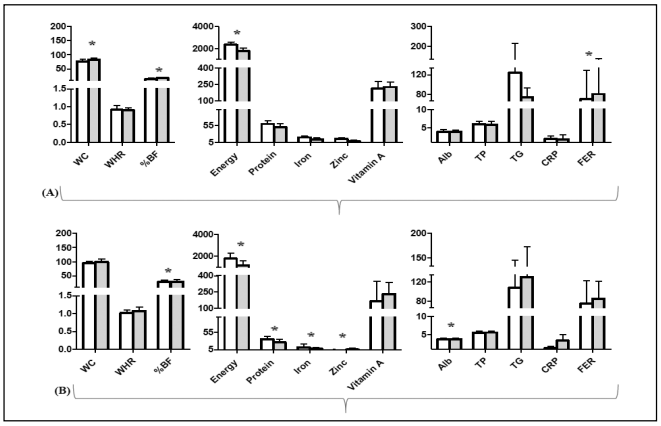

Figure 2. Comparison between young (white bar) and elderly (grey bar) for their anthropometrics, nutrient intake and plasma concentrations. (A) Obese young vs. Obese elderly; (B) Overweight young vs. Overweight elderly. The asterisk (*) shows significant differences at p<0.05.

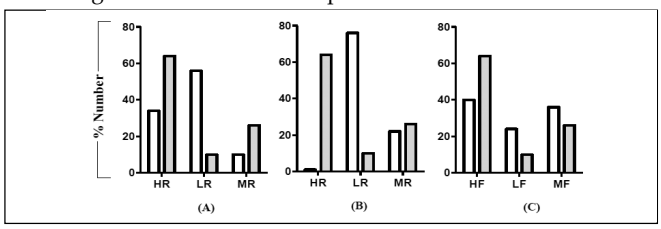

Figure 3. Comparison between young (white bar) and elderly (grey bar) for their anthropometrics, nutrient intake and plasma concentrations. (A) Normal weight young vs. Normal weight elderly; (B) Underweight young vs. Underweight elderly. The asterisk (*) shows significant differences at p<0.05.

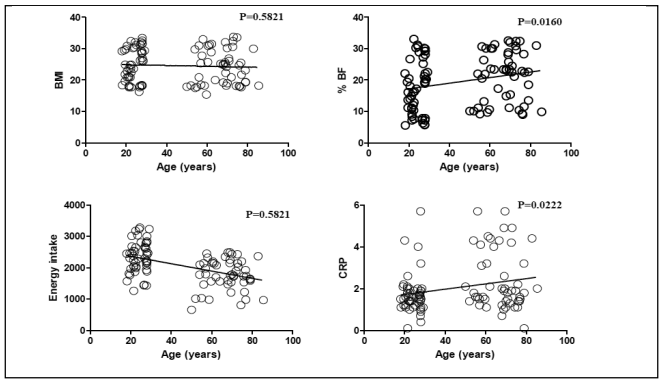

Figure 4. Correlation analyses of age with BMI, % BF, energy intake and CRP. Correlations significant at p<0.05.

Discussion

The effects of aging on nutritional status have been extensively investigated but almost exclusively in so-called "WEIRD" subjects (Western, educated, industrialized, rich, and democratic). It is not well established whether changes in nutritional status with aging found in these populations are representative of the rest of the elderly, who live in developing countries. The main objective of this study was to compare anthropometric measurements, intake of selected nutrients and plasma clinical chemistry of young and elderly rural Pakistani men. The exclusion of a female group in our study is due to difficulties to access this population mainly arising from traditional constraints. Most of the young participants (70%) were selected as close family members of the elderly subjects living in the same household. This selection criterion was adopted purposefully in an attempt to minimize the effects of genetic variations, socioeconomic differences and access to nutrients which may affect the nutritional and health status. A second objective was to compare these parameters within the four BMI categories of young and elderly subjects in order to investigate any possible differences in these parameters within different BMI categories. In the third objective, we wanted to see whether these age-associated changes in nutritional status of the elderly in a developing society are comparable to those seen in "WEIRD" populations. Finally we will be correlating these parameters with assessments of immune status (manuscript in preparation).

All the anthropometric measurements included in this study are related to their expected associations with food habits, health and well-being (21). Weight, height, WC, and WHR are useful indices for the assessment of nutritional health (5, 21-24). We already reported relatively high percentage not only of underweight, but also of overweight and obese elderly subjects (2, 15). There are several other reports (25, 26) that Asian adults have higher risks of developing certain diseases even at lower BMI and WC, which warrants studies to establish

and report the prevalence of malnutrition in the elderly and to target interventions.

We note here that elderly and young individuals in the same family tend to have very similar trends of nutritional status independent of age. For example, 85% of the obese or overweight young belonged to families where their elderly counterparts were also either obese or overweight (data not shown). Similarly, 75% of the normal weight young belonged to families where their elderly relatives were also of normal weight. This coincidence might be due to genetic factors. Family and twin studies have shown that genetic factors account for 40–70% of the population variation in BMI (27, 28). But it might also be explicable on the grounds that with the same dietary practices and within the same socioeconomic background, family members are more likely to have more or less identical nutritional status (29). However, we need more data from a larger sample size to generalize these observations.

Taken as a whole, there were little differences in the anthropometrics except % BF, which differed significantly between young and elderly (Table 1). Young and elderly as a whole had significant differences in almost all nutrients studied (Table 1). Young and elderly were divided into four BMI groups for comparison. These BMI groups also showed large differences in anthropometrics, nutrient intake and clinical chemistry (Tables 2 and 3). Energy intake in OB young subjects was the highest followed by OW, NW, and UW (Table 2). However, statistically significant differences in the energy intake could only be shown between OB vs. NW, and UW vs. NW young subjects (p=0.007 and 0.001, respectively). Regarding protein, the OW and UW young subjects had significantly lower intake compared to the NW young (p=0.075 and 0.001, respectively). In the four BMI groups of the elderly (Table 3), the energy and protein intake of OB, OW and UW differed significantly from NW elderly (p, for all trends <0.05).

The mean values of plasma albumin, total protein (TP), triglycerides (TG), C-Reactive protein (high sensitivity; hsCRP) and ferritin of the four BMI categories of young subjects were not significantly different (p, for all trends ≥0.05), although CRP in young OB (2.1±0.4) and elderly OB (3.39±1.51) were higher compared to their respective NW categories (Table 2 and Table 3). Although an increasing CRP level associated with central obesity has been reported previously (30), results of the current study failed to show any such relationship. The serum CRP level in OB elderly tended to be higher than in NW elderly but this did not reach statistical significance. In the elderly, there were significant differences in serum albumin of UW (p=0.0005) and ferritin levels (p=0.0501) as compared to NW elderly. Serum albumin is the best-studied serum protein having prognostic value for subsequent mortality and morbidity in community-dwelling older persons and is extensively used for nutritional assessment (31, 32). A plasma level of <3.4 mg/dL is considered as an indicator of malnutrition (33). The mean albumin levels in our sample of young (mean (g/dL) ±SD: 3.7±0.48) and elderly (3.6±0.64); Table 1) are fractionally above this threshold. However, serum albumin level is of limited utility in detecting acute nutritional changes owing to its long half-life (18 days) (34). Instead, total protein (TP) has a relatively longer biological half-life and therefore it is a rather late indicator of protein malnutrition (35). The mean plasma protein levels both in young (5.9±0.56 g/dl) and the elderly (5.7±0.76 g/dl) were, however, below the normal standard mean value (7.5 g/dl) (37), which might imply a chronic protein deficiency in both young and the elderly. Some recent data show that elevated serum ferritin has been reported to be significantly associated with several CVD risk factors including BMI, waist circumference or waist-to-hip ratio (36). In our results (data not shown), ferritin was positively and significantly correlated with WC (p=0.0311; r=1820) and WHR (p=0.0254; r=2037), while there was a borderline significant correlation with % BF (p=0.0503; r=1350). Ferritin levels increase with BMI (p=0.2718; r=0.0309) and age (p=0.0651; r=0.0957) but non-significant. Our results are in agreement with some other data suggesting that serum ferritin concentration is associated with WHR and other indices of body fat distribution and obesity (37). In our current study, we failed to find any significant correlation between triglycerides and the anthropometric indices for central obesity although triglycerides have been reported to have a close positive relationship with WC and WHR (38). The only reason our subjects did not show any such correlation might be relatively small sample size. We need large studies to investigate such correlations in Pakistani subjects. Many epidemiological studies have demonstrated a univariate association between triglycerides and cardiovascular risk, particularly in relation to coronary heart disease (CHD) (39).

Comparing anthropometrics, nutrient intake and plasma clinical chemistry of young and elderly men in the same BMI category suggests greater variations (Figs 1 and 2). A summary of these differences is also shown in Table 4. WC and % BF differed significantly between young and elderly (p, for all trends <0.05) across all BMI matched pairs, while WHR did not differ significantly (p, for all trends ≥ 0.05). Similarly, young and elderly in the same BMI categories (Figs 2 and 3; Table 4) show that energy intake differed significantly (p, for all trends <0.05) across all BMI matched pairs of young and the elderly, while protein intake differed significantly (p, for all trends <0.05) across the BMI matched pairs of OB, OW and UW but did not differ significantly (p=0.1081) between NW young and NW elderly. Of particular note, significant differences were observed only for albumin (UW young vs. UW elderly; p=0.0496), CRP (OB young vs. OB elderly; p=0.0014) and ferritin (NW young vs. NW

elderly; p=0.0432). These large variations in anthropometric measurements, nutrient intake and to some extent in plasma values across the matched BMI pairs of young and the elderly may suggest the effect of aging across the same BMI categories. People of different age but of the same BMI may present differences in their overall health, an effect of aging physiology. For example, energy and protein intake have been reported to generally decrease with age adjusted for BMI (40).

Risk assessment for obesity-related conditions (e.g., type-II diabetes, hypertension, CVD etc) by using WC and/or WHR are well established and documented (16-18). The results of the current study report a relatively large number of elderly placed in any of the high risk (HR) categories based on WC or WHR (HR-WC or HR-WHR) (Fig 3 A, B). Furthermore, we found that 24% of young obese and overweight were in the moderate risk (MR-WC) category; only 2% were in the HR-WC category, while 44% of young obese and overweight were in the HR-WHR category. In the elderly, 10% of obese and overweight were in HR-WC category. None of the normal weight young or elderly (BMI=18.5–24.9 kg/m2) had a high WC and hence were at low risk (either LR-WC or LR-WHR). These results are partially in agreement with previous data (12), which reported that only 1% of men with a normal BMI had a high WC and were in the overweight range (BMI=18.5-24.9 kg/m2), whereas 25% of men had a high WC.

With respect to body fat (Fig 3 C), 79% of the young obese and overweight had high body fat (HF-%BF of ≥25%), while 100% (24 of 24) of overweight and/or obese elderly had high body fat (HF-%BF of ≥25%). Also, 9 of 14 (64%) elderly with a normal BMI nonetheless had high body fat (HF-%BF of ≥25%), whereas all young subjects with normal BMI had healthy/normal body fat (10 – 20%). These results suggest that beyond the upper normal BMI threshold (i.e., 24.9 kg/m2) an increase in BMI may be an indication of increased WC, WHR and %BF. These results further suggest that the elderly have increased likelihood of central obesity (defined by high WC or WHR value) not only at higher than normal BMI (i.e., 24.9 kg/m2) but also even at normal BMI. These findings are in agreement with previous observations that although BMI is a reliable measure of fatness (41) better results can be obtained in conjunction with WC and WHR; thus these latter values are better for discrimination of obesity, particularly the central or abdominal variety (9). Here we see that central obesity and body fat increase with age.

Of our particular interest and as we expected (Fig 4), age was positively correlated with %BF (p=0.0160; r=0.2404) and CRP (p=0.0222; r=0.197) but negatively correlated with energy intake (p<0.0001; r=-0.4705). BMI had negative but no significant correlation with age (p=0.5821;r=-0.0559). These results are in agreement with previous studies on relationships between age and body fat (42), age and CRP (43), and age and energy intake (44). A decrease in BMI with advancing age has also been reported (44). There are changes in body fat with advancing age as several studies have reported age-related increases in body weight and fat mass and decreases in lean body mass (44, 45). A number of studies have quantified the gain in adiposity, with an approximate doubling of body fat between 20 and 50 years of age. The Fels Longitudinal Study (FLS) found that total body fat increases with age by 0.37 kg/year in men and by 0.41 kg/year in women. Thus, the percentage of body fat in the FLS was 23.6% in men at 40 years of age, reaching 29.3% at 60 years of age (46). Other studies have determined that fat increases at a rate ≥7.5% per decade in both genders (47), and that older subjects have a mean fat tissue 7 kg higher than young (48). An increase in body fat is usually associated with a progressive increase in total abdominal fat (and especially visceral fat), as well as a progressive loss of lower body subcutaneous fat. What is more, these changes can occur even without changes in body weight or waist circumference (49). We need further longitudinal studies to confirm these observations in elderly Pakistani men.

Our study has several strengths but also several limitations: The major strength was the use of validated tools for anthropometric measurements and nutrient intake through thorough interview sessions and careful evaluation. The major limitations included a relatively small sample of young and elderly representative of only a small locality and only one gender; thus the results can-only be generalized with cautions. . However, the results of this study may represent a vast majority of elderly of the low-middle socio-economic rural segment of the Pakistani population. Another limitation was the possibility that BMI cut-off points used in this study may understate health risk. The cut-off points are those recommended by the WHO 2000 (1). Although these cut-offs have been proven to be fairly robust for classifying obesity across populations, they are based primarily on the association between BMI and mortality in European and North American populations. Furthermore, as a small cross-sectional study, the present analysis is limited in its ability to elucidate causal relationships between risk factors and overweight. BMI can overestimate body fat in individuals who are very muscular and underestimate body fat in individuals who have lost muscle mass, such as many elderly (27). However, estimates from these potentially misclassified groups likely had little overall impact on the analysis. Although we have not carried out any special studies of the validity or reliability of data for this analysis, we made sure to check consistency and, where possible, to ensure completeness of data. Our experience with other parts of the datasets reported (2, 15) gives us some confidence that data quality is sufficient for this type of study and that our results provide useful additional evidence on the prevalence of and risk factors for underweight, overweight, and

obesity. Despite the above limitations, the findings presented here may add substantially to our understanding of malnutrition in the elderly Pakistanis that has been under-represented in past studies.

In conclusion, our data show that young and elderly have differences in their anthropometrics and nutrient intake. Overall, elderly men seem to have nutrient deficiencies as compared to young. The nutritional status of the elderly in the present study is characterized by relatively lower body weight (a possible indication of loss of lean body mass) and higher % body fat. Their nutrient intake seems to be unbalanced in terms of higher energy intake and disproportionately lower intake of other important nutrients. These data suggest that elderly people in developing countries like Pakistan have almost the same dietary malpractices as observed in most of the WEIRD populations surveyed. However, the results of this present pilot small-scale cross-sectional study are difficult to compare with those of larger studies conducted across Europe, mainly due to methodological and study type differences. Nonetheless, in general the findings from the present study are broadly in agreement, for example, with the findings of the SENECA study (Survey in Europe on Nutrition and the Elderly: a Concerted Action) reviewed by de Groot et al., (50). That study was designed to assess regional or cross-cultural differences in nutrition, lifestyle, health, and performance of elderly Europeans in different countries. The survey concluded that nutritional deficiency is more common at older ages than at other periods in life and dietary intake of elderly people was reported to decline over time. Finally, we suggest that BMI, WC and WHR should be used in combination to define nutritional status.

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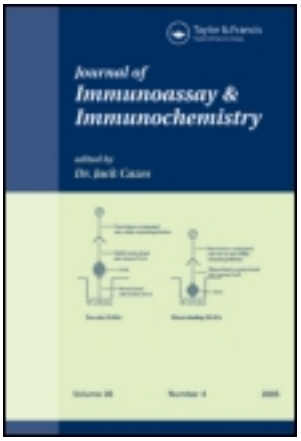

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FLOW CYTOMETRIC LYMPHOCYTE SUBSET ANALYSIS USING MATERIAL FROM FROZEN WHOLE BLOOD

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FLOW CYTOMETRIC LYMPHOCYTE SUBSET ANALYSIS USING MATERIAL FROM FROZEN WHOLE BLOOD

Iftikhar Alam,[1,2] David Goldeck,[1] Anis Larbi,[3] and Graham Pawelec[1]

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□ *Multicenter immune monitoring programs commonly rely on storing and shipping cryopreserved peripheral blood mononuclear cells (PBMC), isolated from whole blood before freezing. However, under many conditions in the field, facilities to separate PBMC are absent. Here, we investigate the feasibility of using whole blood (WB) frozen at −80°C as a source of viable lymphocytes for use in immunological studies. We compare the percentage of CD4 and CD8 T lymphocytes and their subsets from frozen WB with results from cryopreserved PBMC in five random healthy blood donors (three female, two male). We report that CD4 and CD8 values in lymphocytes from WB frozen up to 120 days were very similar to those of PBMC frozen up to 10 days. These data suggest that within the limits of parameters investigated in this study, contrary to our original assumptions, whole blood frozen at −80°C may in fact be an appropriate source of viable lymphocytes for T cell enumeration assays in immunological and epidemiological studies.*

Keywords flow-cytometry, lymphocyte subset analysis, whole frozen blood

INTRODUCTION

Cryopreserved peripheral blood mononuclear cells (PBMC) are routinely used in immunological studies, mainly because of the logistical constraints imposed by multicenter monitoring studies, as well as to facilitate longitudinal assays on the same subject. However, successful cryopreservation of PBMC requires closely controlled conditions in a well-equipped

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laboratory. In studies in the field, these requirements may prevent the inclusion of assays requiring viable PBMC. Collection of these samples is further discouraged by a limited understanding of factors that affect the viability of the cryopreserved lymphocytes, which are collected in the field and transported or shipped over long distances. Factors such as temperatures, times between collection and processing and between freezing and transfer to long-term storage in liquid nitrogen, and potential variation in the final concentration of cryopreservative in each sample are but some of the likely variables.[1]

There are several previous reports of whole blood samples being frozen successfully, with good viability on recovery.[2–7] However, most of those studies[2,5–7] focused on investigating similarities between cryopreserved samples and fresh samples regarding the viability of cells and their suitability for viral transformation to establish cell lines. Additionally, the influence of delayed blood processing on cell viability has also been studied extensively.[4,7] However, very few studies have compared the number of viable lymphocytes and their subsets in cryopreserved PBMC and whole blood, and to the best of our knowledge, none have applied sophisticated modern polychromatic flow cytometry (FC) technology to such samples. The use of cryopreserved separated PBMC is such standard practice that there is skepticism as to whether modern immunological assays are possible using material obtained from frozen WB. On the other hand, fresh whole blood is commonly employed for routine clinical analysis, making comparisons between immune monitoring trials difficult when both techniques are applied. A whole blood sample, which is likely to be more cost-efficient to collect and could reduce variability in lymphocyte subset quantification, is impractical, or impossible in most circumstances, unless stored frozen blood could be used. The present studies were, therefore, designed to develop and test a protocol to cryopreserve whole blood samples at −80°C for immunological studies involving enumeration of lymphocytes and their subsets using polychromatic FC.

MATERIALS AND METHODS

Collection of Blood Samples

For the present study, blood samples from five healthy young individuals were collected aseptically by venipuncture. Approximately 15 mL of whole blood (WB) was obtained from each subject, drawn into two 9 mL EDTA vacutainers (Becton Dickinson, Franklin Lakes, NJ, USA), and processed within 2 h of collection. From each sample, 10 mL of fresh WB was used for PBMC separation and 5 mL for whole-blood freezing and storage at −80°C of one aliquot for 10 days and another for 120 days.

Lymphocyte Freezing Procedure

Lymphocytes were isolated from WB using the Ficoll–Hypaque gradient method[8] according to standard protocols. Briefly, blood was diluted in Hank's Balanced Salt Solution (1:1), carefully layered onto Ficoll-Hypaque (Linaris, Wertheim-Bettingen, Germany) (20 mL), and centrifuged at 810× g for 30 min without applying the brake at room temperature. Cells at the interface were aspirated carefully, transferred to a 15 mL tube, and washed three times with Hank's Balanced Salt Solution (HBSS; Invitrogen, Karlsruhe, Germany). Viable PBMC were counted in a hematocytometer using trypan blue (Sigma, Munich, Germany). The isolated PBMC were resuspended at 10×10^6 cells/mL in RPMI 1640 (Roswell Park Memorial Institute formulation 1640) with 40% fetal calf serum (FCS; Invitrogen, Karlsruhe, Germany). Half that volume of 20% dimethylsulfoxide (DMSO; Serva, Heidelberg, Germany) in RPMI-1640 was mixed with the cell suspension at room temperature and then another half after 5 min. The cells were then transferred to 2 mL cryovials (Cellstars®, GmbH) and immediately placed in a −80°C freezer for 24 h before transfer to liquid nitrogen storage until use.

Whole-Blood Freezing Procedure

DMSO was added to each tube (10%, v/v) of the remaining 5 mL WB samples in a dropwise manner; the mixture was gently agitated and mixed well. The mixture was pipetted into 1.0 mL cryovials, which were then immediately put in a −80°C freezer.

Thawing of Cryopreserved Lymphocytes and Frozen Blood

After storage for 10 or 120 days, the paired samples of frozen purified PBMC and WB were removed from the liquid nitrogen or −80°C freezer, respectively, and quickly thawed in a 37°C water bath. PBMC was washed immediately with RPMI, shaken carefully to mix well and spun down at 300× g for 5 minutes. The cells were resuspended in X-Vivo™ 15 (Lonza, Wuppertal, Germany) and counted in a hematocytometer using trypan blue. Similarly, one cryovial of the thawed WB samples was immediately washed with a 24-fold excess of cold PBS supplemented with 2.0 mM EDTA (Serva, GmbH) and 2% FCS. The diluted blood was shaken gently by inversion by hand 3–4 times to mix well and then centrifuged 300× g at room temperature for 5 min. A relatively large reddish pellet was obtained containing PBMC and some remaining RBC debris. The supernatant was aspirated by pipette.

Lysis of Erythrocytes

To remove remaining erythrocytes in the thawed blood samples, lysis was carried out as follows: To a 1.0 mL blood sample, 10 mL of pure water (ampuwa®, Fresenius Kabi, Germany) was added and mixed well by pipette for 50 s. Then 10 mL 1.8 M NaCl solution was added in two equal halves with a brief interval between each. The samples were centrifuged at 300× g for 5 min, and the supernatant was discarded; cells were then washed twice with PBS.

Staining with Antibodies

Before staining, for FcR blocking, Gamunex® (Immunoglobulin, Bayer, Germany) was added to cell pellets in each tube and incubated for 15 min at room temperature, followed by washing with PFEA (PBS, 2% FCS, 2 mM EDTA, and 0.01% sodium azide), according to standard procedures. The cells were then stained with 50 µl of an antibody cocktail, incubated for 30 min at room temperature in the dark, washed with PFEA, and finally resuspended in PFEA for FC. The monoclonal antibodies and fluorescent conjugates used in the cocktail were as follows: CD3 (Alexa Fluor 700; BD Pharmingen), CD4 (PerCP; BD), CD8 (APC-H7; BD Pharmingen), CD27 (Qdot-605; Invitrogen), CD28 (PE; BD), CD57 (FITC; Immuno-Tools), PD-1 (PerCP-Cy5-5; BioLegend), TCRγδ (APC; BD Pharmingen), and CCR7 (PE-Cy7; BD Pharmingen). RedVid dye (Invitrogen) was included to identify dead cells.

Flow Cytometer (FC) Measurement and Data Analysis

Figure 1 illustrates the gating strategy used to analyze PBMC (Figure 1A) and WB (Figure 1B) samples and shows an example of light scatter and fluorescence dot plots obtained by 10-color FC. To avoid the inclusion of debris during the WB measurement, we set a threshold in the FSC and SSC channels as well as the CD3 channel for WB samples. The lymphocytes were difficult to see as a separate population in the WB sample as compared to the PBMC sample. However, this threshold was set in such a way as to include the whole possible lymphocyte population. The FC data were analyzed by the conventional manual method of visual inspection using commercial software (FACSDiva, BD Bioscience, Inc., San Jose, CA, USA). Cell populations were identified visually and were gated. The gates were then used to filter the population for subsequent analysis. The gating strategies for PBMC and WB are shown in Figures 1A and 1B, respectively. For both PBMC and WB, the first step of analysis consisted of filtering out the

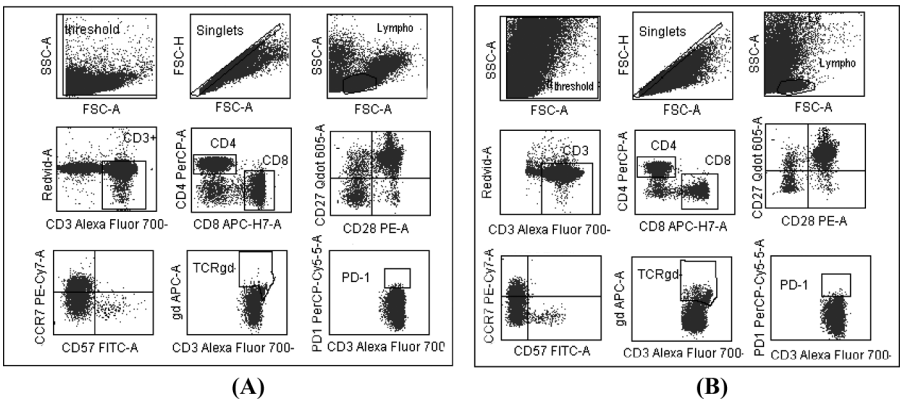

FIGURE 1 The gating strategies for PBMC (A) and whole blood (B).

doublets. For this, a singlet gate was defined using the FSC-H and the forward scatter (FSC-A) plot. Lymphocytes were gated. Then debris and dying or dead cells were identified by their relatively lower FSC and SSC values. RedVid was plotted against CD3 for the discrimination of CD3+ cells (living cell populations). Further analyses were done on these CD3+ populations: CD4+ and CD8+; CD27, CD28 separately on CD4 and CD8; CCR7, CD57 separately on CD4 and CD8; PD-1 on CD4 and TCR$\gamma\delta$ on CD8.

Statistical Analysis

Percent means ($\pm$SD) of CD4 and CD8 T cells and their subsets were calculated. The % mean values obtained for these parameters from PBMC and WB were compared for differences using Dunnett's t-test. In this test, % mean values of CD4 and CD8 T cells and their subsets in the PBMC (10 days in storage) sample were considered as reference, and the corresponding values of the PBMC (120 days) and WB (10 and 120 days) analyses were compared with them. Agreement between methods for CD4 and CD8 and their subsets was evaluated by Bland–Altman plots.[9] All differences were accepted as significant at $p < 0.05$. All statistical analyses were performed using JMP 8.0 (SAS, USA) and GraphPad Prism (Version 4.0, GraphPad Software, Inc., USA).

RESULTS AND DISCUSSION

The present study was carried out to establish a protocol for T cell and subset enumeration using whole blood frozen at -80°C and stored for a

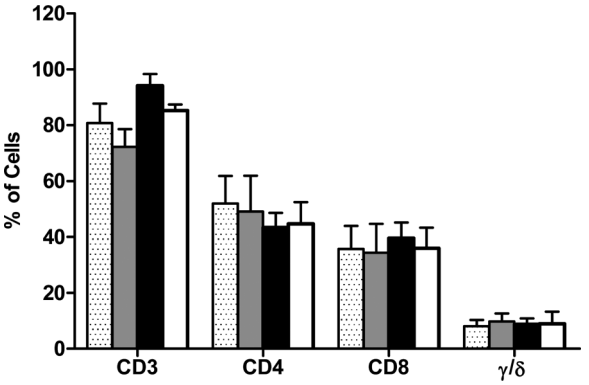

FIGURE 2 Frequency of the main T cell (CD3, CD4, CD8, and TCRγδ) populations. The dotted, gray, black, and white bars represent PBMC (10 days), PBMC (120 days), WB (10 days), and WB (120 days), respectively.

short period (10 days) or a longer period to assess potential deterioration over time (120 days), systematically compared with the standard method of carrying out all such analysis on cryopreserved PBMC. Figure 2 shows a comparison of CD3, CD4, CD8, and γ/δ T cell frequencies in PBMC and WB frozen for 10 and 120 days compared with PBMC frozen for 10 days, expressed as percentage of the mononuclear cells in the sample. There were no significant differences in the frequencies of CD4 and CD8 T cells in the WB samples compared to PBMC (10 days) (Figure 2). Due to the presence of residual red cells in the blood, we gated on lymphocytes using a back-gating strategy of the CD3+ cells. For this reason, more than 90% of the lymphocytes were detected as T cells. As expected, approximately 60% of the PBMC were CD3-expressing cells. The same applies to low frequency populations such as γ/δ T cells. However, despite the frequencies being very similar, a disadvantage of using WB was the poorer recovery of cells compared to PBMC. The loss of CD3+ T cells can be greater than 50% (data not shown), and for this reason, frozen whole blood cannot be used for absolute CD3 cell counts. Nonetheless, the frequency of CD4 and CD8 T cells within the recovered CD3+ T cells was in perfect agreement with values obtained using isolated frozen PBMC. This indicates that frozen whole blood can be used to identify parameters such as the CD4/CD8 ratio.

Next we tested whether not only CD4 and CD8 frequencies were maintained within CD3+ T cells, but other subsets also could be identified in frozen WB as with PBMC. Naïve and memory cells were distinguished using a range of surface markers including CD27, CCR7, CD57, CD28, and PD-1. The p-values calculated using Dunnett's t-test were in the range of 0.074–0.998 for CD4 T cells and their subsets, indicating that the differences between CD4 subsets analyzed in these samples were not significant

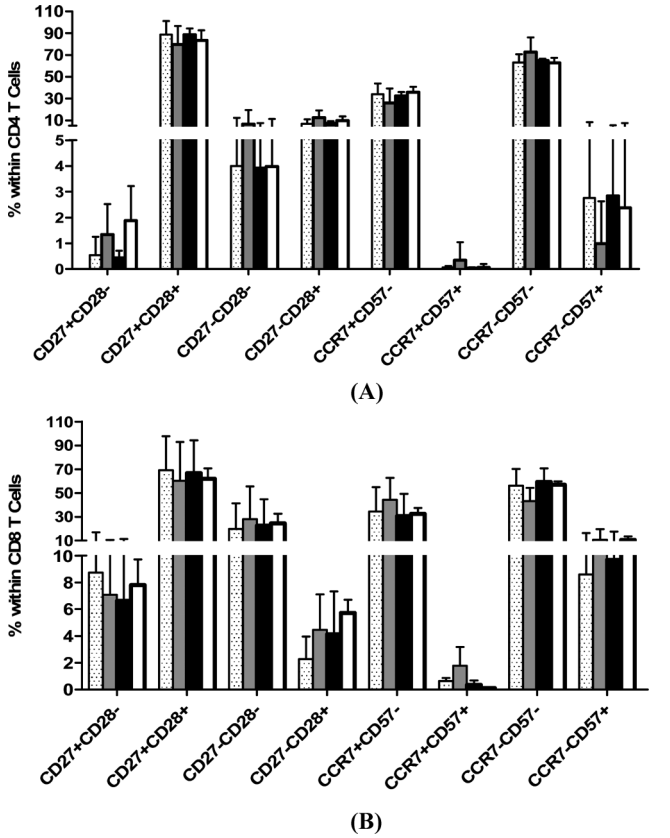

FIGURE 3 Frequency of T cell populations. Frequency of subsets within CD4 (A) and CD8 (B). The dotted, gray, black, and white bars represent PBMC (10 days), PBMC (120 days), WB (10 days), and WB (120 days), respectively.

($p \geq 0.05$) (Figure 3A). Similarly for the comparison of CD8 T cells and subsets analyzed, none of the slight differences observed were statistically significant ($p \geq 0.05$) with a Dunnett's p-value ranging from 0.093–0.993 (Figure 3B). Nonetheless, although the percentages of the subsets were very similar no matter how the samples had been processed and frozen, the density of expression of some surface molecules did vary. Thus, Figures 4A, 4B, and 4C summarize the mean fluorescence intensities (MFI) for parameters showing slight differences, but lacked any statistical significance except CD28 within CD3 of WB (120 days), which was significantly ($p = 0.0377$) reduced as compared to PBMC (10 days).

In general, the results of the Bland–Altman test comparing frozen WB with PBMC (10 days) (Figure 5) provided strong evidence that samples of WB frozen at −80°C for shorter and longer periods (10 and 120 days, respectively) storage yield almost identical results compared to those of

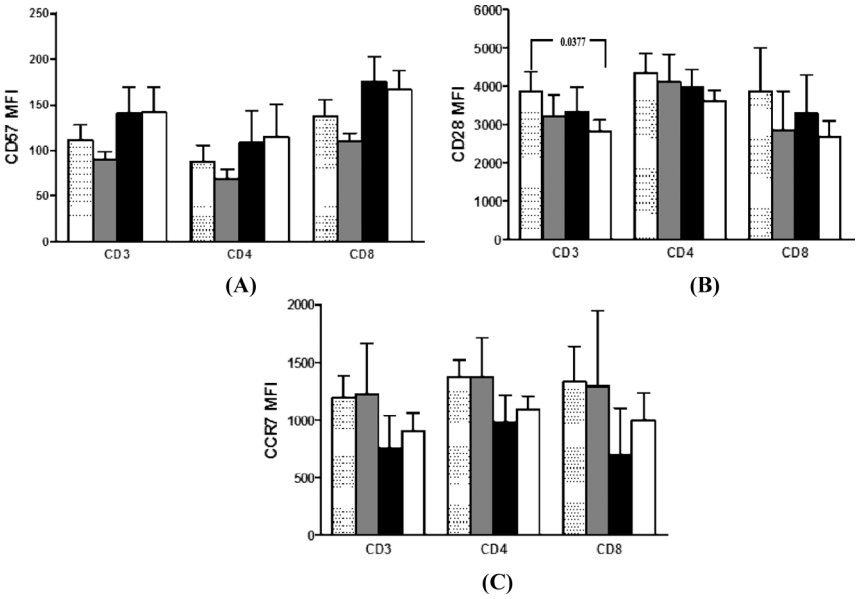

FIGURE 4 Mean fluorescence intensities (MFI): effect of freezing conditions. MFI of CD57 within CD3, CD4, and CD8 (A); MFI of CD28 within CD3, CD4, and CD8 (B); and MFI of CCR7 within CD3, CD4, and CD8 (C). The dotted, gray, black, and white bars represent PBMC (10 days), PBMC (120 days), WB (10 days), and WB (120 days), respectively.

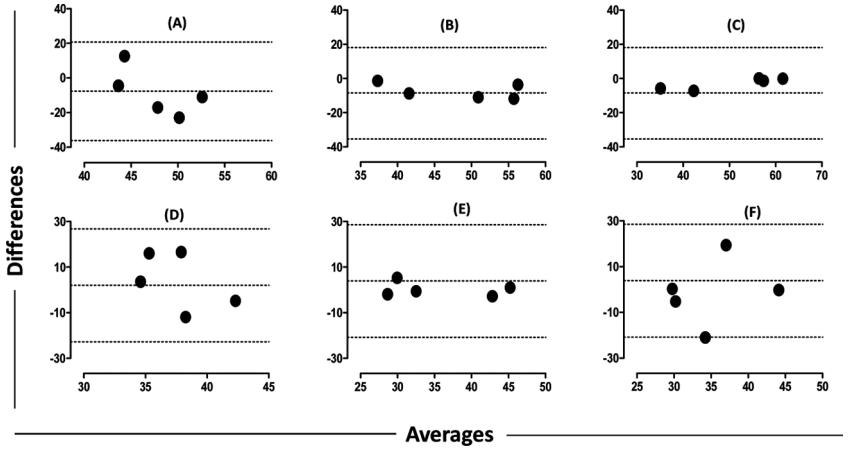

FIGURE 5 Results of the Bland–Altman test for estimates of total bias for % of CD4 (A, B, C) and CD8 (D, E, F) for PBMC and three whole blood samples processed in different ways. (A), (B), and (C) show, respectively, a comparison of CD4 between PBMC (10 days) vs. WB (10 days); PBMC (10 days) vs. WB (120 days); and PBMC (10 days) vs. PBMC (120 days). (D), (E), and (F) show, respectively, a comparison of CD8 between PBMC (10 days) vs. WB (10 days); PBMC (10 days) vs. WB (120 days 1 mL); and PBMC (10 days) vs. PBMC (120 days). The central line in each plot shows the mean, while the upper and lower dotted lines show ±2 SD.

PBMC for both CD4 (Figures 5A–5C) and CD8 (Figures 5D–5 F) cells. The biases (differences between the means) were −7.66, −8.6, and −10.3 for CD4 cells when comparing PBMC (10 days) vs. WB (10 days), PBMC (10 days) vs. WB (120 days), and PBMC (10 days) vs. PBMC (120 days), respectively. These values were for CD8 cells were 3.90, 0.200, and −2.90, respectively. Bland–Altman plots are intended to assess whether the variability of differences between measures is roughly constant across the range of measurements.[9] The very small mean differences indicate that assays on PBMC and WB provide acceptably similar results for CD4 and CD8 cells, and their subsets (data of Bland–Altman test on subsets of CD4 and CD8 not shown here).

While processing frozen WB, we encountered certain procedural problems, but found that these could be overcome by careful handling of the samples. The biggest problem with blood samples frozen at −80°C was that they become sticky and form clumps of dead cells, almost certainly due to granulocyte death.[10] These clumps may pose potential problems during washing, pipetting, and FC measurement steps. However, we were able to minimize these problems by washing the thawed WB in PBS supplemented with EDTA, which is an effective anticoagulant, probably by virtue of its ability to bind divalent cations (Mg^{2+} and Ca^{2+}). Elimination of clumping allows preparation of viable cell suspensions, the concentrations of which can be accurately determined, and avoid blocking the flow cytometer. These preparations have been found to be extremely useful for immunological studies such as in vitro lymphocyte enumeration, activation, and transformation. We also investigated the effect of erythrocyte lysis; although most of the erythrocytes die when frozen under these conditions, appreciable numbers are still present upon thawing.[11] The lysis procedure employed here had no effect (neither positive nor negative) on the yield of CD4 and CD8 cells and their subsets. Inclusion of this lysis step resulted in a great reduction in the time taken for FC measurement of the samples (data not shown), which might be because of further reduction in the number of possible surviving erythrocytes and the washing away of their dead cells and debris. This step is therefore to be recommended when using frozen WB.

By using CD3, CD4, CD8, CD28, CD27, and CCR7 staining, it is possible to establish a subject's immune signature reflecting the immunological history of that individual (Figure 6). CD8+ T cells lose CD28 expression with cell division and differentiation, while the same may apply for CD27 in CD4+ T cells. It is therefore important to note that CD27 and CD28 were proven to be reliable markers when using frozen WB samples. While CD28+ CD27+ T cells may represent naïve and central memory (CM) cells, the CD27–CD28+ and CD27+ CD28– T cells may represent the first stage of effector memory cells (EM), including recently activated cells

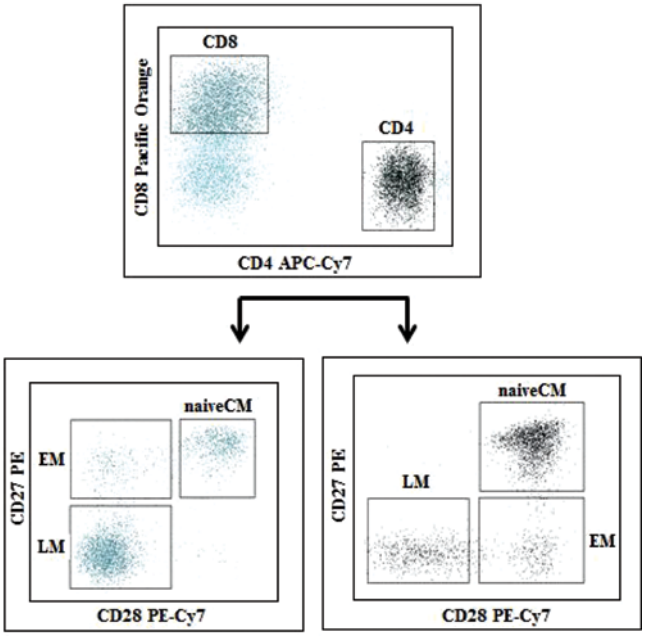

FIGURE 6 CD28CD27 expression as a marker for immune profile in whole blood. A general example showing CD27 and CD28 within CD8+ and CD4+ T cells CD28+ CD27+ T cells may represent naïve and central memory (CM) cells, and the CD27–CD28+ and CD27+ CD28– T cells may represent the first stage of effecter memory cells (EM) including recently activated cells (color figure available online).

(short-term memory). The CD27–CD28– T cells represent the late-differentiated CD4 and CD8 T cells or late memory cells (LM), some of which have been identified as dysfunctional. CCR7 may be useful to identify naïve (CCR7+CD45RA+) vs. memory cells. The extracted information from these subsets is very valuable to identify immune profiles, which, as shown here, can be established from frozen WB using these antibodies.

For many applications, especially multicenter and longitudinal studies, PBMC are separated from whole blood and cryopreserved prior to immunological studies, such as T or B cell enumeration and subset monitoring. Separation of PBMC is quite simple, but requires careful handling, multiple centrifugation steps, and laboratory access.[12] Whole blood is particularly useful when a large number of blood samples must be processed on a given day, when blood volume is limited, or when facilities and expertise are limited, as in many studies in the field.

As argued previously,[5] blood itself is the best medium for storing viable lymphocytes. There are several advantages of this approach over the traditional PBMC method, in addition to the logistical considerations mentioned above. For example, a smaller blood sample is needed for assays

requiring viable lymphocytes, which is particularly important when blood is the only biologic specimen available for biomarker studies, e.g., from elderly frail individuals or infants. Moreover, less blood will be used in one assay, which leaves more blood available for other complementary assays. In addition, the storage of viable lymphocytes in frozen whole blood allows functional assays to be performed in batches with an adequate control for assay variation due to samples being assayed on different days.[5]

CONCLUSIONS

The results of the present study suggest that frozen whole blood may be used to evaluate T cell subset frequencies when logistical matters do not allow PBMC separation following good laboratory practice. The results further indicate that the analyses of T cells are independent of volume (either 1.0 or 2.0 mL) of the frozen blood used, suggesting that even small amounts can be successfully analyzed. Further, the results show that although red blood cell lysis of the frozen whole blood has no effect (positive or negative) on the percentage of T cells analyzed, including this step, however, is useful for minimizing the time required for FC measurement. While the frequencies are very similar, a possible disadvantage of using WB observed in this study was the poorer recovery of cells compared to PBMC. The loss of CD3+ T cells can be considerably high, and for this reason frozen, whole blood cannot be used for absolute CD3 cell counts. While some markers may be affected by freezing conditions, most appeared to be very reliable (including CD28 and CD27), and are thus recommended for use on frozen WB samples.

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IMMUNITY & AGEING

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Nutritional status influences peripheral immune cell phenotypes in healthy men in rural Pakistan

Iftikhar Alam[1,2*], Anis Larbi[3] and Graham Pawelec[1]

Abstract

Immune status is influenced by malnutrition, but how this factor interacts in developing countries and whether these differences are similar to those determined in industrialized countries, is unclear. To establish whether malnutrition-associated immune profiles in a developing country are similar to those in industrialized countries we analyzed peripheral blood immune cell phenotypes by polychromatic flow cytometry in 50 young and 50 elderly subjects. Data on anthropometrics and diet were collected through interviews. Plasma samples were analyzed for common clinical chemistry variables. Subjects in 4 BMI categories differed in their immune parameters demonstrating influence of nutritional status on immunity. This was greater within the young group and affected the CD4 subset more profoundly than the CD8 subset. No nutrition-associated differences were seen in B or NK cells. CD8+ cells as a percentage of CD3+ T cells were positively associated with plasma CRP levels but not other factors. We conclude that there are differences in the immune signatures of obese, overweight and underweight versus normal-weight young and elderly, which seem broadly similar to the more extensively-documented state reported in industrialized countries, despite the marked societal, nutritional and many other differences.

Keywords: Aging, Nutrition, Immunity, T and B cells

Introduction

The effects of malnutrition on immune functions are well established as many studies in the recent past have demonstrated that age-associated malnutrition contributes to immunodeficiency [1,2]. Malnutrition is accompanied by a decrease in immunity and an increase in susceptibility to many infectious diseases. Particularly in the aged subjects, underweight or overweight and/or obesity confer increased risk of mortality [3]. Undernutrition affects many elderly not only because their nutrient intake, in general, may be inadequate [4], but also because older adults have altered requirements for several nutrients due to the effects of aging on absorption, utilization, and excretion of nutrients [5] as well as specialized nutrient needs associated with medication use, metabolic disorders, and chronic disease [6]. The effects of undernutrition on immune status may be judged from the fact that a substantial part of the lean mass of human body is comprised of lymphocytes [7]. Underweight may contribute to increased mortality caused by viral infections due to inability to meet the enhanced energy requirements associated with the immune response [8].

On the other hand, overweight and obesity are forms of malnutrition at epidemic proportions globally [1]. The comorbidities associated with obesity affect virtually every physiological system including the regulation of immunity and inflammation [2]. The relationship between obesity and immunity is logically to be expected mainly on the basis of three lines of evidence. First, obesity is linked to increased risks of virtually all types of cancers [2]. Second, obesity is closely associated with chronic, systemic inflammation, which may contribute to the development of obesity-related co-morbidity [9]. Third, a number of hormones including leptin, which is a satiety factor dysregulated in obesity, have been shown to play an important role in regulating immune functions [10]. In addition, some recent studies have shown a link between alterations in lymphocyte functions and the pathogenesis of a number of obesity-related

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comorbidities including atherosclerosis, steatohepatitis, and diabetes [11,12], the presence of which may underlie a number of obesity effects on the immune system.

Obese and underweight individuals may present with a difference in their immune profile as compared to people of normal weight. Our objectives were to determine the effects of malnutrition in a sample of obese and underweight young and elderly individuals in order to determine whether malnutrition has any association with alterations in the frequency of peripheral blood lymphocyte subsets. Any such study of possible associations would provide further supporting evidence for the presence of an immune diathesis of obesity and underweight, which will help to identify specific lymphocyte subsets as those most important to monitor in further studies in nutritional immunology. Furthermore, this pilot study was conducted in a rural population in a developing country in order to ascertain the general applicability of the immunological findings worldwide.

Subjects and methods

Study subjects

Participants in the current study were recruited from Peshawar, Pakistan. Potential subjects expressing interest in blood donation were first screened by obtaining a verbal medical history to rule out any health conditions or medication use that could affect immune response. We selected 100 subjects (50 each young and the elderly) for this study from a large number of young and elderly subjects, who were previously visited and interviewed for their anthropometric measurements and nutritional data [13]. Based on their BMI values, these subjects fell into four BMI categories, obese (N = 12), overweight (N = 12), normal weight (N = 14) and underweight (N = 12). Anthropometric measurements were carried out with the subject barefoot, wearing light clothing, and after an overnight fast. Body weight, height, and percent body fat (% BF) were measured. Body mass index (BMI) was calculated as body weight divided by the squared height (kg/m^2). There are well-documented links between high levels of central adiposity in adults as measured by waist circumference (WC) and/or waist-to-hip ratio (WHR), and risk of obesity-related conditions including type-2 diabetes, hypertension and heart disease. These links remain even once BMI is adjusted for age, demonstrating that measures of central adiposity are independent predictors of future obesity-related diseases [14]. We, therefore, further divided our subjects into different risk categories relative to normal weight and WC. For the purpose of the present study, for WC the risk categories were defined as: low risk (LR, WC; <89 cm), moderate risk (MR, WC; 90–99 cm) and high risk (HR, WC; ≥100 cm). For WHR, the risk categories were defined as low risk (LR, WHR; < 0.89), moderate risk (MR, WHR; 0.90–0.94) and high risk (HR, WHR; ≥0.95).

Dietary intake data

Habitual dietary intake was assessed through 24-hr Dietary Recalls (24-hr DR). Each item of food eaten during the previous 24 hrs was recalled by the subject and the amount of food eaten was used for estimation of nutrient intake using accepted food composition tables [15].

Immunological studies

For assessment of immune signatures, blood samples were collected aseptically by venipuncture. Approximately 18 ml of whole blood (WB) was obtained from each subject, drawn into two 9 ml EDTA vacutainers (Becton Dickinson, Franklin Lakes, NJ). The samples were processed following a procedure especially developed and validated for analyses on whole frozen blood [16]. Another 10 ml blood sample was used for plasma separation for biochemical analysis. Blood and plasma samples were stored in a −80°C freezer until further analysis. After thawing in a 37°C water bath, red cells in blood samples were lysed with saline and water. The cells were then stained with 50 μl of the antibody cocktail and incubated for 30 minutes at room temperature in the dark. For intracellular FOXP3 staining, cells were resuspended in 1.0 ml FOXP3 Fix/Perm (Biolegend, San Diego), vortexed and incubated for 20 minutes at room temperature in the dark, followed by consequent washing with PFEA and FOXP3 Perm buffer. The cells were resuspended in 50 μl FOXP3 Perm buffer containing FOXP3 antibody, incubated for 30 minutes at room temperature in the dark, washed with PFEA and resuspended in PFEA. The monoclonal antibodies and fluorescent conjugates used were CD3 (Pacific Orange); CD3 (Alexa Fluor 700), CD4 (PerCP), CD8 (APC-H7), CD8 (Qdot 705); CD27 (Qdot-605), CD28 (PE), CD28 (PercP-Cy 5-5); CD45RO (Alex Flour 400); KLRG1 (Alexa Fluor 488); CD45RA (Qdot 655); CD127 (APC e-Fluor 780); CD57 (FITC); FOXP3 (PE). Cell populations were measured using an LSR-II flow cytometer and the acquisition commercial software BD FACSDiva (Becton Dickinson).

The study was approved by the Board of Studies, Department of Human Nutrition, Agriculture University Peshawar. Written informed consent was obtained from all the participants before the start of the study.

Statistical analysis

All the data were statistically analyzed using JMP (Version 8.0. SAS, USA). As the current study involved four BMI categories, the mean values of CD4, CD8 and B cells and their subsets were taken for one-way analysis of variance (ANOVA) and post-hoc comparisons using Dunnett's test with the normal BMI category as

reference after adjusting for multiple comparison. BMI-adjusted partial correlation coefficients were calculated to establish associations between anthropometric measurements, nutrients and blood bio-chemicals with T and B cells and their subsets. To establish WC-, WHR- and % BF- associated differences in the number of CD4+, CD8+ and B cells, the percentages of these cells were compared in each of the three WC and WHR risk categories (considering low risk category as reference) and the three body fat categories (considering normal fat category as a reference). We took $p < 0.05$ to denote a significant difference.

Results

Age and anthropometric characteristics

Age and general characteristics of the study subjects are shown in Table 1. Age of young and the elderly subjects ranged from 18.0–29.2 (mean, SD: 24.2 ± 3.4) and 50–85.5 (mean, SD: 67.3 ± 8.7), respectively. The mean ages of the four BMI groups within young and elderly had non-significant difference (p, for all trends <0.05: data not shown). Compared to the elderly, the young had higher mean BMI and WHR, while the elderly had higher mean weight, WC and % BF. However, all these differences lacked statistical significance except % BF. There were significant differences in the nutrient intake between young and elderly: the young had a significantly higher intake of energy and protein (Table 1).

T cell distribution in the four BMI categories of the young and elderly

When subjects were grouped according to the four BMI categories, both young and elderly showed certain differences in the percentages of CD4+ and CD8+ cells, and their subsets, as well as in B and NK cells. Tables 2 and 3 show, respectively, the percentages of CD4+ and CD8+ cells, and their subsets, according to BMI categories in the young. There were significantly lower percentages of CD4+ cells in young OB *vs.* NW. OW and UW young also tended to have lower frequencies of CD4+ cells compared to NW. The percentage of CD4+CD45RA+CD27+ cells in NW young was significantly higher than in OW. The percentages of CD4+ CD45RA-CD27- cells in OW and UW were significantly higher than in NW young (p for all trends < 0.05). The frequency of CD4+CD28-CD27-KLRG1+CD57+ cells in UW was also significantly higher than in NW young. The differences in the frequencies of all other subsets within CD4+cells among the three BMI groups *vs.* the NW group did not achieve statistical significance.

Regarding CD8+CD45RO-CD27+ cells, the difference between UW *vs.* NW young was highly significant. OW young had a higher frequency of CD4+CD45RO+CD28- cells compared to NW young and UW young had significantly lower CD8+CD28+CD27+KLRG1-CD57- cells than NW young. There tended to be some further differences in the frequencies of other subsets within CD8+ cells among the three BMI groups *vs.* NW group of young, but none of these reached significance (p for all trends ≥0.05).

Tables 4 and 5, respectively, show the frequencies of CD4+ cells, CD8+ cells, and their subsets in the four BMI categories of the elderly. The frequencies of all other subsets within CD4+ cells among the three BMI categories *vs.* NW did not differ significantly except for CD45RA+CD27+, CD45RA-CD27-, and CD45RO-CD28+ cells. The percentage of CD45RA+CD27+ cells in OB was significantly higher than NW. Similarly, CD4+CD45RO-CD28+ cells in OB were higher than in NW. The percentage of CD8+ cells in UW elderly was significantly lower than in NW. None of the other differences in the frequencies of subsets within CD8+ cells among these three BMI categories *vs.* NW achieved significance.

B cell and NK cell distribution in the four BMI categories

Tables 6 and 7, respectively, show a comparison of the percentages of B cells and their subsets (IgD+CD27-, IgD-CD27+), and NK cells (CD56+CD16+), among the three BMI categories *vs.* the NW category of young and

Table 1 General characteristics and nutrient intake of the subjects

Anthropometry	Young		Elderly		*P-value*
	Mean (SD)	Range	Mean (SD)	Range	
Age (years)	24.2 (3.43)	18.0–29.2	67.3 (8.77)	50.1–85.5	-
Weight (Kg)	67.6 (14.02)	45.3–92.4	68.7 (14.57)	46.0–97.0	0.7329
BMI (Kg/m2)	25.0 (5.37)	16.3–33.4	24.2 (5.47)	15.4–33.8	0.5056
WC	82.1 (11.20)	64.1–102.2	86.7 (12.39)	62.1–113.2	0.0735
WHR	1.0 (0.11)	0.70–1.17	0.9 (0.12)	0.67–1.21	0.3676
% BF	17.7 (8.48)	5.5–33.1	21.3 (7.99)	9.0–32.6	**0.0200**
Energy intake (Kcal)	2344 (498.8)	1262–3280	1778 (479.9)	659–2487	**<0.0001**
Protein intake (g)	48.8 (11.39)	29.5–77.2	37.6 (12.19)	14.6–68.6	**<0.0001**

Mean (STD); p significant at á = 0.05.

Table 2 CD4+ T cells and subsets in the 4 BMI categories of young subjects

	BMI Category				p-value		
	NW	OB	OW	UW	OB-NW	OW-NW	UW-NW
CD4+	37.2 (6.71)	24.3 (11.51)	24.4 (11.60)	26.8 (10.11)	**0.0014**	0.316	0.180
CD28+CD27+	54.3 (11.12)	45.3 (14.19)	45.1 (13.72)	54.1 (15.81)	0.2339	0.2213	0.2121
CD28-CD27-	12.3 (6.11)	14.1 (10.61)	14.7 (8.21)	15.0 (7.43)	0.9090	0.8150	0.7434
CD45RO+CD27-	21.2 (15.21)	17.9 (9.21)	16.1 (12.45)	22.5 (14.27)	0.8616	0.6492	0.9892
CD45RO-CD27+	27.6 (16.12)	30.9 (15.9)	31.8 (15.31)	30.3 (15.11)	0.9159	0.8439	0.4312
CD45RA+CD27+	33.6 (14.21)	27.1 (8.81)	19.6 (16.61)	21.8 (19.71)	0.5412	**0.0466**	0.1139
CD45RA-CD27-	14.1 (9.31)	36.3 (10.71)	42.1 (24.40)	54.7 (35.62)	0.3971	**0.0072**	**0.0032**
CD45RO+CD28-	8.8 (4.71)	9.5 (7.32)	6.2 (3.11)	12.6 (4.72)	0.9784	0.4521	0.1656
CD45RO-CD28+	44.4 (14.81)	31.2 (14.12)	38.1 (17.51)	30.9 (8.11)	0.5733	0.5449	0.0511
CD45RA+CD28+	33.8 (17.81)	27.1 (20.62)	17.5 (25.01)	31.3 (14.21)	0.7261	0.1053	0.1797
CD45RA-CD28-	16.7 (12.71)	19.2 (20.21)	23.4 (21.60)	23.4 (14.32)	0.9682	0.6445	0.6512
TRegs	3.9 (4.53)	5.9 (5.72)	4.8 (3.90)	3.2 (4.31)	0.5819	0.9172	0.9713
CD28+CD27+KLRG1-CD57-	37.1 (18.10)	34.6 (16.61)	30.1 (15.61)	22.4 (20.31)	0.9713	0.6355	0.1065
CD28-CD27-KLRG1+CD57+	0.6 (0.90)	0.6 (1.32)	1.0 (1.51)	2.3 (1.10)	0.5436	0.8415	**0.0028**

Significant Dunnett's test ($p < 0.05$) comparing CD4+ T subsets with the reference group. The reference group defined as having BMI, 18.5–24.9.OB, Obese; OW, Overweight; NW, Normal Weight; UW, Underweight. The values are means (SD).

elderly. There were no significant differences in either of these phenotypes in the young. In the elderly, however, significant differences in the percentages of IgD+CD27- cells were apparent between UW and NW; and in IgD-CD27+ cells between UW *vs.* NW; and finally, in NK cells between NW and OB.

T and B cell distribution in WCC, WHR risk categories

Table 8 shows a comparison of the mean percentages of CD4+, CD8+ and B cells in the three WC risk categories (i.e. HR, MR and LR) of young and elderly. For young subjects, CD8+ and B cells, respectively, in HR-WC and MR-WC groups differed significantly from those in the LR-WC group (the control group for comparison). However, no significant differences were observed either in CD4+ cells, CD8+ cells or B cells between the two risk groups (HR-WC, MR-WC) and the control group (LR-WC) in the elderly. Similarly, there were no significant differences in the mean percentages of CD4+ cells, CD8+ cells or B cells for the three WHR categories of young and elderly. Young low fat (LF) subjects had significantly lower CD4+ cells compared to normal fat (NF) subjects.

Table 3 CD8+ T cells and subsets in the 4 BMI categories of young subjects

	BMI Category				p-value		
	NW	OB	OW	UW	OB-NW	OW-NW	UW-NW
CD8+	21.2 (2.76)	17.4 (7.04)	21.5 (4.88)	19.1 (5.06)	0.1549	0.5221	0.5324
CD28+CD27+	37.4 (13.1)	32.0 (8.08)	33.4 (8.86)	32.2 (8.18)	0.3832	**0.1133**	0.4239
CD28-CD27-	29.0 (5.25)	27.0 (8.03)	28.6 (8.87)	25.6 (4.26)	0.7898	0.5432	0.4436
CD45RO+CD27-	6.4 (4.48)	8.8 (6.91)	5.7 (5.32)	7.7 (4.03)	0.5117	0.7566	0.8489
CD45RO-CD27+	47.9 (7.74)	43.2 (10.23)	45.0 (13.11)	32.6 (7.86)	0.4886	0.8057	**0.0008**
CD45RA+CD27+	32.8 (12.31)	29.8 (11.89)	32.3 (9.0)	28.7 (13.77)	0.8651	0.8774	0.7262
CD45RA-CD27-	10.4 (9.18)	15.5 (9.40)	12.7 (9.36)	15.3 (10.98)	0.4219	0.8945	0.4625
CD45RO+CD28-	11.4 (6.80)	14.1 (5.57)	17.3 (5.46)	12.5 (4.90)	0.5072	**0.0360**	0.9321
CD45RO-CD28+	44.1 (13.06)	43.0 (16.10)	35.7 (10.53)	38.7 (11.45)	0.6588	0.2479	0.5923
CD45RA+CD28+	41.9 (14.50)	43.5 (17.43)	46.2 (17.45)	45.4 (15.10)	0.6466	0.8344	0.9074
CD45RA-CD28-	20.1 (4.80)	17.1 (5.54)	20.5 (8.17)	17.1 (9.24)	0.5764	0.6455	0.5887
CD28+CD27+KLRG1-CD57-	24.3 (22.76)	20.9 (15.45)	22.0 (19.53)	12.8 (12.42)	0.3883	0.4239	**0.0133**
CD28-CD27-KLRG1+CD57+	1.0 (1.45)	1.0 (1.71)	2.4 (1.32)	3.4 (1.41)	0.7898	0.5468	0.0487

Significant Dunnett's test ($p < 0.05$) comparing CD8+ T subsets with the reference group. The reference group defined as having BMI, 18.5–24.9. OB, Obese; OW, Overweight; NW, Normal Weight; UW, Underweight. The values are means (SD).

Table 4 CD4+ T cells and subsets in the 4 BMI categories of elderly subjects

	BMI Category				p-value		
	NW	OB	OW	UW	OB-NW	OW-NW	UW-NW
CD4+	26.9 (12.21)	23.8 (9.67)	26.2 (9.91)	22.5 (7.35)	0.7925	0.5421	0.6352
CD28+CD27+	17.9 (10.04)	13.0 (9.87)	16.6 (10.03)	48.1 (14.3)	0.4205	0.9944	0.4745
CD28-CD27-	43.2 (12.73)	45.3 (16.01)	47.4 (9.37)	39.3 (12.45)	0.9611	0.7724	0.6809
CD45RO+CD27-	39.3 (10.46)	31.6 (12.75)	31.0 (17.88)	22.5 (14.21)	0.5734	0.2912	0.5154
CD45RO-CD27+	13.3 (9.36)	22.1 (13.02)	20.5 (15.92)	30.3 (15.19)	0.1882	0.3334	0.9878
CD45RA+CD27+	14.0 (10.41)	26.3 (14.89)	19.9 (10.98)	15.7 (12.71)	**0.0303**	0.4581	0.988
CD45RA-CD27-	56.2 (19.32)	33.6 (21.56)	45.6 (17.47)	49.5 (26.31)	**0.0269**	0.4559	0.7671
CD45RO+CD28-	29.7 (12.89)	20.5 (9.97)	21.3 (14.16)	27.4 (17.8)	0.2348	0.3035	0.7543
CD45RO-CD28+	16.6 (13.32)	33.3 (13.28)	21.0 (13.58)	21.4 (14-8)	**0.0093**	0.7072	0.7521
CD45RA+CD28+	16.0 (12.23)	19.2 (10.49)	13.3 (11.20)	14.8 (12.8)	0.8368	0.8951	0.3426
CD45RA-CD28-	47.2 (16.28)	36.8 (16.17)	40.9 (13.92)	48.6 (14.31)	0.2274	0.6194	0.5461
Tregs	9.4 (4.81)	7.9 (4.24)	9.0 (4.66)	14.0 (7.67)	0.8512	0.4776	0.0918
CD28+CD27+KLRG1-CD57-	8.5 (8.60)	8.3 (6.73)	7.9 (5.71)	13.2 (10.36)	0.5123	0.4521	0.3249
CD28-CD27-KLRG1+CD57+	10.7 (4.60)	10.4 (4.67)	9.8 (3.57)	13.0 (7-08)	0.5412	0.3421	0.4512

Significant Dunnett's test ($p < 0.05$) comparing CD4+ and subsets with the reference group. The reference group defined as having BMI, 18.5–24.9. OB, Obese; OW, Overweight; NW, Normal Weight; UW, Underweight. The values are means (SD).

Correlation between age, anthropometrics and CD8+, CD4+ cells and subsets

Figure 1 depicts correlation analyses. While BMI and % BF had no significant effect on the percentage of CD8+ T cells (p for all trends ≥ 0.05) (Figure. 1 A, B), these values were inversely correlated with CD4+ cells (Figure 1 C, D). None of the nutrient values assessed here correlated significantly with either CD8+ or CD4+ T cells or their subsets (p for all trends ≥ 0.05). There was a significant increase in both CD8+ and CD4+ cells with an increase in plasma CRP. The other plasma factors (albumin, total protein, triglycerides, and ferritin) had no significant correlations with the percentages of CD8+ and CD4+ cells.

Discussion

The effects of nutrition on immune functions have been extensively investigated, but almost exclusively in so-called "WEIRD" subjects (Western, educated, industrialized, rich, and democratic) and rarely in concert. It is not established whether immune alterations found in these populations are representative of the majority of

Table 5 CD8+ T cells and subsets in the 4 BMI categories of elderly subjects

	BMI Category				p-value		
	NW	OB	OW	UW	OB-NW	OW-NW	UW-NW
CD8+	28.5 (4.8)	26.9 (5.8)	25.0 (7.31)	22.5 (6.31)	0.8523	0.3514	0.0443
CD28+CD27+	31.4 (7.32)	27.9 (11.42)	34.6 (6.42)	32.7 (6.68)	0.6545	0.5134	0.4219
CD28-CD27-	12.1 (6.52)	14.4 (6.63)	11.9 (6.01)	8.4 (4.42)	0.5696	0.648	0.9613
CD45RO+CD27-	15.1 (11.21)	9.7 (5.86)	9.2 (5.21)	12.1 (10.01)	0.1541	0.7298	0.5421
CD45RO-CD27+	36.9 (7.21)	34.2 (8.31)	38.9 (9.61)	35.2 (13.61)	0.3854	0.5975	0.8712
CD45RA+CD27+	18.3 (7.31)	16.0 (9.02)	17.8 (11.31)	14.7 (7.91)	0.3043	0.4237	0.6577
CD45RA-CD27-	44.9 (9.31)	46.8 (13.01)	40.9 (12.61)	48.9 (14.23)	0.3175	0.8401	0.2411
CD45RO+CD28-	28.8 (10.01)	35.2 (6.71)	34.3 (14.91)	30.3 (9.01)	0.8421	0.5411	0.6240
CD45RO-CD28+	22.1 (8.0)	17.2 (7.71)	19.9 (9.21)	19.9 (8.31)	0.4954	0.1544	0.7662
CD45RA+CD28+	11.2 (8.61)	12.5 (7.21)	13.9 (9.91)	10.9 (5.81)	0.4954	0.1544	0.7662
CD45RA-CD28-	51.0 (13.91)	59.5 (12.71)	57.4 (19.21)	54.8 (15.61)	0.2732	0.2153	0.7001
CD28+CD27+KLRG1-CD57-	10.5 (9.51)	9.4 (4.31)	8.3 (5.21)	7.9 (7.01)	0.8250	0.1931	0.5442
CD28-CD27-KLRG1+CD57+	9.0 (6.11)	6.5 (2.11)	5.3 (2.21)	9.6 (8.31)	0.6545	0.5134	0.2775

Significant Dunnett's test ($p < 0.05$) comparing CD8+ and subsets with the reference group. The reference group defined as having BMI, 18.5–24.9. OB, Obese; OW, Overweight; NW, Normal Weight; UW, Underweight. The values are means (SD).

Table 6 B cells and NK cells in the 4 BMI categories of young subjects

	BMI Category				p-value		
	NW	OB	OW	UW	OB-NW	OW-NW	UW-NW
B cells	2.9 (2.45)	3.8 (2.44)	3.0 (2.10)	1.9 (1.80)	0.6368	0.5421	0.5520
IgD+CD27-	39.5 (13.0)	39.9 (11.27)	30.2 (11.35)	32.2 (16.20)	0.8581	0.1896	0.3731
IgD-CD27+	16.3 (14.80)	18.3 (12.11)	22.9 (7.11)	16.8 (13.82)	0.7645	0.3172	0.8066
NK Cells	1.0 (3.05)	0.6 (0.71)	0.5 (0.64)	0.5 (1.36)	0.331	0.8060	0.7704

Significant Dunnett's test ($p < 0.05$) comparing B cells and NK cells with the reference group. The reference group defined as having BMI, 18.5–24.9. OB, Obese; OW, Overweight; NW, Normal Weight; UW, Underweight.The values are means (SD).

the world's peoples. The possible detrimental effects of aging on the immune system are further aggravated by numerous physiological and physical conditions, which in many cases can be associated with malnutrition. In the present study, we investigated the effects of nutritional status on immune signatures in a group of healthy young and elderly individuals from a rural area in a developing country where nutritional issues tend to be even more extreme than in developed countries (both in terms of over-as well as under-weight).

The primary findings regarding relationships between nutritional status and immune parameters investigated in this study were that there were some significant differences in the subsets of CD4+ and CD8+ cells but only tendential differences in percentages of B cells and NK cells between young and elderly men based on their nutritional status. While the current study observed changes in both CD4+ and CD8+ compartments, the impact of nutritional status on CD4+ cells and their subsets was more profound than on CD8+ cells and subsets (Tables 2, 3, 4 and 5). Others have previously suggested that fat (one of the predictors of nutritional status) has a more direct effect on CD4+ T cell count, total lymphocyte count, and WBC count than on CD8+ T cells [17]. CD4+ subtypes are directly stimulated by various cytokines including tumor necrosis factor-α, which contributes to the differentiation of CD4+ T cells into the TH1 subset [18] and leptin. In contrast, cytokines produced by fat tissue are not central to CD8+ activation. Therefore, whereas fat directly influences CD4+ cell counts via the action of various adipokines, it may influence CD8+ counts only indirectly via its ability to activate CD4+ T cells.

In contrast to what we initially expected, the current study did not identify many differences among the three BMI categories versus normal. Nevertheless, our findings on the comparison between OB and NW subjects are in agreement with Lynch *et al.* [19], who reported significantly more CD8+ and NK cells in lean controls compared to obese individuals. They further reported no differences in CD4+ levels between obese and lean individuals. In addition, they observed that the phenotypes of immune cells were also different between obese and lean individuals with regard to expression of activation markers and that obese individuals expressed significantly less CD45RA on their T cells. However, when obese individuals were further divided into metabolically healthy (MH) and unhealthy (UH) groups, it was found that circulating NK cells and CD8+ T cell levels were significantly reduced only in the UH obese group. In our study, all the overweight and obese individuals seem more likely to be metabolically healthy as our inclusion criteria excluded all those who had a present or recent past history of diseases including diabetes, hypertension, CVD etc. We, therefore, need further studies in elderly Pakistani subjects including both metabolically healthy and unhealthy obese elderly individuals to investigate the differences as reported previously [20]. It has been suggested that the unique metabolically healthy subgroup of obese individuals appear to be protected or more resistant to the development of comorbidities associated with obesity [20]. Despite having excessive

Table 7 B cells and NK cells in the 4 BMI categories of elderly subjects

	BMI Category				p-value		
	NW	OB	OW	UW	OB-NW	OW-NW	UW-NW
B cells	3.8 (2.19)	4.3 (2.24)	4.0 (1.57)	2.7 (1.81)	0.8483	0.8128	0.3471
IgD+CD27-	14.5 (6.14)	7.8 (6.68)	5.4 (4.35)	25.1 (8.12)	0.4307	0.2002	**0.0159**
IgD-CD27+	24.6 (16.33)	29.9 (12.8)	34.5 (11.21)	12.4 (13.08)	0.6357	0.1720	**0.042**
NK Cells	2.0 (2.2)	0.7 (1.16)	1.0 (1.59)	1.0 (1.70)	**0.0488**	0.3822	0.3614

Significant Dunnett's test ($p < 0.05$) comparing B cells and NK cells with the reference group. The reference group defined as having BMI, 18.5–24.9. OB, Obese; OW, Overweight; NW, Normal Weight; UW, Underweight. The values are means (SD).

Table 8 Multiple comparisons between the low risk group and other groups for percent CD4+, CD8+ and B cells

	Cell type	WC (Cat)	Mean Diff. (LR-cat)	*p* value[1]	WHR (Cat)	Mean Diff. (LR-cat)	*p* value[1]	BF (cat)	Mean Diff. (NF-cat)	*p* value[1]
Young	CD4+	MR	−15.1	0.070	MR	−7.84	0.172	HF	−1.47	0.113
		HR	−0.93	0.738	HR	−0.64	0.078	LF	1.24	**0.020**
	CD8+	MR	−4.11	0.346	MR	−3.04	0.990	HF	−1.11	0.195
		HR	0.35	**0.014**	HR	−4.57	0.632	LF	−1.92	0.343
	B cells	MR	−1.0	0.944	MR	−0.92	0.434	HF	−1.45	0.943
		HR	0.41	**0.006**	HR	−1.04	0.969	LF	−0.72	0.281
Elderly	CD4+	MR	−9.75	0.983	MR	−5.78	0.912	HF	−6.80	0.489
		HR	−7.67	0.727	HR	−6.46	0.844	LF	−7.16	0.550
	CD8+	MR	−3.62	0.822	MR	−6.41	0.125	HF	−4.03	0.932
		HR	−6.39	0.919	HR	−2.41	0.484	LF	−2.56	0.242
	B cells	MR	−2.18	0.793	MR	−1.46	0.921	HF	−1.02	0.707
		HR	−2.07	0.873	HR	−2.23	0.421	LF	−0.77	0.226

[1]The mean difference is significant against low risk (LR) subjects at á = 0.05 level; MR, moderate risk; HR, high risk. For % BF, the mean difference is significant against normal fat subjects at the 0.05 level; LF, low; HF, high fat; Cat, category; [1]p-values were calculated using Dunnett's test. Positive values of the mean difference show pairs of means that are significantly different.

body fat, these individuals display a favorable metabolic profile characterized by high levels of insulin sensitivity, no hypertension, normal lipid, inflammation, and hormonal profiles and, importantly in the context of the present study, a favorable immune profile [19].

In the present study, young and elderly subjects were separated into high, medium and low risk categories on the basis of their WC and WHR values. The comparisons between the low risk (LR) and the other risk groups (high risk, HR; medium risk, MR) show that HR-WC young subjects had significantly ($p = 0.0145$) lower percentages of CD8+ cells ($p = 0.014$) and B cells ($p = 0.0201$) compared to the LR-WC category. In the elderly subjects, there were also differences between these categories but none of them was significant (p for all trends ≥0.05). In the present study, the high body fat

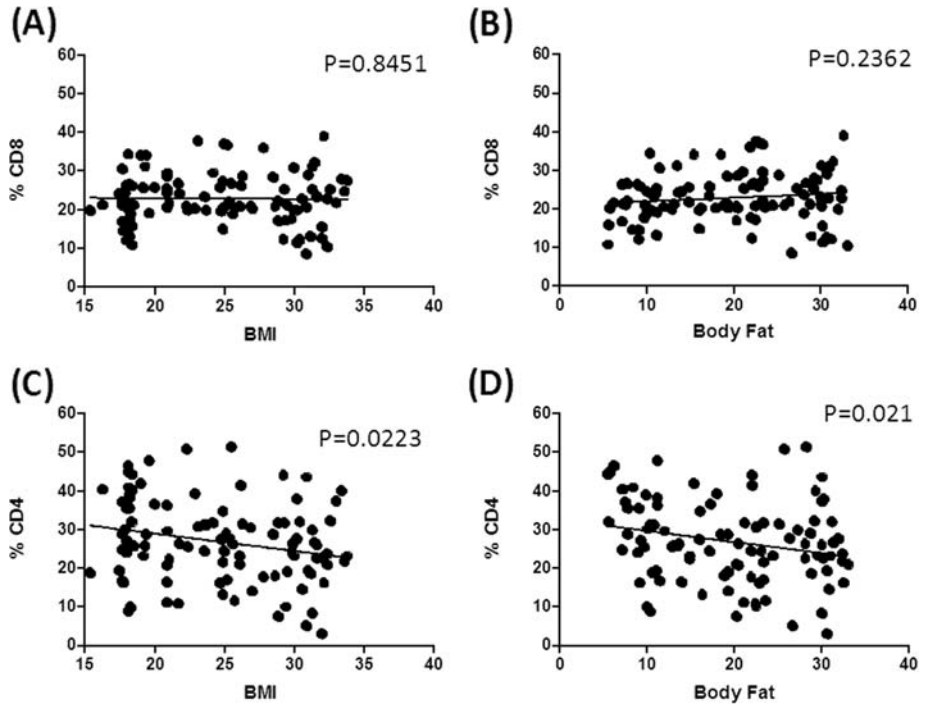

Figure 1 Correlations between percentages of CD8+ and CD4+ cells and BMI and body fat. Significant at p < 0.05.

(HF-% BF) category in young had significantly (p = 0.0201) lower percentages of CD4+ cells compared to the normal fat (NF) category. In general, it has been shown that a decrease in the lean body mass is related to the decrease in body cell mass [21] and that body cell mass depletion is out of proportion to loss of body weight for fat [22]. Furthermore, these results may suggest that compared to the elderly, in young people, WC, WHR and % BF are more sensitive anthropometric measurements influencing the percentages of circulating CD4+, CD8+ and B cells. We are not aware of concrete evidence from previous studies to support the present observations. Some conflicting results, however, demonstrated an increase in CD4+ cells and a decrease in CD8+ cells in obese people (based on BMI) [9]. Others have attempted similar studies of lymphocyte subset frequency in obesity, with conflicting results. Total circulating lymphocytes and monocytes were reported as increased [23] or the same [24] as in the lean controls. Lymphocyte subsets have also been studied, and some investigators have found no differences in numbers of circulating T-cells, B cells, and NK cells [25], while others have shown increased or decreased lymphocytes and T-cells in obese people, and correlated the magnitude of these differences with increasing BMI [26]. Still other investigators have demonstrated a relationship between morbid obesity and CD8+ count only, but not mere overweight and/or obesity when compared with normal weight [23]. Despite these conflicting results, a preponderance of evidence suggests alterations in the immune system of both underweight and obese individuals. For example, monocyte function has been shown to alter in obese humans resulting in increased oxidative burst and phagocytic activity [23]. Such alterations in monocyte function could contribute to the state of systemic inflammation associated with obesity. T-cell phenotypes are likewise altered in obesity as reported previously [23].

No significant differences were found in the four BMI categories of young and the elderly regarding the number of B cells (Tables 6 and 7). In the IgD+CD27-, IgD-CD27+ and NK cells (CD16/CD56) significant differences were noted, respectively, between UW vs. NW; UW vs. NW and OB vs. NW elderly (Table 7). Previous research concerning differences in these cellular phenotypes in the well- and malnourished elderly is scarce. Some studies on the relationship between weight and NK cell number in other age groups, however, have shown a close but conflicting link between body weight and NK cells. Kelley *et al.* [27] reported that individuals who reported losing weight had fewer and less effective NK cells than those who had never lost weight. Likewise, Scanga and co-workers [28] pointed out that obese women consuming a restricted diet had apparent decrements in NK cell cytotoxicity. However, another experimental trial by concerning the influence of obesity on immune response in an adult population indicates that obesity is related to lower T and B-cell mitogen-induced lymphocyte proliferation but normal numbers and function of NK cells [23], while Lynch *et al.,* have shown decreased NK cell levels only in metabolically unhealthy obese compared to a similarly obese but metabolically healthy group [19]. This study had also shown that the levels and phenotypes of NK cells in metabolically healthy obese were similar to lean healthy controls. This decrease in number of NK cells in the unhealthy obese suggests that the immune system is altered in obese people who are at risk from obesity-related comorbidities. In children, a number of studies demonstrated lower proportions of B cells in malnourished compared to well-nourished individuals (nutritional status assessed by weight) [29]. Similarly, a study on overweight children found elevated counts in most types of circulating immune cells, including CD19+ B Lymphocytes, suggesting the presence of low-grade systemic inflammation [30]. In contrast, some other researchers have reported no differences between the percentages of B lymphocytes in malnourished versus well-nourished subjects [31]. If obesity is considered an inflammatory disease [32] then one might expect that NK cell number, or activity, should be increased. In contrast, results on adults suggest that NK cell number and activity may not be changed by obesity [23]. Thus, our results (Table 6) showing no significant difference in NK cells between OB and NW young are in agreement with these previous findings. Taken together, all these studies confirm that malnutrition (whether obesity or underweight) has an effect on the circulating B and NK cells, particularly in the elderly.

In the present study (Figure 1 C D), BMI and % BF were inversely correlated with percent CD4+ cells (p = 0.0223; p = 0.021, respectively), while none of the nutrients (including energy intake) correlated significantly with either CD8+ or CD4+ cells. Although energy intake has been shown to have some effects on the number of circulating lymphocytes, results are disparate. For example, some studies have shown that low energy intake is associated with a reduction in lymphocyte number and proliferation [33], while others have shown no effect of energy on the number of lymphocytes [34,35]. Similarly, except for plasma CRP, none of the plasma factors measured (albumin, total protein, triglycerides, and ferritin) had a significant relationship with either percent CD8+ or CD4+ cells, although a positive correlation between serum albumin and CD4 count and between triglycerides, CD4 and CD8 counts has been reported in a previous study with a group of dialysis patients [36]. The same study also reported non-

significant correlations between CD4, CD8 and BMI, as shown in another recent study in Korean elderly [34]. Yet another study [37] demonstrated serum albumin concentrations correlated positively with some lymphocyte sub-populations.

Strengths and limitations of the current study

This is a pilot study with relatively few individuals and small group sizes; many of the tendencies noted here could likely achieve statistical significance in larger groups. This needs exploring. Potential gender differences could not be addressed because only men could be accessed for this study, but comparisons with women need to be made. We used frozen whole blood rather than isolated PBMC for enumeration of immune cells after careful validation of this method before actual analysis [16]. Other major limitations of this study include a lack of data on immune functions; however, this was outside the scope of this pilot study. Thus, although percentages of certain immune cells were associated with weight, BMI and body fat, we could not evaluate potential differences in the functionality of these cells, but hypothesize that they are present. This remains to be tested.

Conclusions

It is evident from the results of the current study that a state of malnutrition that is nonetheless not overtly associated with diseases such as diabetes, arthritis, and cardiovascular diseases, may still markedly affect cells of the immune system of otherwise healthy young and elderly men. Additionally, future studies will need to measure the activity of immune system cells as well cell counts, because increases or decreases in cell numbers may not necessarily mean a higher or lower associated activity. Even with its limitations, a striking finding of this study was that despite the great differences between the rural Pakistani population tested here and the commonly-reported studies on "WEIRD" subjects in the literature, the overall differences in immune profiles between younger and older people were generally very similar.

Competing interests

All authors declare no competing interests.

Authors' contribution

IA wrote the manuscript. All authors edited the paper and approved its final version.

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Aging affects the proportions of T and B cells in a group of elderly men in a developing country—a pilot study from Pakistan

Iftikhar Alam · David Goldeck · Anis Larbi · Graham Pawelec

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Abstract Immune status is different in the elderly and the young, but whether age-associated differences are similar in developing and industrialized countries is unclear. To approach this question, peripheral blood immune cell phenotypes were analyzed by polychromatic flow cytometry in 50 young and 50 elderly men in a pilot study in a rural area of Pakistan. As a group, the elderly had a significantly lower CD4:CD8 ratio, a lower percentage of CD8+ naïve T cells, and significantly higher percentage of late-differentiated memory cells than the young. No age-associated differences were seen in B cells or NK cells. CD8+ cells as a percentage of CD3+ T cells were positively associated with plasma CRP levels but not other factors. We conclude that there are differences between the peripheral immune cell phenotypes of young and elderly Pakistani men and that these seem broadly similar to those more extensively documented in industrialized countries, despite the marked societal, nutritional, and many other differences in these populations.

Keywords Aging · Immunity · T cells · B cells · Immunosenescence · Developing country

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Introduction

Life expectancy has increased globally over the past two centuries by almost 30 years and by almost 20 years in the last five decades alone (Christensen et al. 2009). This very recent phenomenon emerged as a consequence of improvements in nutrition, hygiene, antimicrobial therapy and vaccinations, and other medical interventions (Pawelec et al. 2009; WHO 2002). Changes in the immune system with aging have been studied extensively over the past few decades, although formally most have investigated differences between young and old people in cross-sectional studies and only imputed changes. The conclusion from the majority of these studies is that aging leads to marked detrimental changes in the composition, function, and competence of the human immune system, commonly termed '*immunosenescence*' (Larbi et al. 2008; Aw et al. 2007). At least partly as a consequence, older populations experience increased morbidity and premature

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mortality, for example, from respiratory tract pathogens (Jartti et al. 2011), an increase in gastrointestinal infections (Larbi et al. 2008), and diminished antigen-specific responses to orally administered vaccines (Grubeck-Loebenstein et al. 2009; Hagiwara et al. 2003). Major age-related phenotypic and functional changes to the T-cell arm of adaptive immunity occur, while B-cell function (Frasca et al. 2008) and the innate immune system (Solana et al. 2006) may be less markedly affected.

The accepted hallmark of '*immunosenescence*' is a decrease in the proportions and numbers of peripheral naïve T cells, especially CD8+ T cells, and reciprocal increases in memory cells (Derhovanessian et al. 2009). Naïve T cells are those that have not yet encountered their cognate antigen, so that it is assumed that during normal aging such encounters gradually decrease the numbers of naïve cells and increase the memory T cells. In addition to the conversion of naïve to memory cells, there is also a gradual loss of functional capacity, cellular integrity, and diversity of both the CD4+ and CD8+ T-cell repertoire with aging (Larbi et al. 2008). The effects of these changes on T cell properties may be further increased if the elderly person is also suffering from a state of malnutrition (Alam et al. 2011).

With the current shifts in demographics resulting in an ever-increasing fraction of elderly people also in developing countries, we need a clear understanding of the relationships between aging and immunity. This is relatively well established for the populations of industrialized countries but not for developing countries. Here, our objectives were to determine the effects of aging in a sample of young and elderly individuals in a developing country in order to determine whether normal aging has any association with alterations in the frequency of peripheral blood lymphocyte subsets in such a less well-studied population. Furthermore, this pilot study was conducted in a rural population in order to ascertain the general applicability of the immunological findings worldwide.

Materials and methods

Study subjects

Participants in the current study were recruited from Peshawar, Pakistan. Potential subjects expressing interest in blood donation were first screened by obtaining a verbal medical history to rule out any health conditions or medication use that could affect immune responses. For the current study, we used a subsample from our previous study conducted in Peshawar, Pakistan (Alam et al. 2011). We selected a convenience sample of 50 families and from each family we selected one young and one elderly subject fulfilling the inclusion criteria. Clinically, healthy subjects were included when they had no history of disease and were not regularly taking any drugs.

Data collection

Age and anthropometrics

Detailed procedures for collection of data on anthropometric measurements and body composition are reported elsewhere (Alam et al. 2011). Briefly, age was assessed from the official records of the subjects (the National Identity Card). Weight and height were measured and body mass index (BMI) was calculated as: weight/height^2 (kilograms per square meter). Waist circumference (WC) and hip circumference were measured in accordance with standard procedures, and waist to hip ratio (WHR) was calculated. Percent body fat (%BF) was assessed using Futrex-5000 according to the procedures recommended by the manufacturer (Futrex®, Hagerstown, MD).

Immunological studies

For assessment of immune phenotypes, blood samples were collected aseptically by venipuncture between 9:00 and 11:00 am. Approximately 20 ml of whole blood was obtained from each subject, drawn into two 9-ml EDTA vacutainers (Becton Dickinson, Franklin Lakes, NJ). The samples were processed following a procedure especially developed and validated for analyses on frozen whole blood (Alam et al. 2012). Blood samples were stored in a −80 °C freezer until further analysis. After thawing in a 37 °C water bath, red cells in blood samples were lysed with saline and water. The cells were then stained with 50 μl of an antibody cocktail and incubated for 30 min at room temperature in the dark. For intracellular FOXP3 staining, cells were resuspended in 1.0 ml FOXP3 Fix/Perm (Biolegend, San Diego, CA), vortexed and incubated for 20 min at room temperature in the dark, followed

by washing with PFEA and FOXP3 Perm buffer. The cells were resuspended in 50 μl FOXP3 Perm buffer containing FOXP3 antibody, incubated for 30 min at room temperature in the dark, washed with PFEA, and resuspended in PFEA. The monoclonal antibodies and fluorescent conjugates used were CD3 (Pacific Orange); CD3 (Alexa Fluor 700), CD4 (PerCP), CD8 (APC-H7), and CD8 (Qdot 705); CD27 (Qdot-605), CD28 (PE), and CD28 (PercP-Cy 5-5); CD45RO (Alex Fluor 400); KLRG1 (Alexa Fluor 488); CD45RA (Qdot 655); CD127 (APC e-Fluor 780); CD57 (FITC); and FOXP3 (PE). Cell populations were measured using an LSR-II flow cytometer and the acquisition software BD FACSDiva (Becton Dickinson).

Clinical chemistry analysis

Plasma was separated from another set of blood samples (approximately 10 ml) by centrifugation at 1,200×*g* and stored in a −80 °C freezer in the Department of Human Nutrition, Agriculture University Peshawar. These samples were shipped on dry ice to the Center for Medical Research (ZMF), Tübingen University, Germany, where they were stored at −80 °C until further analysis. Albumin, ferritin, C-reactive protein (CRP), triglycerides, and total protein concentrations were measured on a Modular Analytics SWA automated analyzer system according to the manufacturer's recommendations (Roche Diagnostics, Mannheim, Germany). All the clinical chemistry analyses were performed in the facilities of Department of Clinical Chemistry, University Medical Center Göttingen, Germany.

The study was approved by the Board of Studies, Department of Human Nutrition, Agriculture University Peshawar. Written informed consent was obtained from all the participants before the start of the study.

Statistical analysis

All the data were statistically analyzed using JMP (Version 8.0. SAS, USA). We took $p<0.05$ to denote a significant difference after correction for the number of comparisons.

Results

Age and anthropometric characteristics

Age and general characteristics of the study subjects are shown in Table 1. Age of the young and elderly subjects ranged 18.0–29.2 (mean, SD, 24.2±3.4) and 50–85.5 (mean, SD, 67.3±8.7), respectively. Compared to the elderly, the young had higher mean BMI and WHR, while the elderly had higher mean weight, WC, and %BF. However, all these differences lacked statistical significance except %BF.

Peripheral immune cell distribution in the young and elderly

T cells

The distribution of CD4+ and CD8+ T cells and their subsets in the young and elderly are depicted in Figs. 1, 2, 3, 4, and 5. Figure 1a shows that the percentage of CD4+ T cells as a fraction of all CD3+ cells in the young tended to be higher than in the elderly but this difference did not achieve significance. However, the elderly did have significantly greater percentages of CD8+ cells than the young ($p<0.0001$). As a result of this, the mean CD4:CD8 ratio was significantly higher in the young than elderly ($p=0.02$; Fig. 1b). Most of the young (80 %; 24.1±

Table 1 General characteristics the subjects

Anthropometry	Young		Elderly		*p* value
	Mean (SD)	Range	Mean (SD)	Range	
Age (years)	24.2 (3.43)	18.0–29.2	67.3 (8.77)	50.1–85.5	–
Weight (kg)	67.6 (14.02)	45.3–92.4	68.7 (14.57)	46.0–97.0	0.7329
BMI (kg/m^2)	25.0 (5.37)	16.3–33.4	24.2 (5.47)	15.4–33.8	0.5056
WC	82.1 (11.20)	64.1–102.2	86.7 (12.39)	62.1–113.2	0.0735
WHR	1.0 (0.11)	0.70–1.17	0.9 (0.12)	0.67–1.21	0.3676
%BF	17.7 (8.48)	5.5–33.1	21.3 (7.99)	9.0–32.6	0.0200

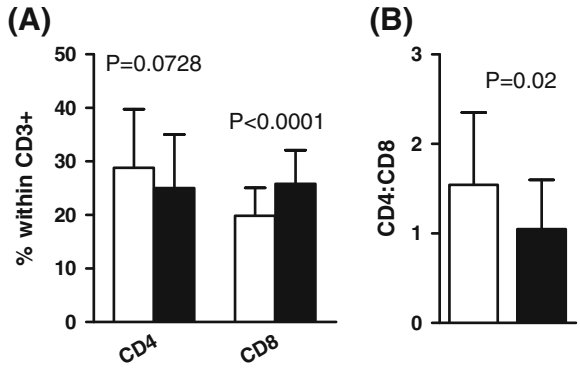

Fig. 1 Percentages of CD4+ and CD8+ cells in young (*white bars*) and elderly (*black bars*) and average CD4:CD8 ratios

3.3 year) had a CD4:CD8 ratio ≥1, while only 46 % of the elderly (65.8±8.4 year) had a normal CD4:CD8 ratio. This is even greater than the ca. 15 % of very elderly Swedes (85 year) with an inverted CD4:CD8 ratio, established in studies giving rise to the concept of the "immune risk profile" (IRP) predictive of 2-, 4-, and 6-year mortality (Derhovanessian et al. 2009) and consistent with the notion of the chronologically earlier occurrence of '*immunosenescence*' in this Pakistani population. There was no difference in the mean age of those young with a CD4:CD8 ratio ≥1.0 or <1.0 (24.1±3.3 and 24.9±3.6 year). Similarly, in the elderly, there was no difference in the mean ages of those with a CD4:CD8 ratio ≥1.0 or <1.0 (65.9± 8.2 year and 68.6±8.8 year).

There was a significantly greater percentage of CD27−CD28− cells both within the CD8+ (Fig. 2a) and the CD4+ (Fig. 3a) subset in the elderly. These surface markers are informative for the state of differentiation of the T cells. Within the CD8+ subset, the young tended to have a higher percentage of CD27+CD28+ cells (less differentiated, more naïve) than the elderly, and did have significantly fewer CD8+CD27−CD28− cells (more differentiated, memory; Fig. 2a). This significantly greater percentage of memory cells and tendency for fewer naïve cells is also exactly a characteristic of the IRP seen in very elderly Swedes (Wikby et al. 2005). The frequency distributions of CD8+ cells with other phenotypes in young and elderly people were as follows: significantly more memory CD45RO+CD27− cells in the elderly (Fig. 2b); significantly more naïve CD45RA+CD27+ cells in the young; significantly less memory CD45RA−CD27− cells in the young (Fig. 2c); significantly more memory CD45RO+CD28− cells in the elderly (Fig. 2d); and significantly more naïve

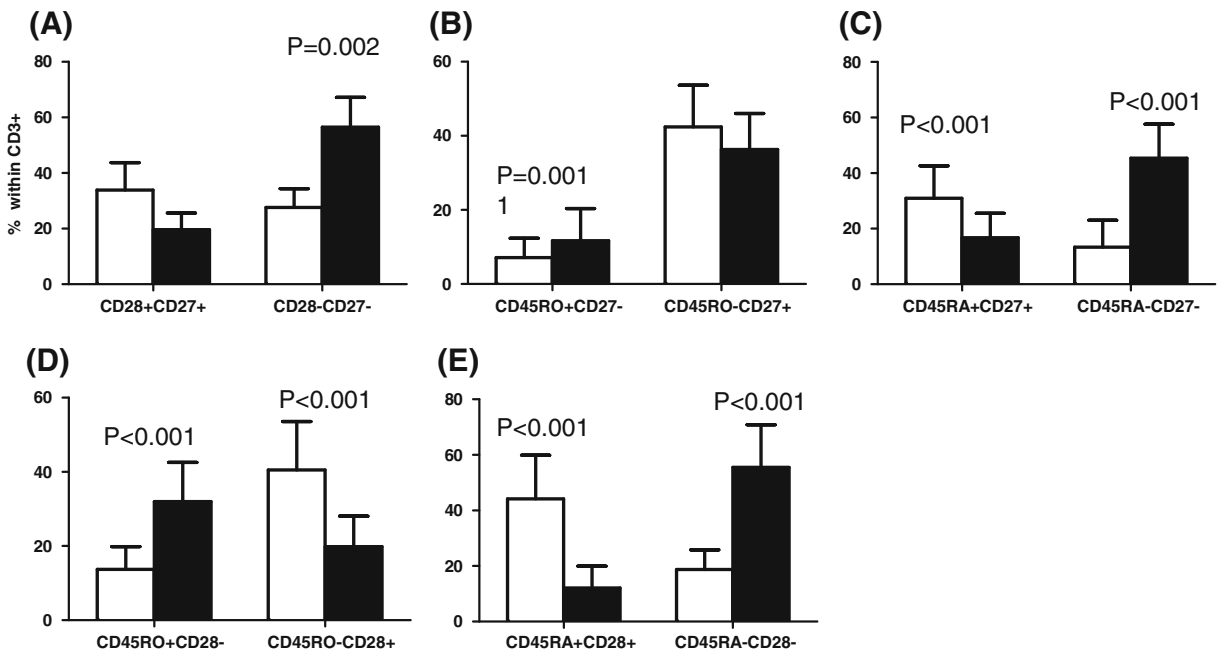

Fig. 2 Distribution of CD28+CD27+, CD28−CD27−, CD45RO+CD27−, CD45RO−CD27+, CD45RA+CD27+, CD45RA−CD27−, CD45RO+CD28−, CD45RO−CD28+, CD45RA+CD28+, CD45RA−CD28− cells within the CD8+ subset in young (*white bars*) and elderly (*black bars*)

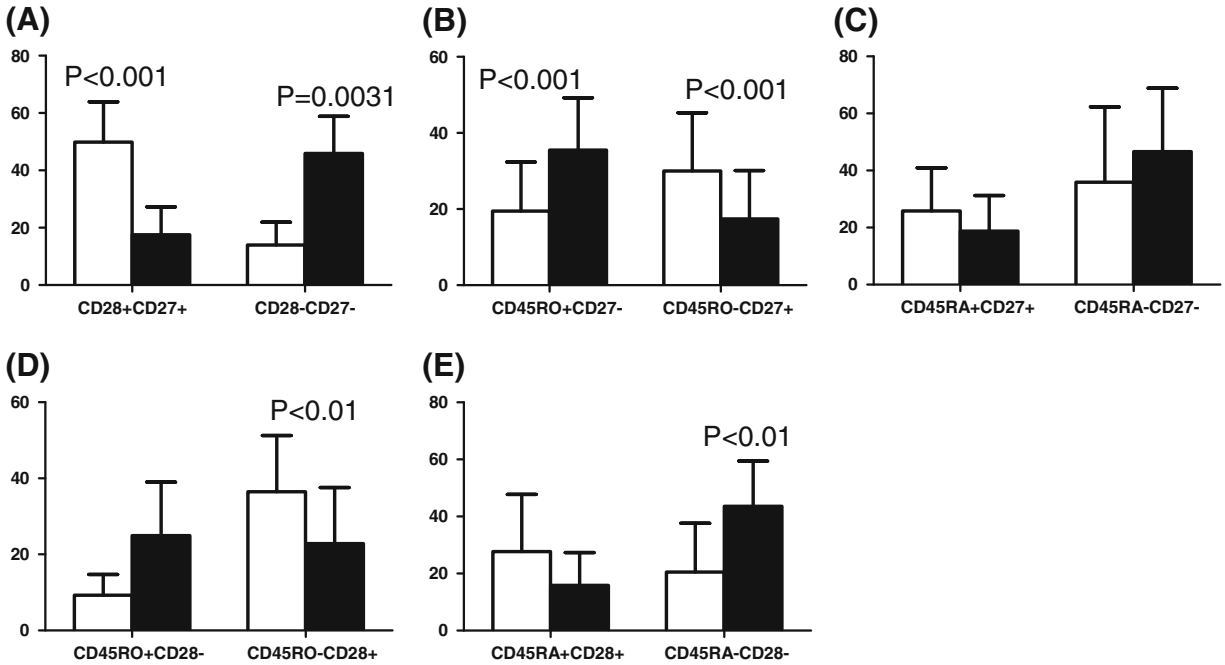

Fig. 3 Distribution of CD28+CD27+, CD28−CD27−, CD45RO+CD27−, CD45RO−CD27+, CD45RA+CD27+, CD45RA−CD27−, CD45RO+CD28−, CD45RO−CD28+, CD45RA+CD28+, CD45RA−CD28− cells within CD4+ cells in young (*white bars*) and elderly (*black bars*)

CD45RA+CD28+ cells in the young and more memory CD45RA−CD28− cells in the elderly (Fig. 2e). Thus, when comparing T cell differentiation stages using different constellations of surface markers, very similar results emerge from this analysis, suggesting its overall robustness.

Age-associated differences within the CD4+ T cell subset are generally less marked than in the CD8 subset, according to most published studies of Western populations. Here, however, we found that the frequency of CD4+CD27+CD28+ naïve cells was significantly higher in the younger than in the elderly (Fig. 3a) and, as with CD8+ cells, there were significantly more memory CD4+CD27−CD28− cells in the elderly compared to young (Fig. 3a). Significantly more CD45RO+CD27− cells were also present in the elderly compared to the young but there were more naïve CD45RO−CD27+ cells in the young (Fig. 3b). Other CD4+ cell phenotypic comparisons showed more CD45RO−CD28+ cells in the young

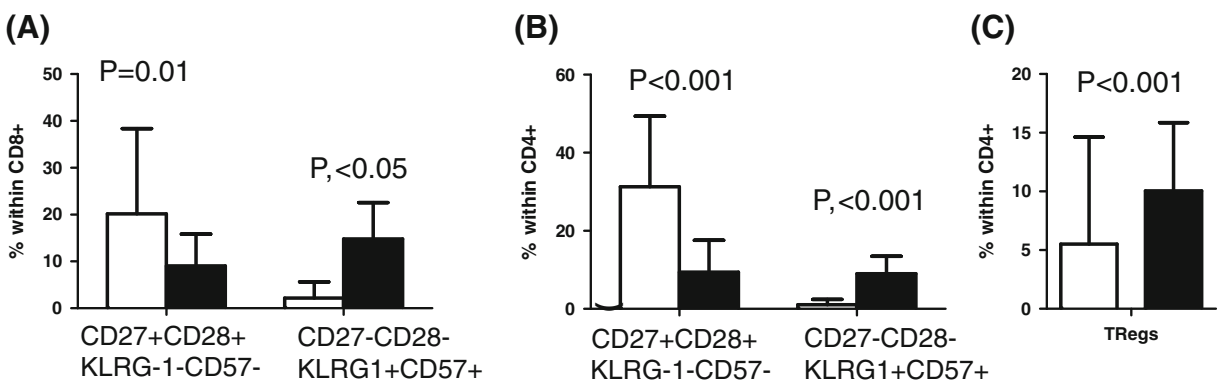

Fig. 4 Distribution of CD27+CD28+KLRG1−CD57−, CD27−CD28−KLRG1+CD57+ cells within CD8+ (**a**), CD4+ (**b**), and Tregs in young (*white bars*) and elderly (*black bars*)

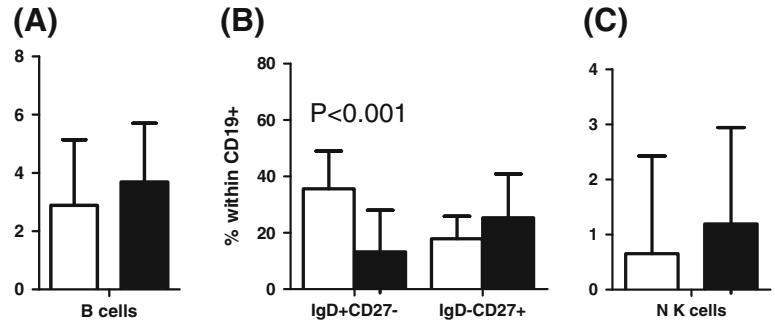

Fig. 5 Distribution of B, IgD+CD27−, IgD−CD27+, and NK cells in young (*white bars*) and elderly (*black bars*)

(Fig. 3d); and more CD45RA−CD28− in the elderly (Fig. 3e). Differences between young and elderly for other phenotypes were not significant. These data are again consistent with the elderly having more memory cells and less naïve cells also within the CD4+ subset, a result somewhat more extreme than commonly reported in the literature on Western populations.

Further detailed analysis of T cell differentiation stages, using more sophisticated polychromatic flow cytometry, showed that many of the CD8+CD27−CD28− cells from the elderly co-expressed the negative NK cell receptors KLRG1 and CD57 (thought to identify the most late-differentiated, possibly dysfunctional, or "senescent" cells). This phenotype was found significantly more frequently in the elderly than the young (Fig. 4a) and was again also true for CD4+ cells with an even higher significance (Fig. 4b). This mirrors what we know about '*immunosenescence*' in Western populations.

A final T cell population that we measured in this pilot study was the so-called T-regulatory cells. These are CD4+ T cells thought to negatively regulate the responses of other T cells, and are identified by the co-expression of high levels of CD25, low levels of CD127, and the presence of the FoxP3 transcription factor in the nucleus. Here, we found that the percentage of these Tregs (CD4+CD25+CD127−FOXP3+) in the elderly was significantly greater than in the young (Fig. 4c). This would perhaps contribute to a state of diminished immune reactivity in the elderly and increase their susceptibility to infection.

B cells and NK cells

Figure 5 shows the percentage of naïve and memory B cells (IgD+CD27−, IgD−CD27+), as well as NK cells (CD56+CD16+). The elderly tended to have more B cells overall, more memory IgD−CD27+ B cells and more NK cells than the young. However, these differences were not significant (p for all trends ≥0.05; Fig. 5a–c). Nonetheless, the percentage of naïve IgD+CD27− B cells was significantly higher in the young (Fig. 5b).

Correlation between age and CD8+, CD4+ cells, and their phenotypes

Figure 6 shows correlation analyses. Age had a significant effect on the percentage of CD8+ cells within the T cell population in this cross-sectional study (Fig. 6a), while the percentage of CD4+ cells tended to be lower with age (Fig. 6d). CD27+CD28+KLRG1−CD57− cells within both the CD4+ and CD8+ subsets were highly significantly lower in the elderly. These cells are the least differentiated, most likely true naïve, T cells of both major subsets. Similarly, a significant reciprocal increase in CD27−CD28−KLRG1+CD57+ cells within the CD8+ and CD4+ subsets with age suggests the accumulation of late-differentiated memory cells, at the expense of reduced naïve cells.

Correlation between plasma biochemicals and CD4 and CD8 cells

There was a significant increase in both CD8+ and CD4+ cells with an increase in plasma CRP (p=0.003 and 0.0041, respectively). The other plasma factors (albumin, total protein, triglycerides, and ferritin) had no significant correlations with the percentages of CD8+ and CD4+ cells (p for all trends >0.05).

Discussion

The effects of aging on immune functions have been extensively investigated, but almost exclusively in

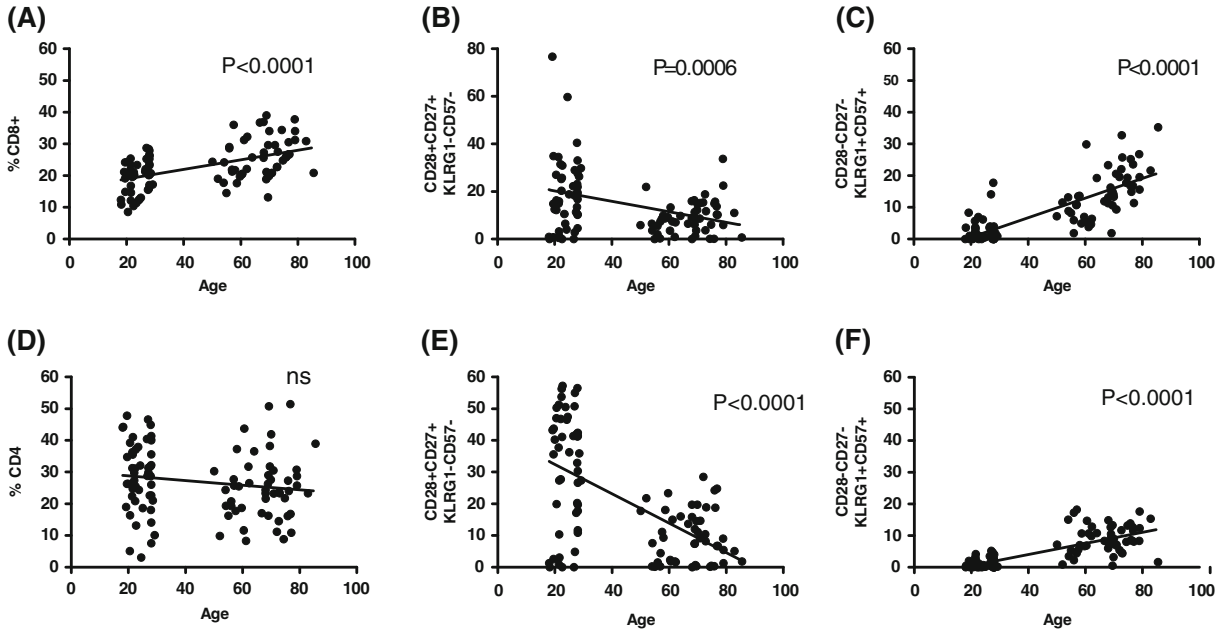

Fig. 6 Correlation analysis of age with CD4+ and CD8+ cells and their subsets

"WEIRD" subjects (Western, educated, industrialized, rich, and democratic). It is not established whether immune alterations found in these populations are representative of the majority of the world's peoples. Even in medically healthy elderly, the overall impact of aging on immune status is that the proportion of memory cells versus naïve cells increases. In the process of aging over time, more memory cells are generated; the body has only a limited capacity of cells it can sustain and the immune system favors memory over naïve cells, which is the basis of adaptive immunity. These age-associated alterations and their consequences have been reviewed elsewhere by us (Derhovanessian et al. 2008; Larbi et al. 2008; Pawelec et al. 1999) and many others.

The present study found a tendency towards the presence of lower percentages of CD4+ cells and reciprocally, highly significantly more CD8+ ($p<$ 0.0001) cells in the elderly compared to the young in a rural population of Pakistani men. There was also a significantly (p=0.02) lower CD4:CD8 ratio in the elderly than young people (Fig. 1a). In analogy to older Western (Swedish) populations, these values would put many Pakistani elderly in the IRP group which is found in a minority of people over 65 in Sweden, and which is known to become predictive of incipient mortality from 85 years of age (Wikby et al. 2008). The definition of "aged" in Pakistan is considerably lower than 65 year (Alam et al. 2011), and in parallel with this, possibly the IRP also occurs at an earlier age. Whether this is indeed predictive of mortality in this Pakistani population remains to be established in longitudinal follow-up studies.

Consistent with many previous studies, there was a reduction in CD28 expression by CD8+ T cells with ageing, presumably as a result of increased antigen exposures over time and lack of expression of CD28 on CD8+ memory cells. In a more extreme manner than usually reported in Western populations, however, there was also a decrease in CD4+ naïve T cells. This may be due to exposure to different constellations of more pathogens, and is similar to what we have observed in Western Alzheimer patients, where amyloid-β may be driving naïve CD4+ T cells to differentiate to memory cells (Larbi et al. 2009).

CD45RA and CD45RO are also often-studied cell surface markers informative for the differentiation state of the cell; thus, they have been reported to be expressed on reciprocal subsets of T lymphocytes in humans and have been used to help delineate naïve and memory phenotypes, respectively (Akbar et al. 1988; 1991; Koch et al. 2008). As alluded to above,

the reduced proportion of naïve T cells commonly seen in the elderly has been assumed to be the result of thymic involution (and the accompanying decreased capacity to generate new naïve T cells) and prolonged exposure to different antigens throughout life (Akbar et al. 2004). Initial activation of the T cell results in a loss of CD45RA and acquisition of the shorter isoform CD45RO on the cell surface (Hamann et al. 1997). Later in the differentiation pathway, effector memory cells may re-express CD45RA, which can be co-expressed with cell surface markers of late-stage differentiation, or even "senescence," especially when co-expressed with KLRG-1 (Voehringer et al. 2000). Consistent with this scenario established in Western populations, in the present study, compared to the young, elderly people had more CD8+CD27−CD28−KLRG1+CD57+ ($p<0.001$) (Fig. 4a) and CD4+CD27−CD28−KLRG1+CD57+ cells ($p<0.001$; Fig. 4b). It has previously been shown that older individuals have relatively higher frequencies of KLRG1+, CD57+, and CD28− cells in the peripheral blood in CD4+ and/or CD8+ T lymphocyte subsets compared to young individuals (Brzezinska 2005; Koch et al. 2008). Cells with these phenotypes have been reported to be incapable of proliferation in response to antigenic stimulation (Ibegbu et al. 2005). A larger proportion of CD45RO+ cells has also been noted in this population in older individuals (Gabriel et al. 1993). It has previously been suggested that the KLRG1+CD57+ population is a senescent phenotype and the KLRG1+CD57− subset is a population of effector or central memory cells destined to become senescent (Ibegbu et al. 2005).

Although B and NK cells seem to be the least affected by aging as also demonstrated in the current study of non-significant differences in percent B cells, IgD−CD27+ and NK cells (Fig. 5a, b, c), changes in the peripheral B cell number with aging have been reported in Western populations (e.g., Caruso et al. 2009) with reductions in naïve B cells and increases in memory cells (Weksler and Szabó 2000; Gibson et al. 2009). However, discrepant results have been reported for memory B cells (Agematsu et al. 2000; Colonna-Romano et al. 2009).

In general, it seems that most or all of the differences between young and old Pakistani men that we have established here appear to be very similar to results from studies conducted in industrialized countries, although the impact on CD4+ as well as CD8+ T cells seems more notable (Derhovanessian et al. 2009) and there are more people in the IRP at a younger age. The data are consistent with chronologically earlier onset of immunosenescence in Pakistani men than in Western populations. They may thus represent true reflections of the impact of ageing on immunity, independently of a plethora of differences between the different populations tested, including nutritional and socioeconomic, as well as potentially genetic and psychological factors, but with different kinetics in different populations.

Limitations of the current study

This is a pilot study with relatively few individuals and small group sizes; many of the tendencies noted here could possibly achieve statistical significance in larger groups. This needs exploring. Potential gender differences could not be addressed because only men could be accessed for this study, but comparisons with women need to be made. Other limitations of this study include a lack of data on immune functions; however, this was outside the scope of this pilot study, and will be pursued in follow-up. Most important will be the follow-up of this population over time. This remains to be tested. The blood samples in the present study were collected following a tight schedule, i.e., between 9:00 and 11:00 am, in order to minimize the possible effects of circadian rhythmicity shown by certain immune cells (Mazzoccoli et al. 2011a, b; 2010; Plytyzc and Seljelid 1997). In humans, total white blood cell counts have been reported to peak at different times of day (Plytyzc and Seljelid 1997) and there has been some seminal research work reporting the effects of circadian rhythms on the numbers of certain T and B cell subtypes (Mazzoccoli et al. 2011a, b; 2010). Statistically significant difference in the observed values of CD20 and TSH serum levels (higher in the young and middle-aged) and CD25+ and HLA−DR+ T-cells (higher in the elderly) have been reported (Mazzoccoli et al. 2010). The need to give due consideration to potential effects of circadian rhythmicity with reported effects on some of the circulating lymphocytes has therefore been recommended (Mazzoccoli et al. 2011a, b; 2010). The 2-h window for blood sampling practiced here was most likely short enough to avoid any of these problems.

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AGING, NUTRITION AND IMMUNITY – THEIR RELATIONSHIP AND INTERACTION

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ABSTRACT

Demographic shifts worldwide are resulting in ever-increasing numbers of the elderly in both developed and developing countries. Malnutrition (both under-nutrition or underweight and over-nutrition or obesity) can affect everyone, and the elderly are no exception. Changes in physical and physiological integrity in the elderly are accompanied by a gradual decline in immunocompetence, commonly termed '*immunosenescence'*. Marked differences between young and old subjects in the proportions of naïve and memory T cells have been reported, as well as less marked differences in B cells and other immune cells. The number and proportion of late-stage memory T and B cells commonly increases, being particularly prominent in the CD8+ cytotoxic T cell pool. The accumulation of late-stage potentially "terminally" differentiated CD8+CD27−CD28−CD45RA+ cells is often considered a hallmark of immunosenescence. Malnutrition in old age can further add to the severity of this age-associated remodeling of the immune system. Age-associated obesity, in particular, is accompanied by greater chronic inflammation, as reflected in increased plasma concentrations of C-reactive protein (CRP), IL-6, TNF and other factors which may mark compromised immunity. These physiological and immunological changes accompanying aging, markedly affected by nutritional status, are likely to be different in different parts of the globe. Data suggest a gradual decline in both nutritional status and immune functions with aging, but the details of these processes, and potential differences in different societies are unclear. In the following review, we will discuss the hallmarks of age-associated immune system changes and consider how these might be affected by nutritional status.

KEYWORDS: aging, physical changes, nutrition, immunity, immunosenescence

1.0 PHYSICAL AND PHYSIOLOGICAL CHANGES ASSOCIATED WITH THE AGING PROCESS

Demographic shift: The elderly population is increasing in both developed and developing countries; demographic studies show that overall improvement in living conditions and better health-care facilities have led to an increased average life expectancy, resulting in increased numbers of elderly people [1-3]. Worldwide, life expectancy has increased markedly during the last few decades [1]. This very recent phenomenon emerged as a consequence of improvements in nutrition, hygiene, antimicrobial therapy and vaccinations [4,5].

Who is an elderly person? A universal definition of "elderly" is impossible, depending as it does on the conditions and circumstances in different countries and societies. Nonetheless, most countries in the developed world have thus-far accepted the chronological age of 65 years as a definition of an "elderly" or "old" person [6]. However, this cut-off derives from conditions of over a century ago, and nowadays, redefining the elderly in industrialized countries, where most studies are carried out, as those $\geq$ 75 years [7] may be more appropriate. But in developing countries, it is still appropriate to consider "elderly" as 65 years of age, and we will use this arbitrary cut-off throughout this review.

Changing immunity as an aspect of the multiple physiological changes with aging: Changes in the immune system with aging have been studied extensively over the past few decades, although most have investigated differences between young and old in cross-sectional studies in developed countries and only imputed changes. The conclusion from the majority of these

studies is that aging leads to marked changes in the composition, function and competence of the human immune system, commonly termed '*immunosenescence'* [8-10]. At least partly as a consequence of an altered immune system, older populations experience increased morbidity and mortality from infectious disease, e.g., respiratory tract pathogens [11,12], gastrointestinal infections [13], and antigen-specific responses to orally administered vaccines are diminished [14]. Major age-related phenotypic and functional changes to the T-cell compartment of adaptive immunity occur [9], while B-cell function [15] and the innate immune system [16] may be less markedly affected.

Age-associated physical and physiological changes, weight, and nutritional status: During normal aging, a decline in lean body mass (LBM) with a simultaneous increase in fat tissue usually occurs. Adverse changes in physiological functioning of the body would therefore not be unexpected given such large alterations in body composition. Increased fat infiltration into muscles with aging is associated with reduced lower extremity performance. The most obvious physiological changes include reduction in muscle strength, sudden fall in basal metabolic rate (BMR), impaired nutritional status, reduced ability to resist infections and a decline in immunity [17].

Changes in body weight can be the first warning sign for changes in LBM and fat tissues [18-21]. Importantly, weight loss in the elderly is typically unintentional and is associated with increased risk of functional impairment [21], mortality [18,22], and other related complications [23]. Perhaps rather surprisingly, the exact prevalence of weight loss among elderly adults is not well-documented, and the literature on this topic is limited to a few small studies that utilize a

variety of definitions for weight loss [24,25]. Over the first 3 years of follow-up, more than 15% of the Cardiovascular Health Study cohort experienced ≥5% weight loss while an additional 5% had weight loss of ≥10% [25]. In a separate study of male Veterans living in Seattle, one quarter of the sample experienced ≥4% weight loss over a 2 year period [26].

Gender differences in nutritional and health status of elderly have also been investigated extensively. Of particular note are studies reporting gender-associated differences in total body fat distribution and content; with women having higher levels of adiposity deposited in the subcutaneous compartment compared to men [27]. Particularly, after menopause the fat deposition in women shifts toward a more central/male-like pattern of fat distribution [28]. It has been previously shown that females between 12 and 80 years of age have a higher percentage of body fat as compared to males. This difference starts notably with puberty [29] and varies between 6 - 11% higher for every decade studied [30] with variations due to ethnicity, genetic, and environmental factors [31]. Sex hormones, including oestrogen, progesterone and androgen, have been reported primarily responsible for these differences [32-37]. Importantly, these sex differences in the distribution of BF are significantly associated with differential risks for various chronic and immune diseases [38].

With such marked physical and physiological changes with aging, the overall nutritional well-being of the individuals is compromised. Consequently a number of nutrient deficiencies emerge in old age including zinc [39], vitamins A, B6, folate and B12 [40,41]. Data from the third National Health and Nutrition Examination Survey [42] in the USA as well as other studies [43-46] clearly demonstrate a linear decline in food intake starting from the age 20 to 80 years in both men and women, particularly a drastic decrease in caloric intake [47]. Average daily energy

intake has been reported to decreases by about 30% between 20 and 80 years [47]. The loss of muscle mass with aging can further contribute to a loss of mobility [48], and decreased physical functioning and activity, which may lead to reduced energy requirement [49]. It is also noteworthy that the rate of the aging process may differ greatly between individuals, resulting in different subgroups within the elderly population **(***e.g.*, the healthy elderly versus the frail older group) [50], implying that nutrient requirements with aging are highly individualized.

2.0 IMMUNITY AND ITS MODULATION BY AGE AND DIET

2.1 COMPOSITION OF THE IMMUNE SYSTEM

Innate Immunity: Innate immunity is comprised of five types of defensive barriers: anatomic, physiologic, endocytic, phagocytic, and inflammatory. Soluble factors such as lysozyme, which is a hydrolytic enzyme capable of cleaving the bacterial cell wall, or complement, also contribute to innate immunity [51-54]. The innate immune system is also composed of protective cells and molecules. Some examples are natural killer cells **(**NK cells), scavenging macrophages [52,53] and the complement system [53,54].

Adaptive Immunity: The other main arm of immunity is termed adaptive because it generates highly antigen-specific memory after encountering target antigens which, unlike those triggering the innate system, are infinitely variable, usually derived from infectious agents. Adaptive immune cells are the thymus-dependent T cells and the antibody-producing B cells, produced in the bone marrow by continuous haematopoiesis, a process requiring stem cell renewal over the whole lifespan, which may be affected by age and extrinsic circumstances [51,52].

2.2 CELLS OF THE IMMUNE SYSTEM

T and B cells: Most of the lymphocytes in the blood are T cells, making up 22–30% of total nucleated white cells (the majority of which are polymorphonuclear phagocytes, part of the innate immune system), whereas circulating B cells represent only 7–10 % of white blood cells. T and B cell differentiation stages can be distinguished by their expression or lack of expression of certain cell surface molecules. For example, approximately 75% of circulating B cells in young people do not express a monomorphic costimulatory receptor called CD27, indicating that they have recently emerged from the bone marrow and have not yet encountered antigen in the periphery [52,55].

While B cells mature in the bone marrow, T cell precursors migrate to and mature in a distinct organ, the thymus, where they go through further developmental stages, to produce mature naïve CD4+ and CD8+ T cells (often called “helper” and “cytotoxic” T cells, respectively). CD4 and CD8 are cell surface co-receptors which assist in binding of the T cells to antigen-presenting cells (APC), required for their activation. After their “education” in the thymus, CD4+ T cells recognize peptide fragments of antigen only when presented in the context of class II self-major histocompatibility molecules **(**MHC class II) by the APC, usually dendritic cells **(**DC). In contrast, CD8+T cells recognize shorter peptide fragments presented on MHC class I molecules. On completion of maturation, i.e. elimination of overtly self-reactive cells and selection of cells recognizing self-plus-antigen complexes, these preprogrammed naïve cells leave the thymus and migrate to the periphery. Clearly, the output of naïve T cells depends on the integrity of the

thymus, but this with changes adversely with age and poor nutrition. Näive T cells are long-lived cells and can circulate for years if not stimulated by their cognate antigen (processed and presented by APC, as mentioned above). They may, however, die before ultimately encountering their specific antigen, and this could happen in old age [56], leaving a "hole in the repertoire" should infection with the relevant specific pathogen occur in later life. Otherwise, upon antigen encounter, näive T cells become activated, proliferate and differentiate into memory and effector T cells to exert their anti-pathogen functions. CD8+ cytotoxic T cells mediate lytic reactivity once activated and can kill infected cells directly by production of cytotoxins such as perforins and granzymes [57]. Activated CD4+ T helper T cells supply cytokines to 'help' B cell maturation and CD8 differentiation, and may also be cytotoxic themselves.

Sub-populations of T Cells: CD4+ and CD8+ T cells are further divided and categorized into sub-populations based on the expression of additional cell surface molecules indicative of differentiation stage. This division is useful when one is interested in studying various cell phenotypes associated with specific functional characteristics. However, it must be borne in mind that these represent a continuum of differentiation stages and are not discrete cell subsets.

There are at least two established models in use for identification of T lymphocyte differentiation stages. The first model utilizes the expression of the TNF-family costimulatory receptor CD27 in combination with the CD45RA isoform of the leukocyte common antigen (a phosphatase). This strategy identifies one population of antigen-inexperienced cells, the naïve cells **(**CD27+CD45RA+**)** and three memory populations designated central memory **(**CM; CD27+CD45RA−), effector memory **(**EM; CD27−CD45RA−), and the most differentiated

‘revertant’ memory cells, which have re-expressed the ‘naïve’ cell marker CD45RA **(**EMRA; CD27−CD45RA+) [58-60].

In a second model, instead of CD27, the immunoglobulin super-family co-stimulatory molecule CD28 or the chemokine/lymphoid homing receptor CCR7 are used in combination with CD45RA [59,61,62]. Identifying cells on the basis of this model also gives three distinct subsets. These subsets also differ along a continuum of differentiation stage; early stage **(**CD27+CD28+), intermediate stage **(**CD27+CD28−), and late stage differentiated cells **(**CD27−CD28−) [61,62]. Nevertheless, these models do not allow absolutely clear distinctions between naïve and memory cells. The reason for this limitation is that due to overlapping dynamic loss and re-expression of CD27, CD28, and CD45RA with cellular differentiation and antigenic stimulation, several of the sub-populations described share similar functional characteristics. Thus, naïve, CM and early-stage differentiated cells show a propensity to migrate to the secondary lymphoid organs. There, the cells interact with APC and rapid proliferation occurs, which produces the required larger numbers of antigen-specific effector cells. These then readily migrate to inflamed tissues [59,60]. CM cells, which have previously responded to infection, have relatively greater capability to produce effector cells more quickly than antigen-inexperienced naïve cells [59]. The EM and EMRA sub-populations strongly overlap with the intermediate and late-stage differentiated cells and they migrate to peripheral tissue **(***e.g.* the skin, mucous membrane etc). In addition, they express cytotoxic effector molecules **(***e.g.* perforin and granzyme B) and readily produce inflammatory cytokines (e.g. IFN-γ) [64,65], which can help in providing immunity to the body in a number of ways but may also inflict collateral damage if not closely controlled. The use of

the terms naïve, CM, EM and TEMRA nonetheless provide a useful conceptual framework in which to view age- and nutrition-associated changes to immune status.

Homeostasis of T Cells: It has now been well established that the homeostasis of näive T-cells remains relatively stable during life [66]. Production of large numbers of näive T cells in early life is followed by their exposure to pathogens, clonal expansion, and differentiation, performance of their function, clonal contraction and death of the majority with retention of some as memory cells. In later life, the numbers of naïve T cells can only be increased by homeostatic proliferation of existing näive T cells in the periphery after age-associated thymic involution [67]**.** A steady loss of näive T cells throughout life is therefore the norm, as they differentiate into memory/effector T cells after antigen challenge. Because thymic output declines significantly with age due to thymic involution [68], there is a decline in the contribution of the thymus to näive T-cell homeostasis over the life-span. This suggests that homeostasis of the näive T-cell compartment in adults may rely mostly on peripheral T-cell proliferation and prolonged survival (longevity) of näive T cells. This contributes to the age-related changes in the immune system, particularly in T cells, as discussed in detail in the next section.

2.2 AGING AND IMMUNITY

Changes to the immune system with aging have been studied and reviewed extensively over the past few years [9, 11, 69, 70] as briefly summarized in Table 1. However, most of the previous studies mainly reported on the so-called WEIRD (white, educated, industrialized, rich and democratic) aged populations in the developed world. Hence, much of our knowledge of

immunity and aging is derived from and may in some ways be limited to this minority of people worldwide.

Although many studies refer to age-associated changes, the majority is cross-sectional and can therefore actually only refer to differences between a current young and old cohort. It is usually difficult to investigate the actual changes in immunity with age in humans in longitudinal studies due to time and resource constraints [71]. However, there is limited number of longitudinal studies on individuals over 85 years of age, the group of people deliberately focused on in the Swedish OCTO/NONA studies. Both OCTO **(**subjects selected for exceptionally good health) and NONA **(**free-living subjects but representative health status) identified a so-called "immune risk profile (IRP)'' for 2, 4 and 6-year mortality at follow-up consisting of an inverted CD4:CD8 ratio of <1, accumulations of CD28-negative CD8+ T cells, and decreased B cells [72]. However, nutritional variables were unfortunately not taken into account in these studies.

Marked differences have been observed between young and old subjects in the proportions of naïve and memory T cells present in the peripheral blood. In newborns, the ratio of naïve to memory T cells is quite high; in adults the ratio is reversed because most of the naïve T cells have been exposed to antigen, and hence converted to memory cells, as mentioned above. As the thymus progressively involutes with age, fewer T cells are produced, and the naïve T cell subpopulation is not replenished. Consequently, the stock of naïve T cells becomes depleted and the aged immune system cannot respond as well as a young person to a new antigen [51]. It is important to note, however, that possessing fewer naïve cells has not actually been shown to be deleterious in aged people. T cell homeostasis ensures that as naive cells are lost, there is a

compensatory increase in the numbers of memory cells. Consequently, the number and proportion of memory T cells with a late-differentiated phenotype **(**e.g. CD27−CD28−CD45RA+) commonly increases [73-76]. These phenotypic changes are particularly prominent in the cytotoxic T cell pool.

The accumulation of CD8+CD27−CD28−CD45RA+ cells is considered a hallmark of immunosenescence [73-75], which implies that young individuals exhibit marked alterations in their T cell repertoire as compared to the elderly [77,78]. As with the naïve cell situation, however, this "remodeling" of immunity, or rather the relative proportions of T cell differentiation phenotypes, has never been shown to be causally related to mortality or any other deleterious clinical outcome, and should thus be considered a hallmark of aging but not necessarily "senescence" per se. It may be viewed as an adaptive change to the requirements of an elderly host, and has been termed "remodeling".

However, as discussed above, it is clear that the involution of the thymus is an important feature of normal anatomical as well as physiological development but nonetheless has profound effects on aging of the immune system [79-81]. Thus, even the apparently normal shrinkage of the thymus may have an active role as a weakening source of näive T lymphocytes and the related thymic hormones [82]. Consequently, this process indirectly results in immunity being greatly dependent on the existing pool of memory T cells and the remaining naïve cells depending on the individual´s "immunological history" of exposures [83].

We now know that young adults thymectomised in the first few years of life exhibit reduced numbers and proportions of naïve T lymphocytes, and sometimes increased numbers of cytotoxic T cells in adulthood [84-86]. These immune profiles of the young after thymectomy are quite similar to those of far older adults, who also show little or no thymic output [84,85]. The thymectomy study also highlighted an important contribution of the individual´s exposure to micro-organisms in determining their immune profile. Sauce *et al.* showed that infection with Cytomegalovirus **(**CMV) exhibited more severe alterations in the T cell repertoire compared to those who were free of CMV infection [84]. This combination of little or no thymic output and selective expansion/maintenance of cytotoxic T cell populations leads to a gradual 'filling of the immunological space' with CD8+ T cells just as is the case with "normal" aging [87]. It is noteworthy that CMV infection was also part of the cluster of parameters making up the immune risk profile in the OCTO/NONA studies, and that in cross-sectional studies of CMV-negative people, no significant age-associated difference in proportions of naive T cells can be found [88].

Most immunosenescence research has focused on adaptive immunity as it was once postulated that innate immunity is better preserved with aging [89]. It is now appreciated, however, that age-related changes are visible in nearly all cells of the innate immune system as well. For example, aging is associated with decreased natural killer cell function and altered neutrophil migration [90,91], which starts as early as adolescence [74,92]. Immunosenescence, by definition, is reflective of the erosion occurring in immune competence over the course of life and is at its maximum in old age [9,93,94].

2.3 NUTRITION AND IMMUNITY IN THE ELDERLY

Under-nutrition and its effects on the immune system: On the other hand, undernutrition also exerts a strong negative effect on immune responses in the elderly [reviewed in ref. 95]. Early work on nutrition and immune functions was primarily based on the findings from studies on nutritional deficiencies in young children from developing countries [96]. Much evidence today points to nutrition as an important determinant of immune functions across all age groups worldwide. Cell-mediated immunity is particularly sensitive to deficiencies in macronutrients [reviewed in ref.97]. In the elderly, immunological dysfunctions may occur because of single nutrient deficiencies, such as of vitamin A, iron or zinc, or because of multiple nutrient deficiencies in conjunction with general malnutrition [47, 98-114] and protein-energy malnutrition [115,116], many of which can be reversed by nutritional supplementation interventions [117,118].

Nutrient deficiencies often result in an increased risk of developing infections [12,13, 119, 120]. This relationship between nutrient deficiencies and infections has been better investigated in studies with the effect of multi-micronutrient supplementation on resistance to infection in the elderly subjects [113, 121]. While some have shown benefits of nutritional intervention in reducing the burden of infectious diseases in the elderly [e.g. 121,122], others have shown no significant effects [e.g. 112]. A multi-center nutritional trial [123] demonstrated a slightly reduced risk of pressure ulcer infections in elderly patients who were given daily protein-calorie supplements. The study concluded that this energy protein intervention was associated with a decreased risk of pressure ulcer incidence. The study of institutionalized elderly persons that

demonstrated clinical benefits [122] also suggests that trace minerals, in particular, may be the key nutritional factors for preventing infection in older adults. Other studies of zinc supplementation in older adults have demonstrated enhanced DTH responses and elevated lymphocyte numbers and function of natural killer cells [e.g. 39]. Some studies have examined the effects of vitamin C (ascorbic acid) supplementation as adjunctive therapy for respiratory tract infections. One such study [116] recruited hospitalized elderly patients with bronchitis or pneumonia to compare vitamin C (200 mg/day) with placebo. The addition of the deficient nutrients back to the diet can restore immune function and resistance to infection [117]. Taken altogether, states of malnutrition and infection can aggravate each other and lead to a vicious circle [100].

Over-nutrition and its effects on the immune system: Like undernutrition (or underweight), overweight and obesity are the other reciprocal forms of malnutrition at epidemic proportions globally [118]. The relationship between obesity and immunity is logically to be expected mainly on three lines of evidence. First, obesity is linked with a multiplied risk of virtually all types of cancers. Second, obesity has a close association with all chronic, systemic states of inflammation, which may contribute to the development of obesity-related co-morbidity. Third, a number of hormones (e.g. leptin and adiponectin), which have been shown to play an important role in regulating immune functions, are likely to be deregulated in obesity [124].

Obesity as chronic inflammation: Obesity in humans is associated with low-level inflammation [125]. The inflammatory response triggered by obesity involves a number of components of the classical inflammatory response to pathogens [126]. These include systemic increases in circulating inflammatory cytokines, adipokines and acute phase proteins, recruitment of

leukocytes to inflamed tissues, activation of tissue leukocytes, and generation of reparative tissue responses. However, the nature of obesity-induced inflammation is unique in comparison to other inflammatory paradigms including infections, autoimmune diseases, and the likes. Qualitatively, for example, in chronic obesity, a low-grade activation of the innate immune system is produced that affects steady-state measures of metabolic homeostasis over time. Obesity-associated inflammation is hence characterized by a low-level but chronic inflammatory state.

The association between obesity and the development of major complications in acute pancreatitis [127], fatty liver diseases [128], vascular inflammation and coronary heart disease [129], chronic obstructive pulmonary disease [130], risk of cerebral ischemia and brain injury [131], atherosclerotic vascular disease and myocardial infarction [132], and cancers [133] are strongly linked to chronic inflammation. In particular, insulin resistance, a direct or indirect result of obesity, is characterized by a chronic state of subclinical inflammation [134] and inactivation of a number of inflammatory mediators [135]. An elevated serum concentration of CRP [136], IL-6, IL-8 and TNF is observed in obese individuals with elevated insulin resistance [137]. In brief, obesity may affect immunity through the mediation of one or more of the aforementioned inflammatory states.

In addition to the findings that obesity leads to inflammation, there are also some recent data showing that the immune system can affect obesity in the same way as obesity can affect immunity. In particular, deficiency of several genes coding for innate immune factors **(**e.g., IL-6, GM-CSF, IL-1RI, and IL-18) has been shown to lead to mature-onset obesity in mice [138,139]. Moreover, combined IL-6 and IL-1 deficiency causes early-onset obesity in mice [139]. Conversely, mice with enhanced IL-1 activity are lean and resistant to diet-induced obesity

[140]. IL-6 has been shown to have obesity suppressing effects by the virtue of its ability to increase energy expenditure in tissues [141]. Furthermore, it is believed that the hypothalamus might be the site of action in the brain since an altered expression of peptides responsible for regulation of energy balance has been found in IL-6-deficient mice. The mechanism whereby IL-1 mediates anti-obesity effects may be partly through leptin as it has been shown that leptin injection specifically increases the hypothalamic levels of IL-1 [142,143].

Caloric restriction (CR), inflammation and immunity: The effects of obesity and its associated inflammation can be reversed through caloric restriction (CR); a state of chronic negative balance achieved in various experimental animals. CR has been successfully exploited for robust, nongenetic means of extending the mean and maximal lifespan in some experimental animals [144]. With a sufficiently large set of research data, mostly from studies with animal models, the CR has been suggested to have significant impact on various components of the immune system [145]. These include responses of T cells to mitogens, NK cell activity, CTL activity, and the ability of mononuclear cells to produce proinflammatory cytokines [145-147]. CR has been suggested to have positive effects on NK cells and CTL as reflected in the much reduced incidence of tumors in caloric restricted mice [148-150].

What is the possible mechanism by which CR may induce the aforementioned effects? An improvement in thymic cellularity has been suggested as a result of CR in old mice [150]. In that study the number of total thymocytes and double-positive T cells was doubled, interestingly, without significantly increasing the size of the thymus It was, therefore, suggested that CR preserves immature T cell precursors in the thymus during aging to maintain higher concentrations of circulating T-helper and naive T cells in peripheral blood. Using aged rats, CR

has been shown to attenuate the age-associated increase in memory:naïve T cell ratios, attributable to a significant reduction in proinflammatory cytokines such as TNF and IL-6 [150]. A CR-associated increase in thymopoiesis and improvement in the TCR diversity with increased naïve:memory T cell ratios in the periphery has also been demonstrated [151]. What is the underlying mechanism of CR-induced effects on the thymus? This remains unclear, but arguably, the neuroendocrine factors responsible for regulating energy balance in the body may be partly having significant effects on immune function during CR by causing an increase in a number of orexigenic factors, importantly, ghrelin [152] and possibly by reducing anorexigenic hormones, such as leptin [153].

Metabolic implications of obesity: It is noteworthy, however, that not all obese individuals may be similarly at risk for adverse inflammatory outcomes and immune compromise and that metabolically healthy **(**MH) obese individuals have a considerable edge over metabolically unhealthy **(**MUH) obese individuals. As an example, Lynch *et al.* reported significantly more CD8+ and NK cells in lean controls compared to obese individuals. The authors further reported no differences in CD4+ T cell levels between obese and lean individuals [154]. In addition the authors observed that the phenotypes of immune cells were also different between obese and lean individuals with regard to activation and differentiation markers and that obese individuals had significantly less CD45RA+ cells. However, when obese individuals were further split into metabolically healthy (MH) and unhealthy (MUH) groups, it was found that circulating NK cells and CD8+ cell levels were significantly reduced only in the UH obese group. There are other studies reporting large health differences between these two distinct groups [154-159]. It has been suggested that the unique metabolically healthy subgroup of obese individuals appear to be protected or more resistant to the development of co-morbidities associated with obesity. Despite

having excessive body fat, these individuals display a favorable metabolic profile characterized by high levels of insulin sensitivity, no hypertension, normal lipid, inflammation, and hormonal profiles and importantly a favorable immune profile [159].

Malnutrition and thymic involution: Thymic involution in normal aging has been established for many years, but the idea that nutrition also plays a vital role predates this realization by a long time: in 1810, J.F. Menkel noted the relationship between the size and functions of the human thymus and malnutrition [16-]. He described atrophy of the thymus in malnourished patients and since then, the term '*nutritional thymectomy*' has been coined and is in common usage today. By 1845, Simon had observed that the thymus is '*a barometer of malnutrition and a very sensitive one*' [161]. Interestingly, these observations were made, however, over almost a century before the role of the thymus in lymphocyte development was truly understood. Currently, it is well-established that malnutrition directly leads to thymic involution, truly making the thymus '*a barometer of malnutrition and a very sensitive one*' [161].

To link malnutrition with immune decline, it has been suggested that general undernutrition with specific deficiencies of some micronutrients (vitamin B6, amino acids, fatty acids, and zinc) results in decreased thymic weight with symptoms of immunosuppression [162-165]. A considerable amount of research work has been conducted in both humans and animals on the impact of protein–energy malnutrition [e.g. ref. 166], and on Zn [e.g. ref. 167] in relation to thymic development. Much of this work has used the simple outcome measure of thymic size, although some of the studies, particularly those on humans, have looked at cell-mediated immunity [168, 169].

On the other hand, nutritional deprivation has been shown to have proportionately greater impact on the size of thymus [170]. However, just as the size of thymus has been suggested as '*a crude index of function*', T cell number and various tests of cell-mediated immunity are also '*crude measures of protection*' [161]. Circulating T-cell levels are homeostatically regulated and hence often maintained or in most of the cases may be even elevated in sick and malnourished individuals, which may mask defective functions [168, 169] or a '*critical hole*' in the T-cell repertoire caused by defective clonal selection in a malnourished thymus [161]. The observation that the thymus is always the organ most vulnerable to nutritional stress also fits with the observation that thymic atrophy represents an ordered process controlled by the induction of apoptosis [171].

Malnutrition-associated thymic atrophy has been reported to be largely due to changes in the lymphoid compartment. Thymocyte depletion appears as an outcome of both acute and chronic experimental protein malnutrition. The main phenotypic feature of this depletion is the loss of immature CD4+CD8+ cells, a finding consistently seen in malnutrition secondary to diets deficient in protein, metal elements (zinc, magnesium and iron) and vitamins [172-174]. As recently demonstrated in rats exposed to deficiencies of Mg or Zn, the consequent thymocyte depletion actually reflects a massive apoptosis of these cells in the organ [171,174]. In addition to the increase in thymocyte death in the thymus of malnourished individuals, thymocyte proliferation seems to be affected. Thus, the numbers of thymic cells expressing the proliferating cell nuclear antigen (PCNA) marker decreases in malnourished rats [175]. This finding is further supported by data showing that thymocytes from animals subjected to distinct protocols of dietary restriction had low mitogen-induced proliferative responses [172]. Thus, the overall

malnutrition-related thymocyte depletion seems to result from enhanced thymocyte death plus decreased thymocyte proliferation.

It would be important to know whether major changes in the thymic lymphoid compartment are also observed in humans suffering from malnutrition. Consistent with this, severe thymic atrophy with cortical thymocyte depletion is a common finding in necropsies of malnourished subjects [176]. In further support of this observation, thymic atrophy was also observed in malnourished children by the technique of echography [177]. Nevertheless, such alterations in the thymus seem to be reversible, at least in the experimental animals, if an appropriate diet is provided [178].

Like undernutrition, overnutrition which leads to obesity, has also been studied extensively with regard to thymic size and function. It has been suggested that obesity-induced accelerated thymic involution and restricted and limited T cell repertoire diversity represents a potent modifier of immunosenescence mechanisms that may further increase the risk and severity of infections in the '*gerobese*' (geriatric obese) population [179]. This situation is likely to leave the subject with potentially greater predisposition to emerging diseases. Although the true mechanistic pathway of obesity-induced thymic involution is still not known, some previous studies examining immune function in extreme monogenic rodent models of obesity have shown clear thymic involution [180] and significant defects in T-cell responsiveness [181]. Interestingly, despite massive replacement of thymic with adipose tissue, the aging thymus still retains limited capacity for generating naive T cells [182], suggesting that restoration of thymic function may be achievable, particularly by the mechanisms of caloric restriction in obesity [151].

3.0 CONCLUSIONS AND OUTLOOK

Decline in immunity with aging is well-established and much of the evidence today supports the notion of an overall impairment of immune functions even with normal “healthy” aging. There is also strong evidence that malnutrition (both under- and overnutrition) impairs elements of adaptive and innate immunity and that nutrition plays an important role in modulating immune functions. The relationship between malnutrition and infection is an intimate one, and it is often assumed that this is because of impaired immune function. There is good evidence of links, particularly between micronutrient deficiencies and immune impairment and obesity and a number of infections. Much of the evidence is suggestive that the size and function of the thymus is affected in the same way both by age and malnutrition; a fact further authenticating the importance of nutrition in the context of immune integrity.

Present day nutritional immunology research is mainly centered around studying the mechanisms underlying the modulation of immune responses by nutrients. Using many sophisticated tools, researchers of nutritional immunology try investigate the role of dietary components and their interactions with immunological parameters. The challenge remains to integrate nutritional immunity with age-associated changes to immune status, and to confirm that knowledge gained in one human population is comparable and informative for different populations. Further work is needed to elucidate the underlying mechanisms and how to perform adequate nutritional intervention for immunologic preservation. For this, larger studies on nutritional supplementation need to be launched and the observations of geriatricians integrated with nutritional immunology. There is still a need to study the effects of nutrients on different components of the

immune system, because we know that immunity depends on multiple components that react differently to nutrients. Considering the complex nature of nutritional immunology, there is a need to dissect the networks of interactions that define the relationships between nutrition, immune function, infections and genetic background in age-associated changes of immune and inflammatory responses. Special emphasis must be given to find how to reverse and/or delay the onset of immunologic and age-related changes by appropriate dietary modifications and to determine the molecular mechanisms by which nutrients modulate immune cell functions. New methods have to be developed to use the immune response as a biologically meaningful index in determining specific dietary requirements.

The main focus of future nutritional immunology will include 1) studying cellular and molecular mechanisms of age and nutrition-induced changes in immune and inflammatory responses, 2) determination of the efficacy of food components (total calories, lipids, micronutrients such as vitamin E, zinc, flavonoids, and pre- and pro-biotics) on improving the immune function and/or dampening inflammatory responses using various techniques (cell culture, animal models and clinical trials), 3) determination of the efficacy of various food components in the prevention of infectious diseases in animal models, clinical trials and observational studies, 4) determination of the impact of reducing caloric intake on immune response of humans.

4.0 List of Abbreviations

APC	Antigen Presenting Cells
BMI	Body Mass Index
BMR	Basal Metabolic Rate
CM	Central Memory

CMV	Cytomegalovirus
CR	Caloric Restriction
CRP	C-reactive protein
DTH	Delayed-Type Hypersensitivity
EM	Effector Memory
GM-CSF	Granulocyte colony-stimulating factor
IL-1RI	Interleukin 1 receptor, type I
IL-6	Interleukin-6
LBM	Lean Body Mass
IRP	Immune Risk Profile
MCH	Major Histocompatibility Complex
MH	Metabolically Healthy
MUH	Metabolically Unhealthy
NK	Natural Killer
PCNA	Proliferating Cell Nuclear Antigen
TLC	Total Lymphocyte Count
TNF	Tumor Necrosis Factor
WEIRD	White, Educated, Industrialized, Rich and Democratic

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Table 1: Selected Age-related changes in B and T cells and their activities

Decrease	Increase
CD28	KLRG1
CD27	ILT-2 (CD85j)
CCR7	CD57
IL-2 production	CD49d
Delayed-type hypersensitivity	KIR-positive T cells
CD45+ T cells	CD244
Thymic output	CD45RO+ T cells
B-cell-derived antibody affinity	Memory B cells
Lymphopoiesis (B and T cells)	CMV-specific CD8+ T cells
Naïve B cells	CMV-specific CD4+ T cells
Generation of immature B cells	

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