Spectrum of autoantibody response to tumour associated antigens in normal population

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Declaration

I hereby declare that this thesis is my personal work, with no material copied or plagiarised from other sources. All information from published data in this thesis has been correctly referenced. This thesis has not been submitted nor being considered for any degree in any other universities or institutes. I have no personal financial interest in the data contained within the thesis.

John Mathew

Ethical Approval

The study was approved by the Medical School Healthy volunteer Committee (Ethics Reference: BT/07/2007)

Abstract

Early detection and diagnosis of cancer has a significant impact on cancer specific mortality as shown in randomised screening trials for breast, colon and lung cancer. However, current screening tests have limitations as they reduce cancer specific mortality for breast, colon and lung cancer by only 24%, 16% and 20% respectively, and partly as a result cancer continues to be one of the common causes of death in the developed world(Aberle *et al*, 2011; Hardcastle *et al*, 1996; Larsson *et al*, 1996). One of the other problems associated with current screening tests is patient compliance (mammography, colonoscopy and CT) (Jonnalagadda *et al*, 2012; Maurer, 1995; Pooler *et al*, 2012).

Diagnosing cancers with a blood test by identifying tumour associated antibodies in serum is a novel method which may allow the identification of early stage cancers and hopefully it would have greater patient acceptability. These tumour associated antibodies represent an indirect amplified signal generated as a response by the immune system to tumour associated antigens secreted early on in development of cancer.

One of the common limitations of many autoantibody studies is the selection of appropriate controls – or the lack of such. One common problem is the use of limited number of normal individuals without cancer as controls, the data from which may not be representative of the normal population as a whole (Stockert *et al*, 1998). In addition not only the numbers of controls are often incorrect but also the age of the controls. Many studies report using 'blood

donors' as controls(Guy *et al*, 1981) and clearly for most tumour types this involves both a younger population and also a relatively health population which may not always be reflective of the individuals to be screened (e.g. compare heavy smokers).

We hypothesised that autoantibody response to cancer associated antigens may alter with demographics (age, sex, and smoking) and the aim of our study was to identify the spectrum of response of tumour associated antigens in a range of demographic groups within the normal population of the East Midlands.

EarlyCDT-Lung™ is a simple commercial blood test which is reported to aid the early detection of lung cancer. The technology was initially developed in the laboratories of the Division of Breast Surgery and subsequently underwent further development by the university spinout company, Oncimmune. EarlyCDT-Lung initially measured autoantibodies (AAbs) to six cancer associated antigens (p53, NY-ESO-1, CAGE, GBU4-5, Annexin1, and SOX2) and was reported to identify up to 40% of lung cancers, at both at early- and late-stage disease(Boyle *et al*, 2011; Chapman *et al*, 2012; Chapman *et al*, 2011; Lam *et al*, 2011; Macdonald *et al*, 2012a; Macdonald *et al*, 2012b; Murray *et al*, 2010).The initial technical (Murray et al 2010) and clinical (Boyle et a;l 2011; Lam et al 2011) validation studies matched high risk individuals to every lung cancer patient. Controls were individually matched to

a patient with lung cancer by age, gender and smoking history. As the 6 antigen panel had been developed and validated (Boyle *et al*, 2011; Murray *et al*, 2010) it was decided to proceed to assess the level of autoantibodies across the population and in particular to look at differences by gender and different decades of life. We used a semi-automated Enzyme linked immunosorbent assays (ELISA) to run the serum samples collected from individuals with no previous history of cancer.

Informed consent was taken prior to a detailed health questionnaire and then a blood sample by standard venipunture. The information acquired in the questionnaire included age, gender, smoking history, any autoimmune disease and family history of cancer.

Serum samples used in this thesis were collected from 2065 individuals. Male to female ratio was 1: 2.6(566:1487). Ratio of smoker versus ex-smokers versus non-smoker was 1: 2.4: 3.8 (285:672:1096). There was a fall in the number of smokers with increasing decade of life. The proportion of smokers and ex-smokers versus non-smokers remained approximately the same in both genders. Almost half the patients (964) had family history of some form of cancer. One hundred and eighty six subjects (9%) had personal history of autoimmune disease.

Analysis of autoantibody levels revealed a small but steady increase with increasing age for 4 out of the 6 antigens (p53, NY-ESO-1, CAGE and GBU4-5). Except for CAGE, there was no significant difference in mean optical densities between males and females. For CAGE, when analysis of variance was used to adjust for run differences, there was no significant difference in mean optical densities between males and females.

Autoantibody response to all 6 cancer related antigens were consistently low in smokers. The rise in autoantibody response was more in the ex-smoker group compared to the other two groups suggesting the possibility of rebound effect when smoking is stopped. It reached statistical significance except in case of NY-ESO-1. Age matched analysis were done, and the differences were statistically significant for p53, GBU 4-5 and Annexin1. To explore further the "rebound" hypothesis further, the year of quitting for ex-smokers were extracted from the database. Any association between AAb levels and time lapse since quitting might provide support to this hypothesis. Very little difference was seen for most antigens back to 1970, but decades before that there was an observed increase in the mean AAb level for all antigens except SOX2. Further work would be required to establish such a rebound effect.

Family history and history of autoimmune disease did not have a significant impact on autoantibody levels.

Analysis of autoantibody levels in a large cohort of the normal population of the East Midlands revealed that age has a small but significant influence on the serum levels of certain autoantibodies to cancer related antigens. However,

this could be confounded by the fact that incidence of cancer also increases with age, and would need further investigation and in particular longer follow-up of patients who have given blood in this research study to see which individuals have developed cancer.

Dedication

I would like to dedicate my thesis to my father Dr Mathew Varughese MS FRCS, for his love, encouragement and support.

Published abstracts from thesis

J. Mathew, G. Healey, W. Jewell, A. Murray, C. Chapman, L. Peek, A. Barnes, W. Wood, J. F. Robertson, P. Boyle Demographics of populations at high risk of lung cancer and results of the Early CDT-Lung test. ASCO (Chicago 06/06/2010) **Poster presentation**, J Clin Oncol 28:15s, 2010 (suppl; abstr 7033)

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Abbreviations

AAb	Antoantibody
AAbs	Autoantibodies
ABC	Advanced breast cancer
ADH	Atypical ductal hyperplasia
AI	Autoimmune disease
APC gene	Adenomatous polyposis coli gene
ASCO	American association of clinical oncology
BMI	Body mass index
BRCA1	Breast cancer 1 gene
BRCA2	Breast cancer 2 gene
CAGE	Cancer associated gene 1
CA15.3	Cancer antigen 15.3
CEA	Carcinoembryonic antigen
CT	Computerised tomography
CT antigen	Cancer testis antigen
CVb	Coefficients of variations between-runs
CVe	Coefficients of variations within-run.

CVs	Coefficients of variations (CVs)
DCIS	Ductal carcinoma insitu
DNA	Deoxyribonucleic acid
EBV	Epstein Barr virus
ECD	Extracellular domain
ELISA	Enzyme linked immunosorbent assays
FAP	Familial adenomatous polyposis
FH	Family history
Fig	Figure
HCC	hepatocellular cancer
HER2	Human epidermal growth factor receptor 2
HHV8	Human herpes virus 8
HIV	Human immunodeficiency virus
HNPCC	Hereditary non-polyposis colorectal cancer
HPV	Human papilloma virus
HRP	Horseradish peroxidase
HRT	Hormone replacement therapy
ICH	Immunohistochemistry
Ig	Imuunoglobulin

IPF	Idiopathic pulmonary fibrosis
MRI	Magnetic resonance imaging
NHS	National health service
NSCLC	Non small cell lung cancer
NY-ESO-1	New York oesophageal antigen 1
OD	Optical density
PAH	Polycyclic aromatic hydrocarbons
PBC	Primary breast cancer
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PET	Positron emission tomogram
PAS	Population antibody study
PSA	Prostate specific antigen
RCT	Randomised controlled trial
RNA	Ribonucleic acid
SCLC	Small cell lung cancer
SD	Standard deviation
SEREX	Serological analysis of cDNA expression
TAAs	Tumour-associated antigens

TMB	3,3'5,5' Tetramethylbenzidine
TNM	Tumour node metastasis
UICC	Union for international cancer control
UK	United Kingdom
VOL	Vector only lysate
%	Percent

Chapter 1. **Introduction**

1.1 Screening for cancer: current modalities and future opportunities

Randomised controlled trials of screening for breast, colon, lung and prostate cancer have shown that early diagnosis has a significant impact on the mortality from each of these tumour types (Aberle *et al*, 2011; Hardcastle *et al*, 1996; Larsson *et al*, 1996; Schroder *et al*, 2012).

In spite of the advances in screening for each of these types of cancer there remains significant opportunities to further improve on early detection since screening in these four types of cancer reduce cancer specific mortality between 15%(Hardcastle *et al*, 1996) and 24%(Larsson *et al*, 1996). It should be acknowledged too that the improvement in cancer specific mortality has not all been due to screening and early detection. It has been shown for breast and colon cancer in particular through randomized trials of adjuvant therapy that systemic therapies have also made a significant impact on cancer specific mortality in these diseases(Boccardo, 2008; Gray *et al*, 2007; Sakamoto *et al*, 1999)

Despite such advances in screening and treatments, there continues to be significant mortality from each of these diseases with the result than cancer remains one of the most common causes of death in the developed world. For cervical cancer there are non-randomised data which show a significant reduction in cancer specific mortality where screening has been introduced (Duggan, 2012; Kitchener *et al*, 2010). For other types of solid tumours such

as gastric and ovarian there is less than level 1 evidence and ongoing randomized trials (Menon *et al*, 2008; Partridge *et al*, 2009). For other solid tumour types such as pancreatic, oesophageal and hepatocellular cancer there are no screening tests which thus far hold much promise and systemic therapies are also of limited effectiveness.

Diagnosing cancers with a blood test would seem a simple and relatively easy approach. However for a blood test to be useful in the diagnosis of primary cancers, it needs to be sensitive enough to detect early disease and specific enough to be effective in a screening context. Majority of blood tests for cancer markers presently available look into level of cancer-associated antigens. Examples of classical 'tumour markers' would be CEA for colorectal cancer, CA15.3 for breast cancer and CA125 for ovarian cancer, all of which reflect a dynamic overview of disease burden (tumour load) in the individual. Thus these markers has been traditionally used in diagnosing recurrences and monitoring treatment of metastatic cancers, and found to be not particularly useful in diagnosising early cancers. As methods of detection of antigens has become more sensitive many researchers have focussed on proteomics using chromatography looking for abnormal patterns associated with different types of cancer. Thus far these techniques have not been proven effective either in detecting early disease or specific enough for screening(Liu *et al*, 2012; Ma *et al*, 2012).

A new approach in improving the early diagnosis of certain cancers especially lung cancers is the use of real time PCR. miRNA expression is involved in

cancer development and progression as a tumour suppressor or oncogene, and its aberrant expression has been used as a biomarker for the diagnosis of lung cancer. Bianchi F and colleagues from Milan have developed a multivariate risk-predictor algorithm based on the weighted linear combination of the 34-miRNA expression levels and they predicted an overall accuracy of 80% (sensitivity 71%, specificity 90%; AUC 0.89) in a group of asymptomatic lung cancer patients detected using LD-CT(Bianchi *et al*, 2012). Studies from other centers on miRNA on lung cancer patients has published similar accuracy in the diagnosis of lung cancer patients (Hennessey *et al*, 2012). Similar research has been published in other forms of cancer (Kang *et al*, 2012). Stathmin1 is a candidate oncoprotein and prognostic marker in several cancers and the above paper supports its oncologic role in gastric cancer.

Another field which has recently attracted significant attention with a large number of publications has been the presence of humoral immune response to cancer (amplified signal) produced in response to the cancer derived antigens. These 'autoantibodies' to cancer modified antigens are present in measureable amounts not only in detectable early stage cancer but have even been reported to be measureable in the peripheral blood months to years before cancers are diagnosed(Li *et al*, 2005; Robertson JFR, 2005a; Robertson JFR, 2005b).

The measurement of autoantibodies in the peripheral blood as a means of diagnosising early stage cancer has been reported previously from our institute, including lung and breast cancers(Chapman *et al*, 2007; Chapman *et al*, 2008) and has eventually led to the establishment by the University of Nottingham of a spinout company (Oncimmune Ltd) whose aim has been to

identify raised levels of such antibodies in individuals with a cancer and provide clinical blood tests as an aid to diagnosis that can be used alongside current imaging techniques. The basis for the research programme has been that in defined high risk populations raised levels of autoantibodies could be identified in the preclinical and early stage disease and that linked to modern imaging technologies this would result in detection of the cancer earlier, leading to not only improved survival but also reduction in cancer specific mortality. These studies have compared the autoantibody signal in sera derived from both cancer and normal individuals (Chapman *et al*, 2007; Chapman *et al*, 2008) . Screening is usually thought to be appropriate only for high risk groups and so these published studies[34 35] like most similar studies were performed in cancer patients and high risk groups of individuals. As such, knowledge of the tumour associated antibody responses in the normal population is largely unknown. The EarlyCDT-Lung™ test (Oncimmune Ltd) measures autoantibodies (AABs) to six cancer-associated antigens (p53, NY-ESO-1, CAGE, GBU4-5, Annexin1, and SOX2) and has been reported to identify up to 40% of lung cancers regardless of grade or stage of the disease. These 6 antigens are described in more detail in the following sections but in brief they include well-recognised cancer-associated antigens like the p53 suppressor oncogene which is mutated in large number of cancers (Linzer & Levine, 1979; Soussi, 2000b) and also two previously described cancer testes antigens, NY-ESO-1 (Chen *et al*, 1997a) and CAGE (Chapman *et al*, 2008). GBU4-5 is a recently identified protein and like CAGE it encodes a DEAD-box domain and has been described to produce antibody response in lung cancers (Cho *et al*, 2003a). Annexin 1 belong to a family of calcium^{2+} and

phospholipids-binding, membrane associate proteins with varied expression in different cancers and antibody response to these antigens has been recorded in significant proportion of lung cancers (Ahn *et al*, 1997; Yeatman *et al*, 1993). Finally, SOX2 is a high mobility group box transcription factor involved in the maintenance of pluri-potency and self-renewal in embryonic stem cells and has been identified in the serum in high frequency in patients with squamous cell and small cell lung cancers (Bass *et al*, 2009; Titulaer *et al*, 2009).

To our knowledge the autoantibody response to cancer related antigens has not been assessed in a large group of normal subjects with no personal history of cancer. We believe this study is the first of its kind looking into the spectrum of autoantibody response in a normal population, with or without a family history. In addition the study will also look at whether benign autoimmune disease might interfere with the autoantibody response to cancer modified antigens by such individuals. We hypothesise that autoantibody response to cancer associated antigens may alter with demographics and the objective of our study to investigate the influence of age, gender, smoking and benign autoimmune diseases on antibody response to the cancer related antigens described above, in a large cohort of subjects with no previous personal history of cancer.

1.2 Review of literature

1.2.1 Cancer statistics

Cancer is one of the leading causes of death, and in United Kingdom it accounts for one in four (27%) of all deaths(2006a; 2006b; statistics, 2006). On an average around 293,000 new cancers are diagnosed each year(Aug 2009) with overall 1:1 ratio in males to females. In males the most common cancers are lung, prostate and colorectal and in females they are lung, breast and colorectal. The incidence of lung cancers are 68% higher in males than in females (62 and 37 per 100,000 respectively) and the incidence of colorectal cancers are 58% higher in males (55 and 35 per 100,000 for males and females respectively). Breast cancer and prostate cancer represent the highest incidence among cancers in females and males respectively, and incidence of breast cancer (122 per 100,000) is 24% higher than the incidence of prostate cancer (98 per 100,000)(Aug 2009).

On average there are approximately 155,000 cancer deaths each year. The age standardized mortality rate for men and women were 211 and 153 per 100,000 respectively. The three most common cancers for both sexes was also the most common cause of death from cancer. In females the mortality from lung cancer was only slightly higher than breast cancer (28 and 31 per 100,000 respectively) although the incidence of lung cancer is much lower- a reflection of the much higher mortality rate for lung cancer compared to breast cancer. In men the mortality from lung cancer was twice that of prostate cancer (53 and 26 per 100,000 respectively), again a reflection of the greater mortality rate from lung cancer. The mortality from lung cancer was 75% higher in males

than females. The mortality rate for colorectal cancer was 59% higher in males than females (20 and 13 per 00,000 for males and females respectively). In the UK, overall Scotland had higher mortality rates for both sexes, about 16% higher than those for the UK as a whole, and overall cancer incidence rates were 8% higher(Aug 2009).

Although cancer deaths are more common in the elderly, cancer causes a greater proportion of deaths in the young and relatively greater years of life lost to cancer (eg breast cancer)(Boyle *et al*, 1995). Cancer is responsible for a third of all deaths under the age of 65 years compared to a quarter of all deaths in over 65's(2006a; 2006b; statistics, 2006).

1.2.2 Screening

Screening is looking for cancer in people who have no symptoms or looking for conditions which could lead to cancer. It may not be useful in all cancers, and to be effective it should have a long occult growth period where they can be detected before the cancer has spread leading to treatment that could improve survival. Thus not all screening tests are useful, and some have risks associated with it ie. Flexible sigmoidoscopy or colonoscopy can injure the bowel wall and can also cause bleeding. False positive results may lead to anxiety and further tests which may themselves lead to complications. False negative results may lead to a false sense of being normal even when presented with symptoms, leading to delay in seeking medical care.

Between 1977 and 2006 the European age-standardised mortality rates for all malignant neoplasms fell by 17% from 215.9 to 178.6 per 100, 000 population(2006a; 2006b; statistics, 2006). Along with the recent availability

of better cancer treatment, early detection through screening has an important part in more recent improved survival(Henschke *et al*, 2006; Labrie *et al*, 1999; Otto *et al*, 2003; Tabar *et al*, 2003). However available screening modalities for cancer have limitations and an ideal screening test should have high sensitivity, specificity, should be reproducible, cheap, non-invasive and acceptable to patients.

1.2.2.1 Lung cancer screening

Lung cancer is the commonest cancer worldwide representing approximately 12% of all new cancers(Youlden *et al*, 2008). It is also most common type of cancer in males and remains the most common cause of cancer related mortality in both sexes. The majority of patients worldwide still present with advanced inoperable stage(Parkin *et al*, 2005; Youlden *et al*, 2008). Overall 5-year survival for lung cancer in men and women are in the range of 13 and 17 percent respectively and this poor outcome is almost uniformly due to late stage of disease at diagnosis(Ries LAG). If lung cancers are diagnosed early, the 5-year survival rate for UICC stages I is reported to be over 70%(Flehinger *et al*, 1992). Cancers which are diagnosed very early, for example those involving only the very few layers of cells in a localized area, the survival is even better and it may approach 80%. The fact that this is not simply due to lead time bias was shown in the recent publication of the NLST study which reported that screening with three annual computerised tomography (CT) scans reduced lung cancer mortality by 20%(Aberle *et al*, 2011)

Previous non-randomised trials with low radiation dose CT had shown CT had a high sensitivity for small non-calcified pulmonary nodules, the most common manifestation of early lung cancer. Screening of high risk group using low dose CT has shown a prevalence rate ranging from 0.4% to 2.7% and incidence rate (results of repeat screening) ranging from 0.3% to 1.2%. In a study by MacRedmond et al looking into 449 high risk subjects undergoing low dose CT, the prevalence rate was 0.4% and incidence rate of 0.7% at 2 years(MacRedmond *et al*, 2006). Overall, 2 of the 9 lung cancers diagnosed were stage 1 non small cell lung cancer. In the Early Lung Cancer Action Project (ELCAP), Henschke et al reported a prevalence of 2.7% (27/1000) and incidence of 0.7% (6/841)(Henschke *et al*, 2001). Eighty one percent (27/33) of the cancers diagnosed were of stage 1 disease. New York Early Lung Cancer Action Project (NY-ELCAP) looking into 6295 high risk subjects undergoing CT screening reported a prevalence of 1.6% (101/6295) and incidence of 0.3% (20/6014)(New York Early Lung Cancer Action Project, 2007). In another study involving 817 high risk subjects undergoing low dose CT screening reported by Diederich and colleagues, the prevalence rate was 1.5%(12/817) and incidence rate of 1.2% (10/792)(Diederich *et al*, 2004). Seven out of the 12 patients (58%) diagnosed with lung cancer in the prevalence group were stage 1 disease and 7/10 (70%) patients in the incidence group had stage 1 disease. In a prospective cohort study by Stephen and colleagues looking into 1520 high risk individuals aged 50 years or older the prevalence rate of lung cancer was reported to be 1.6% (25/1520) and the incidence rate was 0.3% (3/1000)(Swensen *et al*, 2002). Pastorino and colleagues reported the use spiral CT and positron emission tomography in

high risk group of 1035 subjects aged 50 years or over who had smoked for 20 pack-years or more(Pastorino *et al*, 2003). All patients had annual CT with or without PET for 5 years. By year two, 22 cases of lung cancer were diagnosed (11 at baseline, 11 at year 2). Complete resection of the tumour was possible in 21(95%) of lung cancer patients and 17 (77%) of them were of stage 1 disease at pathological diagnosis. It is important to note that true potential of a screening test is not reflected by the prevalence results, but by incidence results (results at repeat screening) which should also give information of screening intervals.

In a recent RCT by van Klaveren and colleagues, in a large group of over 7500 subjects with high risk of lung cancer undergoing CT screening in year 1, 2 and 4, following the first and second rounds of screening, 2.6 and 1.8 of the participants respectively had positive test result. The sensitivity of the test was 94.5% (95 CI; 86.5 to 98) and the negative predictive value was 99.9% (95 CI; 99.9 to 100). Twenty lung cancers were detected in 7361 subjects with a negative screening result in round one after 2 years of follow-up(van Klaveren *et al*, 2009).

Screening high risk populations for early-stage lung cancer using CT scans has therefore been shown to reduce lung cancer mortality. Other potential screening techniques include serum analysis for tumour associated antibodies, advanced sputum analysis in combination with autofluoresent bronchoscopy, and assessment of molecular markers in sputum. None of these techniques have thus far been reported in randomized clinical trials.

1.2.2.2 Breast cancer screening

The breast cancer mortality rate in UK has been falling since the late 1980's(statistics, 2006) and is one area where screening has been extensively evaluated and reported to be successful. Screening trials have taught us that timing of treatment in relation to natural history of the disease is probably more important in the final outcome rather than the choice of treatment(Tabar & Dean, 2008). With the introduction of mammographic breast screening there has been a shift from primary palpable more advanced cancer to non-palpable good prognostic pre invasive and invasive cancers which may not require the adjuvant treatment and thus avoiding its toxic effects. On the other hand some experts believe there is a significant over-diagnosis rate(Moynihan *et al*, 2012) and a recent review of analysis of data from Norway estimate that 15% to 25% of breast cancers identified on mammography would never have been clinically significant in a women's lifetime.(Elmore & Fletcher, 2012)

One of the early RCT of mammographic screening began in Sweden in 1977 in the counties of Kopparberg and Ostergotland. Randomisation into the trial took place between 1977 and 1980 between the ages of 40 to 74 years. Invited to screening group comprised 77092 women and the not invited group comprised of 56000 women(Tabar *et al*, 2002). After an approximately mean follow-up of 10 years, there was a 31% reduction in breast cancer mortality in the invited group (RR 0.69, 95% CI 0.58-0.80; $p<0.001$). These results have been consistent with the results in other mammographic screening trials(Nystrom *et al*, 2002). In England and Wales, based on recommendations of the Forrest committee, the breast cancer screening program was introduced

in 1988 (Quinn & Allen, 1995). By 1994, there was a reduction of 12% mortality compared to prescreening rates in the late 1980s in patients between 55-69 years of age. While the widespread use of adjuvant tamoxifen could account in part for these improved survival results in the later decade, breast cancer screening using mammograms has thought to have played a significant role in reducing mortality(Quinn & Allen, 1995).

In United States, the American College of Surgeons recommends annual mammographic screening beginning at the age of 40 years. In the UK mammographic screening under the National Health Service (NHS) for all women commences at 47 years of age. Screening women younger than 47 years on the NHS is only allowed if the patient can be shown to be above population risk either through family history or having a genetic mutation, such as BRCA 1 or 2 or p53, known to be associated with increased risk of developing breast cancer. A meta-analysis of previous mammographic screening trials has reported a 15% reduction in breast cancer mortality in women aged 40-49 years at entry who were invited to screening(Miller *et al*, 1992). However most of the results included in this meta-analysis were from subgroup analysis of larger trials and there has been a view expressed that a significant proportion of patients in these trials reached 50 years soon after entry into the trial and the mortality benefit could have been due to mammograms performed after the age of 50 years(Smith *et al*, 2004). To overcome this issue the 'Age Trial' was designed and run in the United Kingdom(Moss *et al*, 2006). In a recent report of this trial, following 10.7 year mean follow-up of 16,0921 women aged 39-41 years randomly assigned on a 1:2 ratio to an intervention group (annual screening mammogragms from

entry to age 48 years) or control group showed a reduction in breast cancer mortality in the intervention group relative to the control group of 17% which was not statistically significant (relative risk 0.83, 95% CI 0.66-1.04;P=0.11). There is relatively little follow-up beyond 10 years and results of longer follow-up are awaited. Comparatively low mortality benefit in younger patients could be attributed to low sensitivity of mammograms in this group. The sensitivity of mammograms is shown to be inversely proportional to the density of the breast. In women under the age of 50 years almost half have dense breast tissue and approximately one third of the women over the age of 50 have still dense breast(Stomper *et al*, 1996). Apart from that there appears to be a shift of balance towards number induced by screening compared to the breast cancer deaths prevented by screening in patients using mammograms under the age of 50 years(Beemsterboer *et al*, 1998). Similar trend has been noted in BRCA mutation careers(Berrington de Gonzalez *et al*, 2009).

One of the well documented risk factors for breast cancer is family history, and genetic predisposition which accounts for 5-10 % of all breast cancers(Easton *et al*, 1995). The closer the relative and the younger the relative at first diagnosis, then the greater is the risk of breast cancer. With no first degree relative with breast cancer, the life time risk of breast cancer (up to 80 years) is 7.8% (approximately 1 in 13)(Collaborative Group on Hormonal Factors in Breast, 2001). With one first degree relative the risk becomes almost twice (1 in 7.5) and with 2 first degree relatives the risk becomes almost 3 times (more than 1 in 5). While the optimal time to start screening women with a family history remains to be established some experts have proposed that it is advisable to start screening approximately 10 years before

the age of diagnosis of first degree relative(Dershaw, 2000) with a lower limit of 30 years based on family history alone since the incidence of breast cancer in this group is very small before this age. However women who are known or suspected to carry BRCA1 and BRCA2 mutations are at a particularly high risk of breast cancer, especially at earlier age in BRCA1 carriers. The incidence of breast cancer among BRCA1 carriers in their 20s exceeds those of women with no family history in their 40s and thus it may be reasonable to start screening in this group even in their 20s(Berg, 2009).

Because of low sensitivity of mammograms in younger age group, it has been combined with other screening tools like MRI or ultrasound. In high risk women the combined sensitivity of mammogram and MRI has been reported to be 92.7% compared to 52% with USS and mammogram(Kuhl *et al*, 2005). However MRI is less well tolerated and expensive compared to ultrasound(Kuhl *et al*, 2005). Another disadvantage is that, although MRI is not affected by density of the breast as opposed to mammograms, its comparatively low specificity leads to potentially unnecessary biopsies and anxiety.

1.2.3 Genetic changes in cancer

There has been an explosion in the knowledge about molecular basis of cancer in the last 30 years and it is now recognized that genetic instability (mutation or altered expression of genes) is thought to be the driving force involved in the initiation and progression of cancer. (Shen, 2011). In non cancerous somatic cells, the duplicated genome is evenly divided into the two daughter cells, thus ensuring that they receive the same genetic material as their parent cell. These cell divisions are tightly regulated and failure of this regulatory process or errors during this process will result in various forms of genome alteration in daughter cells. Accumulation of these alterations results in dys-regulation of cell division eventually leading to cancer. In cancer cells, additional genetic alteration can result in sub-population of cells with more aggressive properties than others and thus accumulation of genetic alterations is not only a hallmark but also a driving force in tumorigenesis.

In addition to this, environmental factors and epigenetic changes (changes in the gene caused by mechanisms other than changes in the underlying DNA sequence) play an important role in the development of cancer.

Three classes of genes may play a role in cancer initiation – Oncogenes, tumour suppressor genes and genes involved in DNA repair (Caretaker genes). Genetic alterations (mutation, amplification and deletion, translocation) involving the three classes of genes leads to derangement of mechanisms involved in normal cell growth and differentiation and ultimately culminate in the six biological endpoints which characterize malignant growth i.e. evasion of apoptosis, insensitivity to anti-growth signals, sustained angiogenesis, limitless replicative potential, self-sufficiency in growth signals and tissue

invasion and metastasis(Hanahan & Weinberg, 2000). Genetic instability in cancers is thought to occur at two distinct levels(Lengauer *et al*, 1998; Michor *et al*, 2005). In a small group of cancers instability is observed at the nucleotide level resulting in a base substitution or deletion or insertions of a few nucleotides. In most of the cancers the genetic instability is seen at the chromosome level resulting in losses or gains of a whole chromosome or large portions of it. The consequence is an imbalance of chromosome number (aneuploidy) and an increased rate of loss of heterozygosity (loss of normal function of one allele of a gene in which the other allele was already inactivated) which in turn accelerates the inactivation of tumour suppressor genes.

Oncogenes are mutated forms of normal genes called pro-oncogenes which control normal cell division and become permanently activated or tuned on with mutation leading to abnormal cell division and cancer. Only one of the alleles of the proto-oncogene needs to be overactive in order to have an oncogenic effect. A classical example is the Philadelphia chromosome in chronic myeloid leukemia.

Tumour suppressor genes encodes proteins that normally slow down cell division, repair DNA mistakes and trigger apoptosis, and their inactivation though genetic alteration can lead to cancer. Although it was initially thought that both alleles of the gene needs to be inactivated in order to render it inactive, some tumour suppressor genes needs only one allele to be mutated to produce an oncogenic effect(Knudson, 1971). The best known tumour suppressor gene is P53 gene which is the most frequently inactivated gene in human cancers and is described in more detail later. Another example is the

familial breast cancer associated genes (BRCA 1 and BRCA 2 gene) which act as adaptors that convey the damage signal to various mechanisms that induce repairs, arrest damage or promote apoptosis(Shiloh, 2003; Venkitaraman, 2004). Caretaker genes also known as stability genes are a class of tumour suppressor genes involved in recognizing and repairing DNA damages(Vogelstein & Kinzler, 2004).

It is estimated that the human genome has twenty to twenty-five thousand genes and at present more than 300 are known to play a role in the development of cancer(Lander *et al*, 2001). A study of breast and colorectal cancer cell lines has shown that these cancer cells may have an average of 90 mutant genes (Sjoblom *et al*, 2006). However there is also an argument that the large scale genetic instability seen in cancer could be a consequence of neoplastic process rather than its cause itself and only a few mutations are actually responsible for the development of cancer(Fearon & Vogelstein, 1990). The other insignificant mutations are called bystander(Ilyas *et al*, 1999) or passenger mutations(Sjoblom *et al*, 2006).

Development of cancer is thought to be a multi-step process requiring accumulation of mutation in a number of genes. In sporadic cases the initial genetic alteration occurs in a single cell and if this is not repaired it is passed on to the daughter cell during cell division. If one of the daughter cells acquires another genetic alteration it passes on this as well as the original alteration to its daughter cells. This process eventually leads to population of increasingly abnormal cells and subsequent development of cancer.

There is some dispute about the cell type involved in carcinogenesis. Increasingly scientists believe that cancer arise from stem cells (tumour initiating cells)(Bjerkvig *et al*, 2005; Jordan *et al*, 2006). Evidence of cancer stem cells was first reported for leukaemia and myeloma where it was found that a small subset of cancer cells was capable of extensive proliferation and to form new tumours(Bruce & Van Der Gaag, 1963; Park *et al*, 1971; Wodinsky & Kensler, 1966). Similar cells have been identified in breast and brain cancers (Al-Hajj *et al*, 2003; Singh *et al*, 2003). They are thought to occur as a result of deranged cell division or cell fusion events. It has been defined as a cancer cell that has the ability to self-renew giving rise to another malignant stem cell as well as a cell that will give rise to the phenotypically diverse cancer cells (Houghton *et al*, 2007; Sell, 2004). In this model there could be disruption of genes involved in the regulation of stem-cell self renewal. It is thought that the environment or the "niche" surrounding the stem cells provides signals necessary for the stem cells to continue to self-renew and that upon exit from this niche the stem cells begin to undergo differentiation(Spradling *et al*, 2001).

Majority of the cancers are sporadic and caused by progressive accumulation of genetic mutation and/or epigenic changes during person's lifetime. They are called somatic mutations and are not heritable. In contrast, some individuals are born with increased susceptibility to cancer through inheritance of single genetic mutation and this can be passed on from generation to generation. It is estimated that approximately 1% of cancers are caused by these high risk mutations which generally effect tumour suppressor genes(Aarnio *et al*, 1999). Individuals with high risk mutations have a one in two chance of passing the

mutated gene to their offspring and are copied to every cell in the child's body. Unless both parents are carriers, the child would inherit one mutated and one normal allele. Although both alleles need to be mutated in order to lose the function of the gene and develop cancer, these individuals only need one more mutation to be susceptible and are therefore high risk group. In this group cancers arise at a younger age than the general population.

Tendency of cancers to aggregate in families cannot be wholly explained by the rare inherited high risk mutation. A substantial proportion of such cancers are thought to be due to combined effects of multiple common gene variants known as polymorphisms, each of which is associated with an increased risk of cancer(Peto & Houlston, 2001). Mutation in BRCA 1 and BRCA 2 do not account for all cases of familial breast cancers. In fact most cases are due to families carrying one or more genetic polymorphisms. It has been found that inheriting particular polymorphism called 1100delC a variant of CHEK2 gene would double a woman's risk of breast cancer and increases the risk of cancer in males by ten-fold (Meijers-Heijboer *et al*, 2002). Irrespective of family history, an individual's risk of developing cancer is likely to be influenced by their genetic make-up, most commonly by the combination of polymorphisms they have inherited. The interaction of these gene variants with each other and with environmental risk factors is likely to determine overall cancer risk. HRT and oral contraceptives are some of the common environmental risk factors implicated in breast cancer and will be discussed in detail later (1997; Beral, 2003; Rossouw *et al*, 2002).

1.2.4 Stages of cancer development

Many forms of cancers are thought to arise from premalignant lesions which subsequently progress to invasive cancer. Well known examples of this process are colorectal cancer, breast cancer, bladder cancer, prostate cancer and cervical cancer

1.2.4.1 Adenoma carcinoma sequence (polyp-cancer sequence) in colorectal cancer

It has been widely accepted that adenoma-carcinoma sequence represents the process by which most of the colorectal cancers, if not all develop. There is substantial epidemiological, clinico-pathological and genetic evidence behind this theory. Prevalence of both colonic polyps and colorectal cancers increase with age, but the prevalence of adenomas peak 5 years earlier than that of colorectal cancers (Muto *et al*, 1975). Prior to the era of colonoscopic polypectomy several studies examined the progression of polyps left in-situ and colorectal cancer developing at the site of index polyp was observed (Bersentes *et al*, 1997; Hoff *et al*, 1986; Knoernschild, 1963; Stryker *et al*, 1987). There have also been reports of foci of malignancy within polyps(Colacchio *et al*, 1981; Shinya & Wolff, 1979)and conversely, remnants of adenomatous tissue within invasive colorectal cancers (Muto *et al*, 1975) . Adenomas are also found to coexist with cancers in approximately 30% of cases at endoscopy or in the specimen (Arenas *et al*, 1997; Chu *et al*, 1986; Langevin & Nivatvongs, 1984). It has also been noted that adenomas are encountered more frequently in patients with synchronous tumours than those with single primary cancer (Langevin & Nivatvongs, 1984). Similarly those

patients with colorectal cancer and simultaneous adenomas have been shown to have a higher risk of synchronous and metachronous tumours (Chu *et al*, 1986). The anatomical distribution of adenomas and carcinomas are similar and both occur more frequently distal to the splenic flexure (Chu *et al*, 1986; O'Brien *et al*, 1990). Finally polypectomy reduces the long term risk of colorectal cancer (Atkin *et al*, 1992; Hoff *et al*, 1996; Muller & Sonnenberg, 1995; Selby *et al*, 1992; Winawer *et al*, 1993) and randomised controlled trials have shown that screening reduces the incidence of colorectal cancer (Mandel *et al*, 2000).

Apart from the above epidemiological and clinico-pathological evidence, the genetic evidence behind this theory is also compelling. These genetic alterations often occur in a sequence that parallels the clinical progression of the tumour. However it is the total accumulation of changes rather than the order is the key factor in the progression (Fearon & Vogelstein, 1990; Vogelstein *et al*, 1988). Mutation involving the adenomatous polyposis coli (APC) tumour suppressor gene occurs early in the adenoma-carcinoma sequence and is located on chromosome 5q21 (Bodmer *et al*, 1987; Groden *et al*, 1991). Mutated APC has been demonstrated in adenomas as small as 0.5 cm(Powell *et al*, 1992). APC mutations or allelic losses of 5q are observed in 40-80% of colorectal cancers and found in similar frequency in adenomas (Jen *et al*, 1994; Miyaki *et al*, 1994; Miyoshi *et al*, 1992; Powell *et al*, 1992). The allelic loss or loss of heterozygosity has been shown to increase in frequency from early adenomas through to invasive carcinomas (Miyaki *et al*, 1994). Similarly K-ras genetic alteration occurs early in the adenoma-carcinoma

sequence. 35-42% of colorectal cancers have this mutation (Vogelstein *et al*, 1988) and similar frequencies are observed in large adenomas (Andreyev *et al*, 1998; Bos *et al*, 1987; Forrester *et al*, 1987; Hardingham *et al*, 1998).

P53 is the gene most frequently altered in human cancers and is located in the short arm of chromosome 17(Caron de Fromentel & Soussi, 1992). It is labelled as the guardian of the genome because of its ability to block cell proliferation in the presence of DNA damage, to stimulate DNA repair and to promote apoptotic cell death if the repair is insufficient (Lane, 1992). Alteration in p53 or 17p allelic loss has been reported in 4-26% of adenomas (Darmon *et al*, 1994; De Benedetti *et al*, 1993; Kaklamanis *et al*, 1993; Ohue *et al*, 1994; Scott *et al*, 1993; Vogelstein *et al*, 1988; Yamaguchi *et al*, 1994), approximately 50% of invasive foci within adenomas (Ohue *et al*, 1994; Yamaguchi *et al*, 1994) and 50-75% adenocarcinomas (Boland *et al*, 1995; Darmon *et al*, 1994; Hardingham *et al*, 1998; Kaklamanis *et al*, 1993; Kaserer *et al*, 2000; Vogelstein *et al*, 1988). These observations have led to the belief that loss of p53 function is associated with the transition from adenoma to carcinoma.

1.2.4.2 Premalignant to malignant change in breast cancer

It is widely believed that many breast cancers originate as a premalignant stage of atypical ductal hyperplasia which progress to DCIS and culminates in invasive breast cancers. A proportion of DCIS if not treated progress to invasive disease. From autopsy studies it is estimated that approximately one third of the DCIS progress to invasive cancer (Nielsen *et al*, 1984). The median age of patients with DCIS is reported to be 45-65 which is not

different from that reported from patients with invasive cancer and there is a trend towards lower median age in patients with DCIS detected by screening mammography (Sakorafas & Tsiotou, 2000). The family history of breast cancer in first degree relatives of DCIS patients is similar to that reported from invasive malignancy (10-35%)(Robinson KE, 1999) . Another factor which supports this theory is that a significant proportion of breast cancers (%) are found to have associated DCIS on histopathology. However, the most convincing evidence of DCIS being a forerunner for invasive cancer comes from studies in which patients with DCIS were initially misdiagnosed with benign lesions and were subsequently identified as DCIS (Betsill *et al*, 1978; Eusebi *et al*, 1994; Farrow, 1970; Page *et al*, 1995; Page *et al*, 1982; Rosen *et al*, 1980) . Eventually invasive recurrence developed in a large percentage of women (15-75%). In patients with DCIS the risk of development of subsequent invasive cancer is estimated to be approximately 11 times higher than the normal population. Another convincing piece of evidence is that among patients with DCIS undergoing surgery alone, approximately 50% of the recurrences following surgery are invasive cancers. These recurrences are significantly higher in those patients undergoing wide local excision alone compared to mastectomy.

1.2.5 Risk factors for cancer

1.2.5.1 Age at diagnosis

Cancer is predominantly a disease of the elderly with approximately 75% of cases diagnosed in people aged 60 or over and less than 1% of cases occur in

children (Office of national statistics, 2009; Online, 2009). The common solid tumours including breast cancer, lung cancer, bowel and colorectal cancers are all increasingly commoner in the elderly.

Age is one of the most important risk factors for breast cancer. Breast cancer is uncommon before the age of 30 years but rises rapidly until 40 years and there after continues to increase in prevalence at a slower rate (McKinnell RG, 1998). It accounts for nearly half of all cancer diagnosed in UK women aged 40-59 years (Office of national statistics, 2009; Online, 2009). Prior to 40 years of age the likelihood of developing breast cancer is 1 in 200 and the likelihood increases from 1 in 15 between the ages of 60 to 79 years (McKinnell RG, 1998).

Although the age-adjusted incidence of breast cancer is increasing, the morality has fallen recently. Breast cancer in women is second only to lung cancer as the leading cause of death by cancer in spite being 2-3 times more women being affected with breast cancer (McKinnell RG, 1998). Breast cancer is the leading cause of cancer death in women 15-54 years. Lung cancer has over taken as the primary cancer killer from the age of 55 onwards.

1.2.5.2 Heredity and cancer

Heredity is one of the most important risk factors for breast cancer. A women with a first degree relative with breast cancer before the age of 45 years has a 3.85 times greater risk than women of general population (Houlston *et al*, 1992). Many genes are known to be involved in the development of breast cancer. Deleterious mutation in BRCA 1(Futreal *et al*, 1994; Miki *et al*, 1994)

and BRCA 2(Wooster *et al*, 1994) are well known for their increased lifetime risk of breast and ovarian cancer. Men are at an increased risk of breast (primarily BRCA 2) and prostate cancer. The prevalence of BRCA 1 mutation is estimated to be 1/800 in the general population. Lifetime risk of breast cancer in women with BRCA 1 and BRCA 2 mutations varies from 56% to as high as 80-85% (Easton *et al*, 1993; Easton *et al*, 1995; Struewing *et al*, 1997). The probability of detecting a deleterious mutation in BRCA 1 or BRCA 2 is higher when there is a family history of both breast and ovarian cancer compared with women with breast or ovarian cancer alone(Couch *et al*, 1997). BRCA 1 mutations are seen in 75% of families with both breast and ovarian cancer in a single individual.

Age of onset of breast cancer is also important as younger ages of onset (diagnosed at the ages of 40 years or younger) of breast cancer are associated with a deleterious mutation, especially a mutation in BRCA 1(Berry *et al*, 1997). Women with inherited susceptibility generally have breast cancer at a younger age. As cancer evolves from a multistep process with several mutations rather than one, only fewer genetic changes are required in those patients with inherited genetic change to result in malignancy. Thus the inherited form is likely to occur earlier. In women with sporadic breast cancer the incidence peaks one or two decades later than those who have detectable BRCA 1 mutations.

Approximately 15-20 % of individuals referred for testing are found to have mutation involving either BRCA1 or BRCA 2 gene. Among men with breast cancer, about 25% carry mutations and of these more than one third occur in

BRCA1. BRCA 1 mutations are found to be three times more prevalent than BRCA 2 mutations in families with male breast cancer.

The average age of breast cancer diagnosis is significantly younger in the families with BRCA1 (41.4 years) or BRCA2 mutations (40.1 years) than in families without detectable mutations (50.1 years) ($P < .0001$) (Shih *et al*, 2002). Probands with *BRCA1* and *BRCA2* mutations were diagnosed with breast cancer at an average age of 37.6 and 32.1 years, respectively ($P = .028$). Women who carry an abnormal ataxia telangiectasia (AT) gene are associated with increased risk of breast cancer (Athma *et al*, 1996; FitzGerald *et al*, 1997).

Around 75% of colorectal cancers occur sporadically in people over the age of 50 years. Bowel cancers occur as a result of multiple mutations within a cell in the bowel lining allowing it to escape the normal growth control mechanisms. The step wise accumulation of mutations drives the histological transition from normal tissue to adenoma to carcinoma(Fearnhead *et al*, 2002). Most common genetic alterations in sporadic bowel cancers are activating mutations in the oncogene KRAS and mutation or loss of the tumour suppressor genes APC, SMAD4 and TP53. Evidence suggest that occurrence of APC mutations represents the earliest step in colorectal carcinogenesis and both copies of the APC genes are inactivated in 80 % of sporadic cases (Miyoshi *et al*, 1992; Powell *et al*, 1992).

Familial adenomatous polyposis (FAP) accounts for only 1% of colorectal cancers. However, they have a 100% risk of bowel cancer by the age of 40

years. The gene of FAP is the APC tumour suppressor gene on chromosome 5q21(Bodmer *et al*, 1987). Hereditary non-polyposis colorectal cancer (HNPCC) or Lynch syndrome, is responsible for another 2-5% of colorectal cancers. It is a result of a DNA mismatch repair(MMR) gene (Liu *et al*, 1996). It is often associated with other cancers including endometrial, ovarian, stomach, pancreatico-biliary and urinary tract. Studies on colorectal cancers below 30 years of age show that approximately 41% are carriers of MMR gene mutations(Farrington *et al*, 1998). The risk of people with these mutation to develop colorectal cancer by the age of 70 years is 91%(Dunlop *et al*, 1997). Apart from FAP and HNPCC, hereditary factors account for another 20% of colorectal cancers and a national collaborative study is looking into other genes involved in familial bowel cancer.

1.2.5.3 Smoking

The prevalence of smoking among UK adults is falling, possibly aided by the recent bans on smoking in public places introduced in Scotland and England & Wales(statistics, 2009). In spite of this, smoking causes more than a quarter (29%) of all cancer deaths in United Kingdom. More than one in five adults smoke and men are still likely to smoke than women(statistics, 2009). The average consumption of cigarettes per smoker per day is 14 in men and 13 in women and almost 40% of the regular smokers start smoking regularly at the age of 16 years(statistics, 2009). Around half of all regular smokers die from the habit and half of these in the middle age(Peto, 1994).

Smoking causes 90% of lung cancers in men and 83% of cases in women in the UK(al, 2006). Other environmental pollutants like asbestos could increase the risk of lung cancers in smokers. It is estimated that asbestos increases the

risk of lung cancer by around ten-fold in non smokers, while in smokers exposed to asbestos, there is a 100 fold increase in risk(Lee, 2001).

Exposure to environmental tobacco smoke also increases the risk of lung cancer. A recent meta-analysis showed that exposure of environmental tobacco smoke at work or in the home increase the risk of lung cancers among non-smokers by about a quarter, while heavy exposure double the risk(Stayner *et al*, 2007; Taylor *et al*, 2007). This exposure to environmental tobacco smoke has also been linked to other cancers like bladder and larynx(Bjerregaard *et al*, 2006; Bosetti *et al*, 2008; Jiang *et al*, 2007).

Smoking is also an established risk factor for other cancers like those of the oesophagus, larynx, pharynx, oral cavity, nasal cavity and sinuses, stomach, bowel, kidney and cervix(1986). Alcohol consumption in combination with smoking greatly increases the risk of upper aero-digestive tract cancers(2004). The risk of cancers of the upper aero-digestive tract in ex-smokers becomes lower than that of current smoker within five years, although risk is still higher than someone who has never smoked 20 or more years after stopping, and the risk of bladder cancer is also higher than in never-smokers 20 years after giving up(Bosetti *et al*, 2008; Brennan *et al*, 2000; Brennan *et al*, 2001).

The role of smoking in the pathogenesis of breast cancer is controversial. Within the Million Women Study (a large UK prospective study), 224 917 never smokers who completed a questionnaire were asked if their parents had smoked and if their current partner smoked. After a follow-up 3.5 years, 2518 subjects developed invasive breast cancers. The adjusted relative risk of breast cancer for passive exposure combined as a child and as an adult vs neither

exposure was 0.98 (95% CI 0.88–1.09). The results were similarly null for childhood exposure (0.98, 0.88–1.08) and adult exposure (1.02, 0.89–1.16) separately(Pirie *et al*, 2008).

In a cohort of 78,206 women who were followed up prospectively, 3140 reported diagnosis of invasive breast cancer during follow-up. There was no increased risk of development of breast cancers in active and passive smokers. However it showed a modest 20% increase in risk of breast cancer confined to women who began smoking before the age of 17 years(Egan *et al*, 2002).

1.2.5.4 Anthropometry, exercise and diet

In Britain 67% of men and 52% of women have a BMI of 25 or more and approximately one fifth of the men and women are obese(statistics, 2003/2004). There is convincing evidence that obesity is associated with increased risk of cancers. In postmenopausal women increasing levels of anthropometric variables including height, weight and BMI are associated with increased risk of breast cancer(Lahmann *et al*, 2004; Reeves *et al*, 2007; van den Brandt *et al*, 2000). Obesity may increase levels of sex hormones, insulin and insulin like growth factors which all in turn increase the risk of breast cancer. By contrast premenopausal women with a BMI of 31 or more have shown to have a 46% reduced risk compared to those with BMI of less than 21(van den Brandt *et al*, 2000). This may be because obesity can disrupt ovulation(Key *et al*, 2004).

Breast cancer risk is also affected by age at menarche and height, both of which are influenced by body weight earlier in life(Key *et al*, 2004). Obesity also increases the risk of other cancers like endometrial cancer, colon cancer, oesophageal cancer and cancers of the gall bladder and pancreas (Bergstrom *et al*, 2001; Bianchini *et al*, 2002; Moghaddam *et al*, 2007; Reeves *et al*, 2007).

Although it is difficult to separate the effects of physical activity from those of increased body weight, studies have shown that higher level of physical activity reduced risk of developing certain cancers which are independent of body weight. Two large cohort studies have shown reduced risk of colon cancer (approximately 20-25%) with higher levels of exercise(Friedenreich *et al*, 2006; Wei *et al*, 2004). Approximately 50 studies have looked into association between physical activity and breast cancer, showing that post-menopausal women who are physically active have an 80% reduction in risk(Monninkhof *et al*, 2007). The risk of breast cancer in industrialized countries is higher than in developing countries where the women are characterized with lower energy intake and higher energy expenditure. This could be related to lowering of BMI or change in hormonal levels (Chan *et al*, 2007; McTiernan *et al*, 2004). Similar influence of physical activity in premenopausal women is less clear (2007; Monninkhof *et al*, 2007).

A meta-analysis of 7 cohort studies have shown 20% reduced risk of endometrial cancer in most active women compared to the least active women(Voskuil *et al*, 2007). There is also limited evidence than increased physical activity could influence the risk of developing lung and prostate cancer (Giovannucci *et al*, 2005; Nilsen *et al*, 2006; Patel *et al*, 2005; Tardon *et al*, 2005).

1.2.5.5 Alcohol

Alcohol is a well excepted carcinogen and around 6% of UK cancer deaths could be avoided if people did not drink(PJ, 2003). Alcohol consumption increases the risk of cancers of the oral cavity, larynx, oesophagus, breast, bowel and liver and there is a direct correlation to the amount and the risk of cancer(Boffetta & Hashibe, 2006). Alcohol is thought to have a synergic relationship with tobacco in which the alcohol consuming smokers are 80 times increased risk of developing cancer of the aero digestive tract(Franceschi *et al*, 1990). It is estimated that it would take 16 years for the risk of these cancers in a former drinker to fall to the level of someone who has never consumed alcohol(Rehm *et al*, 2007). There is an estimated 5 fold increase in the risk of liver cancer in people drinking more than 80gm per day for over 10 years(Morgan *et al*, 2004). The risk of rectal cancer is stronger with alcohol compared to rest of the colon with almost doubling of risk in people drinking more than 60g per day(Ferrari *et al*, 2007).

Emerging evidence indicates that drinking alcohol is correlated to the risk of breast cancer and the risk is increased by approximately 7% for every additional 10g/day of alcohol (Hamajima *et al*, 2002). Two meta-analysis of epidemiological studies, one carried out in 1994(Longnecker, 1994) and the other carried out through 1999(Ellison *et al*, 2001) showed an increase in the relative risk of breast cancer with alcohol consumption. Both studies showed a modest relation of alcohol consumption to risk of breast cancer, with a 10% risk being seen in women reporting approximately one alcoholic drink per day as compared to non-drinkers. This could be mediated by an increase in the oestrogen levels(Boffetta & Hashibe, 2006). Another mechanism is thought to

be the stimulation of cytochrome P-450 microsomal enzyme by alcohol which is involved in procarcinogen activation.

As with coronary artery disease, moderate amount of alcohol is thought to have protective effect against certain cancers like kidney cancers(Lee *et al*, 2007).

1.2.5.6 Diet

Fruit and vegetable contain antioxidants and are thought to be protective against certain cancers. Each daily portion of 80-100 grams of fruit or vegetables halves the risk of oral cancer(Pavia *et al*, 2006), reduces the risk of squamous cell cancer of the oesophagus by approximately 20% and stomach cancer by about 30%(Freedman *et al*, 2007). High folate intake has been shown to reduce risk of breast cancer in moderate and heavy drinkers(Larsson *et al*, 2007). High fibre diet is thought to be protective against bowel cancer. This is particularly protective in people who consume high amounts of red and processed meat (Norat *et al*, 2005). High intake of salt and salt-preserved foods are associated with increased risk of stomach cancer (Shikata *et al*, 2006). A recent collective analysis of cohort studies found a 16% reduction in risk of bowel cancer in people with higher consumption of milk, which remained after adjustment for total calcium and vitamin D intake(Cho *et al*, 2004).

Contrary to the earlier belief that dietary fat has no relation to the incidence of breast cancer, recent studies suggest a link. In a recent cohort of pre-menopausal women higher intake of animal fat was associated with increased

risk of breast cancer(Cho *et al*, 2003b) and a pooled analysis of nine breast cancer cohort studies reported a 9% increased risk with each 5% increase in saturated fat(Smith-Warner *et al*, 2001). Lack of association between fat and breast cancer in some of the previous cohort studies has been attributed to inaccuracy in reporting diet(Bingham *et al*, 2003).

1.2.5.7 Exogenous hormones

Abnormal hormone levels have been associated with some of the common female cancers. Early menarche and late menopause has been associated with increased risk of ovarian, endometrial and breast cancers(2002; Albrektsen *et al*, 2005). Each full term pregnancy has a protective effect against these cancers and first full term pregnancy providing more protection than successive ones(Albrektsen *et al*, 2005). Breast cancers are reduced by around 4% for each year a women breastfeeds(2002). In women who breast feed there is also a 50% reduction in the risk of endometrial cancer(Newcomb & Trentham-Dietz, 2000) and 40% reduction in ovarian cancer(Tung *et al*, 2005).

In 1996, a meta-analysis of data from 54 epidemiological studies of oral contraceptive use and risk of breast cancer showed that subjects taking oral contraceptives have a slightly increased risk of breast cancer compared to the risk in non-users(1996). Data of over 53000 women with breast cancer from different countries were collected, checked and analyzed. The risk decreased with increasing years after stopping the oral contraceptives and there was no evidence of increased risk of having breast cancer diagnosed 10 years or more after stopping the use of oral contraceptives and the cancers diagnosed were

less advanced than in never users. Although there is a transient increase in breast cancer when oral contraceptives are taken(1996; Boyle *et al*, 2003), a meta-analysis of European case-control studies indicated that use of oral contraceptive for five or more years results in a 50% reduction in risk of ovarian cancer which remains for more than 20 years(Bosetti *et al*, 2002). Similar reduction in endometrial cancer has been noted in case control studies with reduction in risk increasing with duration of use(J, 1996).

There is extensive data linking hormone replacement therapy to the risk of breast cancer. It is estimated that over the past decade the use of HRT by UK women aged 50-64 years has resulted in an extra 20,000 breast cancers(Beral, 2003). The collaborative group in 1997 published the meta-analysis of approximately 90% of worldwide epidemiologic evidence on the relation between the risk of breast cancer and the use of HRT(1997). It involved over 52,000 cancer cases and 108,000 controls. Among current users and those who used HRT within 1 to 4 years previously, the risk of having breast cancer diagnosed increased by 2.3% for each year of use and the relative risk was 1.35 for women who used HRT for 5 or more years. However the effect disappears 5 years after stopping HRT.

A randomized controlled primary prevention trial involving over 16,000 postmenopausal women aged 50-79 years receiving combined hormone preparation showed an increased risk of breast cancer in the users by 26%. The incidence of coronary heart disease was also increased by 29%(Rossouw *et al*, 2002). The risk of colorectal cancer and hip fractures were reduced by 30%. Overall the risks exceeded the benefits of the use of combined oestrogen plus progestin preparation.

Risk ratio of breast cancer in women taking combined or oestrogen only therapy is 2 and 1.3 respectively(Beral, 2003). The risk increases with duration of use, with risk ratio of 2.3 for 10 or more years of combined HRT, compared with 1.5 for less than 1 year. There is no difference in risk between never users and women who stopped therapy at some point in the previous 5 years.

Oestrogen only HRT are also associated with increased risk of endometrial and ovarian cancers(Lacey *et al*, 2002). The risk of endometrial cancer is doubled after 5 years of their use and similar use produces a 25% increased risk of ovarian cancer(J, 1996; Pike *et al*, 2004). Studies have shown than the use of combined pill does not increase the risk of both these cancers(Lacey *et al*, 2002; Pike *et al*, 2004). Abnormal hormone levels have been associated with male cancers as well. There appears to be a more than two-fold increase in risk of prostate cancer in men with highest testosterone levels(Shaneyfelt *et al*, 2000).

1.2.5.8 Occupation and cancer risk

It is estimated that 11% of all cancer cases in men were caused by occupational exposure and approximately 4% of cancer deaths in men were caused by occupation(IARC, 2008; Winther *et al*, 1997). The cancer most commonly linked to occupation is mesothelioma and 80-90% of mesothelioma cases in men are linked to occupational exposure to asbestos (IARC, 2008; Rushton l, 2008; Winther *et al*, 1997). The other cancer which is strongly linked to occupation is cancer of nasal cavity and sinuses. The important workplace exposure for this cancer is leather dust, wood dust and metals(IARC, 2008; Winther *et al*, 1997). Lung cancer also has a strong occupational component, with up to 17% of deaths in men and 5% in women

caused by workplace exposure(Rushton 1, 2008). The major causes of occupation related lung cancer are metals, asbestos, radon, arsenic, silica and polycyclic aromatic hydrocarbons (PAH)(IARC, 2008; Pelucchi *et al*, 2006; Rushton 1, 2008). A small proportion of bladder cancers are linked to occupation and occupational exposure to PAH and other aromatic amines as in rubber industry. Laryngeal cancer has been linked to PAH and leukemia to benzene and other solvents. Other possible occupational exposures leading to cancer include vinyl chloride to liver cancer(Boffetta *et al*, 2003) and diesel engine exhaust exposure to lung cancer(Lipsett & Campleman, 1999).

The relationship between ionizing radiation and the risk of breast cancer is well established (Evans *et al*, 1986; John & Kelsey, 1993). Increased risk has been observed in patients receiving fluoroscopy for tuberculosis and radiation treatment for medical conditions. The risk is inversely associated with age at exposure. Women treated for Hodgkin's disease by the age of 16 have a subsequent risk of developing breast cancer as high as 35% by the age of 40 years(Bhatia *et al*, 1996; Hancock *et al*, 1993). Higher dose of radiation and treatment between the ages of 10 and 16 corresponded with higher risk. The risk of breast cancer is also raised when radiation therapy was given in the late teens and twenties, but to a lesser degree. In these studies majority of breast cancers (85-100%) developed either within the field of radiation or at the margin(Sankila *et al*, 1996).

In subjects at high risk of developing breast cancer intensive mammographic screening has been advocated. It is unknown whether the additional radiation exposure could produce increased risk. In theory, breast cancer patients treated with lumpectomy and radiation therapy should be at an increased risk of

second cancers compared to those treated by mastectomy. However outcome studies with long follow-up (15 years) does not show any difference (Obedian *et al*, 2000).

1.2.5.9 Infections and cancer

Several infectious agents are considered to be causes of cancer in humans. The estimated total of infection-attributable cancer in the year 2002 is 1.9 million cases or 17.8% of the global cancer burden (Parkin, 2006). The principal agents are the bacterium *Helicobacter pylori* (5.5% of all cancer), the human papilloma viruses (5.2%), the hepatitis B and C viruses (4.9%), Epstein-Barr virus (1%), human immunodeficiency virus (HIV) together with the human herpes virus 8 (0.9%). Relatively less important causes of cancer are the schistosomes (0.1%), human T-cell lymphotropic virus type I (0.03%) and the liver flukes (0.02%). There would be 26.3% fewer cancers in developing countries (1.5 million cases per year) and 7.7% in developed countries (390,000 cases) if these infectious diseases were prevented. Table below shows some of well known infections associated with cancer.

Table 1.1

Infection	Cancer site/type
Human papilloma virus (HPV)	Cervix, anus, lower genital tract, pharynx, other head & neck
Hepatitis B and C virus (HBV, HCV)	Liver, NHL
Helicobacter pylori (Hp)	Gastric cancer, gastric MALT*
Schistosomes and liver flukes	Liver, bladder, colon
HIV	Kaposi sarcoma, NHL, Hodgkinlymphoma
Human herpes virus 8 (HHV8)	Kaposi sarcoma, NHL
Epstein Barr virus (EBV)	Hodgkin lymphoma, Burkitt NHL

1.2.6 Immune response to cancer

Evidence supporting an immune response to cancer is compelling due to the identification of autoantibodies against a number of intracellular and surface antigens in patients with cancer referred to as tumour-associated antigens (TAAs)(Crawford *et al*, 1982; Lubin *et al*, 1993; Winter *et al*, 1992). Studies show antibodies to TAAs identified before symptomatic disease (Li *et al*, 2005; Robertson JFR, 2005a; Robertson JFR, 2005c). Low levels of TAAs can elicit a detectable antibody response and its detecting appears more promising than direct protein screening which is less sensitive and specific(Luborsky *et al*, 2005; Nesterova *et al*, 2006). Our review will look into the present modalities available in the identification of TAAs which initiates the antibody response.

The productive mechanism of these antibodies is less well understood. Usual targets are often cellular proteins which have altered location and/ or post-translational modifications which have led to tumourogenesis(Scanlan *et al*, 2001). How these intracellular molecules acquire immunogenicity is not entirely clear. Antibodies to p53 is one of the earliest and the most intensely investigated and we intend to review some of the important studies in this field which may shed light into the mechanism of production of autoantibodies. We will also look into some of the more recent antigens identified to play an important role in cancer diagnosis.

Hundreds of autoantigens have been cloned with the recognition of autoantibodies in patient's sera (Boon & Old, 1997; Forti *et al*, 2002; Sahin *et al*, 1995; Scanlan *et al*, 2001). However to predict the malignant disease based

on the presence of these individual antibodies have been largely unsuccessful and demonstrating their tumour relevance has been difficult(Brandt-Rauf & Pincus, 1998). Since more than one antigen is often associated with a particular cancer, panels of antigens to improve the diagnostic potential are being evaluated.

1.2.6.1 Tumour associated antigens and their detection.

Old and colleagues initially demonstrated the presence of tumour-specific antibodies in the serum of cancer patients (Old, 1981). However major improvement in identification of various tumour associated antigens did not materialize until early to mid 1990's when a technique to identify T and B-cell epitopes uniquely expressed on tumour cells was developed. Initially Boon and colleagues popularized the T-cell screening of a cDNA expression library made up of m-RNA from the tumour cells(van der Bruggen *et al*, 1991). This technique and its modification led to the identification of more than 50 antigens, most of which were identified in malignant melanoma. In concordance with this genetic approach, a more biochemical approach was found to be successful. Here antigenic peptides pool eluded from the cell-surface HLA molecules of the tumour cells were fractioned by liquid chromatography and individual fractions were screened with tumour-specific T-cell clones(Cox *et al*, 1994; Wang, 1999). However the most recent and popular technique is the SEREX (serological analysis of recombinant cDNA expression libraries) and its implementation dates from 1995(Sahin *et al*, 1995). With the introduction of SEREX a large number of TAAs have been identified (over 2000). In this antibody based method cDNA clones were constructed from tumour tissue specimens recognized as autoantigens by autologous cancer patient sera(Chen *et al*, 2005; Sahin *et al*, 1997; Sahin *et al*, 1995; Tureci *et al*, 1997). An advantage of SEREX over other techniques is that by identifying immunogenic proteins uniquely expressed in tumours, these immunogenic proteins may also contain T-cell epitopes which could be

identified as well. Antigen specific cellular and humoral immune response can occur simultaneously against tumour associated antigen. This has been found to be true with number of antigens including breast cancer antigens HER2 and NY-ESO-1(Jager *et al*, 1998). However it has its own disadvantages and such expression libraries contain not only cDNA clones from tumour cells but also from inflammatory and other cellular infiltrates, and thus being dependent on the particular patient(Fernandez Madrid *et al*, 2005). It fails to employ criteria to select those antigens recognised by multiple patients. Another disadvantage is that since SEREX involves screening tumour-derived autologous cDNA libraries, it may have a bias towards identifying autoantigens that are over expressed but otherwise normal antigens rather than mutated in tumour cells(Fernandez Madrid *et al*, 2005). Thus they may not preferentially select antigens that are oncogenic. As a result antigens which are expressed in low abundance but more relevant to tumorigenesis may be missed.

To negate these problems, researchers have used the approach of using cDNA clones constructed from immortalized cell lines and screening with antibodies from multiple cancer sera (Fernandez Madrid *et al*, 2005; Imai *et al*, 1993a). This method could minimize the confounding effect of irrelevant antibodies and has a potential of revealing a number of proteins that may be involved in cellular functions related to tumourogenesis.

Another method used in the identification of tumour associated antigens is the proteomics approach (Klade *et al*, 2001). This allows individual screening of large number of sera and at the same time identify a large number of autoantigens(Zhang, 2004). Here lysates from cancer cell lines are separated on two-dimensional gel electrophoresis, transferred to membranes, and probed

with cancer sera and immuno-reactive proteins identified by mass spectrometry (Canelle *et al*, 2005; Klade *et al*, 2001; Le Naour *et al*, 2001; Yang *et al*, 2007). This approach allows proteins, in their post-translational modification states as they occur in cells, to be analyzed for their antigenicity(Qiu *et al*, 2008).

1.2.7 Antibodies to Cancer antigens

1.2.7.1 Antibodies to traditional well recognised antigens in the diagnosis of cancer

A number of well recognised antigens to which tumour associated antibodies have been detected will be described in this chapter..

1.2.7.1.1 P53

P53 is a phosphoprotein not easily detectable in the nucleus of normal cells(Benchimol *et al*, 1982). It arrests cell cycle progression following DNA damage caused by cellular stress by activating a series of genes involved in cell cycle regulation(Kastan *et al*, 1991). It thus allows the DNA to be repaired or leads to apoptosis(Yonish-Rouach *et al*, 1991). However cancer cells which bear a mutant p53 are unable to exhibit its normal functions leading to insufficient DNA repair and thus leading to genetically unstable cells(Giaccia & Kastan, 1998; Levine, 1997; Oren & Rotter, 1999; Prives, 1998). In human cancers, p53 tumour suppressor gene is the most frequently identified site for mutations accounting for more than 50% of cases(Hofseth *et al*, 2004; Soussi, 2000b).

In 1979 the importance of p53 in tumourogenesis was reported from several laboratories(DeLeo *et al*, 1979; Linzer & Levine, 1979). Subsequently during the course of a tumour-associated antigens screening, Crawford et al in 1982 identified p53 antibodies against human p53 protein(Crawford *et al*, 1982). In

this study (Crawford et al), 14 out of the 155 (9%) sera from breast cancer patients tested were positive for anti-p53 antibodies and no positives were detected among 164 control sera from normal women. Later, Caron de Fromentel et al found that p53 autoantibodies in wide variety of tumours in children with an average frequency of 12% and in certain tumours reaching approximately 20%(Caron de Fromentel *et al*, 1987). Soussi et al compiled a large group of studies involving serological analysis of p53 in different types of cancer(Soussi, 2000a). They analysed almost 9500 patients with different types of cancer and also included controls composed of approximately 2500 individuals with no evidence of cancer. P53 was found to be strong markers for malignancy which was highly statistically significant ($P<0.001$) with a specificity of 96%. The sensitivity of such detection was 30%.

Mutation in p53 gene has been implicated in the development of immunogenesis. In the above study by Soussi et al involving a wide variety of cancers it was found to be associated significantly with most types of cancers with a few exceptions of testicular cancer, melanoma and hepatocellular carcinoma which were negative for p53 auotoantibodies(Soussi, 2000a). These three cancers are well known to be devoid for p53 mutations(Fleischhacker *et al*, 1994; Lubbe *et al*, 1994; Puisieux *et al*, 1993).

Mutation in the p53 gene or accumulation of mutant p53 protein has been identified in common premalignant lesions and in histologically normal tissue adjacent to tumours. This may suggest that they are early events in the process of carcinogenesis(Downing *et al*, 2003; Harris, 1996; Hill & Sommer, 2002).

Further studies on other cancers since then have shown a good correlation between the presence of p53 autoantibodies in the sera of patients with cancers and the occurrence of p53 mutation or accumulation of mutant p53 protein in the tumour tissue(Soussi, 2000a; Soussi, 2000b). Study on patients with premalignant conditions like leukoplakia and Barrett's oesophagus has shown p53 antibodies in their sera suggesting its potential use in early diagnosis(Soussi, 2000a). Autoantibodies to p53 have also been identified in cases of heavy smokers before their development of lung and other tobacco related cancers and also in certain workers who have been exposed to carcinogens before their development of cancer(Lubin *et al*, 1995; Schlichtholz *et al*, 1994; Trivers *et al*, 1996).

In 1992 an important study by Winter et al on correlating p53 mutations with appearance of antibodies in lung cancer patients was published(Winter *et al*, 1992). Cancer cell lines from patients with lung cancer were analysed for mutations. Here, sera of 13% (6/46) of patients with lung cancer were found to be positive for p53 antibodies and all of them had point mutations (missense mutations), which resulted in accumulation of p53 proteins with altered function and relatively increased stability. These missense mutations occurred in the hot spot regions of p53 in exons 4 to 9. Patients with other forms of mutations like stop codon mutations or frameshift mutations failed to induce antibody response. However contrary to the expectations, the analysis of p53 antibody specificities revealed that the antigenic determinants of p53 were located at the amino-and carboxy-terminal regions and away from the central region of p53 containing the mutations(Labrecque *et al*, 1993; Lubin *et al*,

1993). Absence of antibodies in patients with other mutations (stop codon or frameshift mutations) in the study by Winter et al would be related to the relative absence of p53 protein in the transformed cells. Thus the load of the aberrant protein appears to be an important factor in development of antibody response. The inactive mutant p53 protein is associated with increased stability with half life of several hours compared to 20 minutes for wild type p53(Soussi, 2000a). Another factor which may decide the antigenic stimulus could be the location of the p53 protein. It is normally located in the nucleus, and in cancer cells this protein can be excluded from the nucleus and could appear in the cytoplasm as well(Moll *et al*, 1992). Even with all the information, the mechanism behind the antigenicity of p53 is far from clear. Several studies have shown that despite similar types of cancer, identical p53 mutations, and p53 accumulation, some patients could be either positive or negative for p53 antibodies(Hammel *et al*, 1999; von Brevern *et al*, 1996; Winter *et al*, 1993). This would suggest that other factors are contributing to humoral response and there is appears a possibility that it could be linked to the biological make up of the patients(Soussi, 2000a).

1.2.7.1.2 HER2

Like tumour suppressor genes, oncogeness represent the other class of genes contributing to tumour development(Aaronson, 1991). Human epidermal growth factor receptor-2 (HER-2) is an important member of the oncogene family(Coussens *et al*, 1985). The gene product in HER-2/neu gene function as a normal epithelial protein in cell growth and proliferation(Carney *et al*, 2003). However their gene amplification and protein over expression contribute to converting healthy cells to cancer cells and the oncoprotein is now recognized

as an important factor in breast cancer development(Carney *et al*, 2003). HER-2/neu DNA amplification and protein overexpression occur in 25-30% of women with primary breast cancer(Ross & Fletcher, 1999; Schaller *et al*, 2001). Patients with pre-invasive cancers are associated with high IHC staining for HER-2. Latta et al reported 34 % IHC staining in patients with pure DCIS and sixty percent of cases of high grade ductal carcinoma in-situ showed increased IHC overexpression(Latta *et al*, 2002). The extra cellular domain (ECD) of the receptor protein has been found to be shed in plasma and serum of healthy individuals and patients with breast cancer(Leitzel *et al*, 1992; Pupa *et al*, 1993; Zabrecky *et al*, 1991). In a systematic review by Carney et al the analysis of 1923 primary breast cancer patients from various studies showed increased ECD concentration in 18.5% of patients at the time of cancer diagnosis(Carney *et al*, 2003). Although found to be useful in the diagnosis of primary breast cancers, observations points to their concentration being closely linked to tumour burden. In metastatic disease a review of 45 references and 4622 patients with metastatic breast cancer showed that 43% of patients had raised ECD values(Carney *et al*, 2003). Likewise a report by Anderson et al showed that significantly more patients with recurrent breast cancer have raised ECD (59% - 55 of 93) compared to primary breast cancer patients (8%), and importantly in patients with recurrent disease, ECD concentrations were detected significantly more often in patients with distant metastases than those with local metastasis (68% vs 19%)(Andersen *et al*, 1995).

1.2.7.1.3 c-myc

Almost all types of human malignancy have been reported to have amplification and /or over expression of the c-myc gene(Nesbit *et al*, 1999). It is now known that the c-myc gene participates in most aspects of cellular function, including replication, growth, metabolism, differentiation and apoptosis(Dang, 1999; Dang *et al*, 1999; Elend & Eilers, 1999; Hoffman & Liebermann, 1998; Packham & Cleveland, 1995; Prendergast, 1999). c-myc gene produces three major proteins named c-myc 1, c-myc 2 and c-myc S(Facchini & Penn, 1998; Henriksson & Luscher, 1996). c-myc 2 is approximately 62-kDa protein that is the major form of the three c-Myc proteins and the one referred to as 'c-Myc' in most studies. There is increasing evidence to support a role for c-myc proto-oncogene in tumour onset and progression. In breast cancer, the chromosome 8 region where the gene is localized has been identified as one of the three most commonly amplified regions of the genome(Courjal *et al*, 1997). This region is commonly amplified in small cell lung carcinoma, leukemia and colon carcinoma(Alitalo *et al*, 1983; Dalla-Favera *et al*, 1982; Little *et al*, 1983). Studies utilizing immunohistochemistry show that about 50-100% of breast cancer cases have increased levels of c-myc proteins(Agnantis *et al*, 1992; Hehir *et al*, 1993; Pavelic *et al*, 1992; Saccani Jotti *et al*, 1992).

1.2.7.1.4 Annexins

They are a family of calcium^{2+} and phospholipids-binding, membrane associate proteins found in various organisms and implicated in ca^{2+} signal transduction process associated with cell growth and differentiation(Ahn *et al*,

1997; Brichory *et al*, 2001; Chetcuti *et al*, 2001; Emoto *et al*, 2001b; Mai *et al*, 2000; Maler *et al*, 2002; Srivastava *et al*, 2001; Yeatman *et al*, 1993). Annexins are classified into five groups, A-E, and within each of these groups, individual annexins are identified numerically. Human annexins belong to group A which has been sub-classified from 1 to 13(Mussunoor & Murray, 2008). Some annexins are over-expressed in specific types of cancers, while others show consistent loss of expression. There is increasing evidence that changes in annexin expression and/ or their subcellular localization contribute to development and progression of cancer(Mussunoor & Murray, 2008). Annexin 1 is found to be over-expressed in oesophageal adenocarcinoma, pancreatic adenocarcinoma, colorectal carcinoma and hairy cell leukemia(Mussunoor & Murray, 2008). However in oesophageal squamous cell carcinoma and breast adenocarcinoma and prostatic cancer there is loss of annexin A1 expression(Mussunoor & Murray, 2008). Increased expression of annexin A2 has been described in several types of tumours, including gastric cancinoma, colorectal cancer, pancreatic cancer, breast cancer, high grade gliomas, kidney cancers and vascular tumours(Duncan *et al*, 2008; Emoto *et al*, 2001a; Emoto *et al*, 2001b; Esposito *et al*, 2006; Reeves *et al*, 1992; Sharma *et al*, 2006; Syed *et al*, 2007; Zimmermann *et al*, 2004). In contrast there is loss of expression of this annexin in other types of tumours, including prostatic carcinoma and pulmonary metastasis of osteosarcoma(Gillette *et al*, 2004; Kang *et al*, 2002; Stewart *et al*, 2007; Yee *et al*, 2007). AAbs to annexin 1 and annexin 2 have been investigated in patients with lung cancer. In a study by Brichory et al (methodology), 2001 looking into 54 lung cancers, Annexin 2 were found in 33% of lung cancer patients(Brichory *et al*, 2001).

Over all 60% of lung cancer patients had annexin 1 and/or annexin 2 AAbs present. The reactivity seen in lung cancer was not limited to advanced stages, and 51% of patients with stage 1 cancer had AAbs. In another study by Qui et al, involving 85 patients with lung cancer with preclinical samples collected with 1 year before the diagnosis of lung cancer matched with high risk controls showed significant antibody reactivity against annexin 1 and other two antigens (LAMR1, 14-3-3 theta) with a sensitivity of 55% and specificity of 95%(Qiu *et al*, 2008). When comparing the reactivity of samples taken between 0 and 6 months before diagnosis and the other group between 7-12 months before diagnosis there was equal reactivity between two groups in annexin 1.

Annexin X1A has been reported to be reactive in a high proportion of sera of patients with DCIS, even higher than those with invasive cancer(Fernandez-Madrid *et al*, 2004). A study by Fernandez-Madrid, 2004 micro-arrays containing the T7 phages encoding the potential breast TAAs were probed with serum from 90 patients with breast cancer (15 with DCIS and 75 with IDC), along with 51 non-cancer control sera and sera from 21 patients with systemic autoimmune disease. One of the antigens identified corresponded to annexin XI-A and although the number of patients with DCIS was small, AAbs to these antigens were present in 60% of patients with DCIS suggesting a strong link between annexin XI-A and early development of cancer. Eleven percent of patients with IDC had positive AAbs to annexin XI-A and no reactivity was reported in autoimmune samples.

1.2.7.1.5 PSA

PSA is a kallikein which belongs to the family of serine proteases expressed at high levels in the epithelium of the human prostate gland(Malm & Lilja, 1995). PSA as a tumour marker is invaluable in the diagnosis and post surgical management of prostate cancer. It has been established that prostate cancer patients have a greater fraction of PSA complexed to ACT(alpha 1 antichromotripsin) and the ratio of free to total PSA has become more important for distinguishing benign and malignant prostatic pathology(Armbruster, 1993). In is now evident that hormonally regulated tissues in females like breast can produce PSA(Yu *et al*, 1996). With the use of ultrasensitive immunoassays, PSA has been demonstrated in at least 50% of normal female sera(Melegos & Diamandis, 1998). The concentration of PSA in female sera is approximately 1000-fold lower than that of the breast and is approximately 100-500 times lower than male serum PSA(Black & Diamandis, 2000). The serum PSA in women is inversely correlated with age and is probably related to the hormonal levels and PSA has been found to be differentially expressed during menstrual cycle(Zarghami *et al*, 1997). It is also seen in milk of lactating women and nipple aspirate fluid(Foretova *et al*, 1996; Sauter *et al*, 1996). While level of serum PSA is elevated in both benign and malignant breast disease, molecular form of circulating PSA differs in women with and without breast cancer. The predominant serological form (>50%) of PSA in a significant proportion of females with breast cancer is free PSA(Black *et al*, 2000). In a study by Black et al, in patients with breast cancer, benign breast disease and normal's, the percentage of breast cancer patients with free PSA as predominant (>50% of the total PSA) form was five

times higher compared to benign breast disease or healthy women(Black *et al*, 2000). Free PSA as the predominant molecular form showed a sensitivity of 20% and a specificity of 96%. The study also showed a dramatic reduction of free PSA following surgery, and this is a strong indication that this fraction is produced by breast tumours.

1.2.7.1.6 NY-ESO-1

NY-ESO-1 is classic cancer/testes (CT) antigen discovered during a SEREX analysis of an oesophageal cancer(Chen *et al*, 1997a). NY-ESO-1 is a 22 kD hydrophobic protein coded for by a gene in the Xq28 region(Chen *et al*, 1997a). In normal tissues, expression of NY-ESO-1 mRNA as detected by RT-PCR is predominantly restricted to the testes, whereas in cancer it is found in variable proportion in a wide range of different malignancies including melanomas, cancer of the breast, ovary and lung(Chen *et al*, 1997a). In a report by Sugita et al, using conventional RT-PCR, they found NY-ESO-1 mRNA in 37 of 88(42%) breast cancer specimens(Sugita *et al*, 2004). NY-ESO-1 antigens are strongly immunogenic, and may produce specific hormonal and cellular immunity in patients with NY-ESO-1 expressing cancer(Chen *et al*, 1997b; Jager *et al*, 1998). In a recent study 4% of breast cancer patients produced detectable antibodies against NY-ESO-1(Mischo *et al*, 2006). Melanomas studies have shown a better antibody response and approximately 50% of patients with advanced NY-ESO-1 mRNA-positive melanoma produce NY-ESO-1 antibody(Chen *et al*, 1997b). In a study by Stockert et al involving sera of 234 cancer patients and 70 normal controls,

antibodies to NY-ESO-1 in 19 patients, to MAGE-1 in 3, MAGE-3 in 2, and to SSX2 in 1 patient. No reactivity to these antigens was found in sera from 70 normal individuals(Stockert *et al*, 1998). The frequency of NY-ESO-1 antibody was 9.4% in melanoma patients and 12.% in ovarian cancer patients[324].

1.2.7.2 Antibodies to new antigens useful in the diagnosis of lung cancer

With the introduction of SEREX, hundreds of new cancer related antigens have been identified. However, only a few of them are presently used in the potential diagnosis of early cancer and those used in our panel are described.

1.2.7.2.1 CAGE

It is a cancer testes antigen like NY-ESO-1 which has been reported as capable of producing antibody response to lung cancers(Chapman *et al*, 2008). GBU4-5, like CAGE encodes a DEAD-box domain and has recently been described to produce antibody response in lung cancers(Chapman *et al*, 2008). These DEAD-box-containing proteins are involved in RNA processing, ribosome assembly, spermatogenesis, embryogenesis, and cell growth and division. They seem to be immunogenic, cancer-specific and provide diagnostic and potentially immunotherapeutic cancer targets. Analysis of autoantibodies to the full panel of seven antigens resulted in 75% of the lung cancer samples being identified and 92% of squamous cell carcinomas with a specificity for cancer of 92%(Chapman *et al*, 2008). In this study, autoantibody response to DEAD-box proteins CAGE and the new cDNA GBU4-5 were also highly

sensitive and specific in this study. The DEAD-box cancer testes antigen CAGE has previously been shown to be expressed in a number of other cancers, and autoantibodies has been reported in gastric and pancreatic cancers apart from lung cancers. However, autoantibodies to GBU4-5 have not been investigated in other cancers.

1.2.7.2.2 SOX2

It is an amplified oncogene in lung carcinomas. It is expressed in the developing foregut, where it regulates initial dorsal-ventral patterning, shapes epithelial-mesenchymal interactions and is required for proper differentiation both of the squamous oesophageal and of multiple respiratory cell types(Bass *et al*, 2009). It is also preferentially expressed in squamous cell carcinomas of the oesophagus and anal canal compared to adenocarcinomas from these sites(Long & Hornick, 2009). SOX antibodies have been found to specific serological markers for small cell lung cancers and have a reported sensitivity of 43% in this group which represent 13-20% of lung cancers(Titulaer *et al*, 2009).

1.2.7.2.3 GBU 4-5

GBU 4-5, like CAGE encodes a DEAD-box domain and has been identified to express antibody response in lung cancers(Chapman *et al*, 2008). It has been identified using SEREX technologies(Tureci *et al*, 2005). These proteins have been thought to be involved in embryogenesis, cell growth and division, and may have a role to play in carcinogenesis(Linder, 2006). They are

immunogenic, cancer specific and may provide diagnostic and potentially valuable targets for cancer immunotherapy(Park *et al*, 2003).

1.2.8 Autoantibodies to panel of antigens in cancer diagnosis

Cancer is a heterogeneous disease with variation in individual antigen expression between patients. Although detection of individual antigens have been shown to have diagnostic potential, it is likely that the reliability of a test could be improved by identifying group of antigens which are associated with particular cancer. The first report of using a panel of antigens was from our centre (Robertson et al Blood Borne Tumour Markers, patent W099/58978). Zhang et al in their study used enzyme immunoassay to look into 7 different types of antibodies in sera of 527 patients with 6 different types of cancers(Zhang *et al*, 2003). The antibody frequency of any individual antigens varied from 15-20%. However a stepwise increase in the positive reaction was noted on successive addition of seven different antigens to the panel. There was a rise in the level of antibody against at least one antigen in 51% of cases. For different cancers there was a variation in the percentage of antibodies detected against various antigens. For breast cancer it appeared that c-myc and Koc antibodies were more frequently detected than other antibodies. However for lung cancer antibodies to cyclin B1,p62 and p53 were more frequently detected. For prostate cancer there was a high frequency of antibodies to p62 and cyclin B1.

In another study by Megliorino et al, sera from 1137 patients with different types of cancers were analysed for antibodies to survivin(Megliorino *et al*, 2005). They were positive in 8.4% (96/1137) of cases. From this large group of cancer cases, sera of a subgroup of 546 cancer patients were analysed for antibodies to p53, c-myc and survivin. The study showed that when antibody to any one of the antigens was considered, the cumulative frequency was

increased to 27% from an individual maximum of 12%. This was significantly higher compared to normal sera which was positive in 4.9 % of subjects. Interestingly the rate of antibody frequency was particularly high in certain cancers notably lung (32%), HCC(39%), and gastric cancer (31%). There was a different pattern of antibody frequency in individual cancer types i.e for breast cancer, c-myc was a better reactive antigen than p53 and survivin, in oesophageal and lung cancer both p53 and c-myc appeared more reactive, for colorectal cancer the most reactive antigen was p53.

TAAs p62 and Koc are insulin-like growth factor 11 (IGF-11) messenger RNA binding proteins. In a study by Zhang JY et al, sera from 777 patients with 10 different types of cancers were assessed for antibodies to p62 and Koc(Zhang *et al*, 2001). Individually autoantibodies to p62 and Koc were found in approximately 12% of patients. The cumulative frequency was increased to 21% and compared to normal controls and those with autoimmune disease it was statistically significant ($p<0.01$).

In a similar study by Stockert E et al, sera of 234 patients with different types of cancer and 70 normal individuals were analyzed for antibodies to seven different antigens(Stockert *et al*, 1998). Antibodies to 4 different antigens were found to be raised in cancer patients and none in normal controls. In particular, 7.7% (2/26) of patients with breast cancer and 4.2 %(1/24) of patients with lung cancer had raised antibodies to NY-ESO-1. This was detected in higher frequency in melanoma patients (9.4%; 12/127) and ovarian cancer (12.5%;

4/32). There was no evidence in this publication that a panel of autoantibodies was additive.

Roberson JFR et al in his report involving approximately 200 primary breast cancers, reported a sensitivity of 82% using a panel of 4 antigens compared to normal controls(Robertson JFR, 2005a). In a later study from the same institute, sera of breast cancer patients (PBC-97, DCIS-40) and 94 normal controls were tested for antibodies to a panel of 7 antigens(Chapman *et al*, 2007). Individual antibodies were raised in between 8-34% of PBC sera and 3-23% in DCIS sera. Antibodies to at least one of a panel of 6 antigens was identified in 64% of PBC and 45% of DCIS with a specificity of 85%. One other study has reported on autoantibodies in patients with DCIS(Fernandez-Madrid *et al*, 2004). In this study, to start with sera of 10 patients with invasive breast cancer was used for immunoscreening a library of T7 phage cDNA breast cancer proteins. Microarray was constructed from positive phages cloned with the sera. Ninety patients with breast cancer (PBC-75, DCIS-15) and 51 non cancer controls were used to probe the breast autoantigen microarray. Starting with the phage most significantly associated with cancer versus the non-cancer group, a 12-phage breast cancer predictor group was constructed. Overall, 77% of the patients demonstrated a response to at least one of the antigens. The sensitivity of DCIS group was 60% (9/15) compared to 11% (8/75) in the invasive group. Annexin XI-A, one among the tumour-associated antigen panel suggested a possibility of distinguishing DCIS from invasive breast cancer. In DCIS, 60% (9/15) of the sera was positive for annexin XI-A compared to 11% (8/75) invasive breast cancers. Although the

above results were found to be significant, the sample size of the DCIS group was small.

There are further studies which have looked into other panel of antigens both in primary and advanced breast cancer. In a report by Yagihashi et al, prevalence of anti-survivin and livin antibodies in sera of 46 patients with breast cancer was analyzed in comparison with 10 healthy controls(Yagihashi A, 2005b). Anti-survivin antibodies were detected in 24% of cancer cases and anti-livin antibodies were detected in 33% of cancer cases. ELISA test was positive in 52% (24/46) of patients for one or both the antigens.

There is a further report by Zhong L et al in 87 breast cancer patients reporting high sensitivity and specificity with panel of antigens (Zhong L, 2008).In this study, breast cancer cDNA T7 phage library was interrogated with antibodies in patient sera to identify aberrantly expressed tumour proteins in breast cancer. Six of the phage expressed proteins were isolated and the corresponding antibody activities were measured by ELISA in sera of 87 breast cancer patients and 87 normal subjects. The results showed that 3 of the phage clones reached statistical significance in determining patients from normal (ASB-9, SERAC1, RELT). Combing the measurements of the three phage proteins in logistic regression modal achieved 80% sensitivity and 100% specificity.

Lung cancer is another well researched area with regard to autoantibodies in cancer diagnosis. In a study by Chapman CJ et al from our institute, sera of 104 lung cancers (non-small cell-82, small cell-22) and 50 controls were

analyzed for the presence of antibodies against a panel of 7 antigens(Chapman *et al*, 2008). It showed a sensitivity of 76% (79/104 patients had raised antibodies against at least one of the 7 antigens) and specificity of 92% (4/50 patients had raised antibodies against at least one of the 7 antigens). The highest level of antibody sensitivity was seen to MUC1 peptide antigen for both forms of cancer (non-small cell-34%, small cell-36%), once again underlining the importance of panel of antigens in cancer diagnosis.

In another study by Yagihashi et al, sera of 37 patients with lung cancer were analyzed for antibodies to livin and survivin antigens (Yagihashi A, 2005a). Nineteen of the 37 lung cancer patients were positive for livin antibodies compared to 18 out of 31 (58.1%) for survivin. Sera of 31 lung cancer patients were analyzed simultaneously for both livin and servivin, and 21 patients (71%) were positive for any one or both. It was noted that anti-survivin antibodies were found in all stages of lung cancer, but anti-livin only in advanced stages. This could imply that larger expression of livin is required for inducing an antibody response compared to survivin. In another study on survivin by Rohayem et al, sera of 51 patients with lung cancer, 49 patients with colorectal cancer and 60 age and gender matched normal controls were analyzed for survivin and p53 antibodies(Rohayem *et al*, 2000). Overall sensitivity for both cancers was approximately 28%. In lung cancer patients, none of the sera containing anti p53 antibodies recognized survivin and none of the sera containing anti-survivin antibodies reacted with p53. This would again suggest that the prevalence of cancer patients exhibiting antibody reactivity against TAAs would increase when both anti-p53 and survivin antibodies are determined. There are reports of antibodies identified against

prostate cancer specific antigens(Bradley *et al*, 2005; Minelli *et al*, 2005) (Huntington-interaction protein 1, protasomes, alpha-methyl-acyl-co-enzyme A-recemase (AMACR) and colorectal cancer specific antigens(Line *et al*, 2002). However very few studies have reported on panel of autoantibody assays to different antigens in diagnosis of prostate and colorectal cancers. In a study by Wang et al, reported a technique which combined the use of phage display library derived from prostate cancer tissue with protein microarrays for diagnosis of prostate cancer(Wang *et al*, 2005). Sera from prostate cancer patients and healthy controls were initially tested on phage peptide microarray developed with a phage display library derived from prostate cancer. Peptides with highest level of differentiation between cancers and controls were used to develop specific microarrays. A panel of 22 phage-peptide detectors were developed and used to analyze serum samples from 60 patients with prostate cancer and 68 controls. Eleven serum samples from prostate cancer and 8 from controls and were misclassified resulting in 81.6% sensitivity (95%CI, 0.70-0.90) and 88.2% specificity (95% CI-0.78 to 0.95) in discriminating between the groups of prostate cancer and control group.

In a study by Ran Y et al on colorectal cancer, following immunoscreening of cDNA expression library prepared from colon cancer tissue, clones picked out by the screening were subjected to serological analysis of a training set of 24 colon cancers and 24 controls(Ran *et al*, 2008). A combination panel of 6 markers which got the most satisfactory sensitivity and specificity were identified(Ran *et al*, 2008). A test course was carried out using this panel with an independent set of 24 colorectal cancer sera and 24 healthy controls.

1.2.9 Evidence for preclinical diagnosis with autoantibodies

Development of cancer takes time, and demonstration of autoantibodies to tumour antigens in sera prior to their clinical presentation could have an impact on outcome. That is, antibody response to carcinogen stimulus could form well before the tumour phenotype arises giving a lead time to cancer diagnosis and treatment.

Immune response to TAAs in solid tumours have been increasingly reported recently, and studies show antibodies to TAAs identified before symptomatic disease(Li *et al*, 2005; Robertson JFR, 2005a; Robertson JFR, 2005c). In a report by Robertson et al, serum taken at least 6 months before their clinical diagnosis of cancer in 9 high risk subjects for breast cancer showed autoantibodies to a panel of antigens (MUC1,p53, c-myc, c-erbB2) in 67% of the subjects(Robertson JFR, 2005a).

The lead time to tumour diagnosis ranged from 6-36 months. A further follow-up of a larger cohort from the same institute involving 15 high risk subjects for breast cancer showed autoantibodies detectable up to 4 years before mammographic detection of breast cancer in 60% (9/15) of subjects(JFR, 2006). In another report, Yongliang Li et al looked into 103 high risk cases of asbestos exposure (average of 20 year employment in asbestos related industries) whose follow-up blood samples were collected over an 8 year period(Li *et al*, 2005). Forty nine of this group eventually developed cancer. Antibodies to p53 was found in 31 serum samples and at least one serum sample was positive in 13 out of 49 (26.5%) of those who developed cancer

(11 lung cancer, 1 mesothelioma, 1 lymphoma) compared to 4 out of 54 who did not develop cancer. The average lead time to clinical diagnosis was 3.5 years(range $\leq$ 1-12 years).

Autoantibodies to p53 have also been identified in cases of heavy smokers before their development of lung and other tobacco related cancers, and also in certain workers who have been exposed to carcinogens before their development of cancer(Lubin *et al*, 1995; Schlichtholz *et al*, 1994; Trivers *et al*, 1996). Schlichtholz et al in 1994 reported the study of 2 smokers who were found to have p53 antibodies in their serum(Schlichtholz *et al*, 1994). Follow-up showed that one patient died rapidly from aggressive lung cancer 8 months later. Second patient was detected to have lung cancer 2 years later, before clinical manifestation of the disease. This patient responded to treatment and was tumour free 4 years after treatment. Response to treatment was associated with disappearance of p53 antibodies and it has not reappeared in the last follow-up. In a similar report by Lubin, a heavy smoker developed lung cancer a few years following detection of p53 antibodies and had a cure after early detection and treatment(Lubin *et al*, 1995). In another study, serum samples of 92 individuals who were exposed to vinyl chloride through occupation was analysed for p53 antibodies (Trivers GE, 1995). Fifteen of the above group developed angiosarcoma of the liver and 5 of them were positive for p53 antibodies. Two of these patients had raised antibodies prior to diagnosis with one 11.3 years and the other 4 months before diagnosis. Four of the 77 subjects who did not develop cancer had raised p53.

In a case control study by Trivers et al, 23 patients who developed cancer subsequent to chronic obstructive airway disease were analysed for p53

antibodies(Trivers *et al*, 1996). Five cancer patients had raised p53, of which 80% (4/5) had raised p53 prior to clinical presentation with a median of 7 months (2 lung cancers, 1 prostate cancer, 1 breast cancer). Forty four controls with COPD with no evidence of cancer matched for age, gender and smoking habits were negative for p53. Yongliand Li et al on a later report compiled the results of some of the above mentioned studies to include 13 subjects exposed to carcinogens whose preclinical sera showed elevated p53 with an average lead time to diagnosis of approximately 2 years(Li *et al*, 2005).

Another chronic inflammatory condition associated with lung cancer is idiopathic pulmonary fibrosis. P53 mutation has been implicated in this association. To test this hypothesis, Oshikawa K and his group conducted a case control study where serum samples of 98 lung cancer patients, 46 patients with IPF (14 with lung cancer and 32 without lung cancer), 36 patients with pulmonary emphysema (PE) and 93 healthy controls were analysed for antibodies for p53 antigen(Oshikawa & Sugiyama, 2000). The sensitivity of the test to p53 antibodies was significantly high in lung cancer (61.2%), IPF with lung cancer (57.1%) and IPF without lung cancer (53.1%) compared to those patients with PE (21.7%; P , 0.005) and normal controls (5%; p, 0.00001). No significant differences were found among groups with lung cancer, IPF with lung cancer and IPF without lung cancer. The author suggests a possible mutation occurring frequently in IPF resulting in a high prevalence of lung cancers.

Antibody response to survivin, a tumour associated inhibitor of apoptosis protein had been identified in the sera of 2 lung cancer patients at 12 and 18 months before clinical manifestation of the disease(Rohayem *et al*, 2000).

Pereira-faca et al, in their case control study analysed 18 pre-diagnostic lung cancer sera analysed for autoantibodies 1 year prior to the development of cancer(Pereira-Faca *et al*, 2007). Control group comprised of 19 non cancer subjects matched for age, sex and time of sample taken. Statistically significant greater reactivity was observed for 14-3-3θ antigen (p=0.0042) and annexin 1 antigen (P= 0.0038). Reactivity against these antigens was observed as early as 10 months prior to diagnosis. Another report by Imani H et al on patients with chronic hepatitis and/or liver cirrhosis showed that 1/3rd of patients showed increasing antibody titres and novel autoantibodies at or before transition to hepatocellular carcinoma(Imai *et al*, 1993b).

Study on patients with premalignant conditions like leukoplakia and Barrett's oesophagus has shown p53 antibodies in their sera suggesting its potential use in early diagnosis(Cawley *et al*, 1998; Ralhan *et al*, 1998). In a report by Ralhan R, sera of 183 subjects (70 with oral cancer, 50 with premalignant oral lesions and 63 normal controls with history of heavy consumption of tobacco) were analysed for p53 antibodies. Circulating p53 antibodies were observed in 34% (24/70) of patients with cancer and 30% (15/50) patients with premalignant oral lesions(Ralhan *et al*, 1998). Four out of 63 controls were also positive for p53 antibodies. In another report by Cawley et al, sera of 36 patients with Barrett's oesophagus, 33 oesophageal cancer patients (23 squamous, 10 adenocarcinoma) and 19 controls with normal oesophagus or with oesophagitis were analysed for p53 antibodies(Cawley *et al*, 1998)[177]. Four patients with Barrett's oesophagus including one which progressed to adenocarcinoma had raised p53. Ten patients with cancer also had raised p53

(8 squamous and 2 adenocarcinomas), of which 2 had p53 prior to cancer diagnosis. The sensitivity and specificity of the assay were 83.3% and 87.5% respectively.

1.2.10 Autoimmune disease and immune response

Autoimmune response can be systemic (rheumatoid arthritis, systemic lupus erythematosus) or organ specific (Graves disease, autimmune thyroiditid, type 1 diabetes meletes). Autoantibodies are often detected many years before the onset of disease(Scofield, 2004).

Some autoantibodies like antinuclear factors which are positive in diseases like hushimotos thyroiditis, idiopathetic thrombocytopenic purpura, dibetetes meletes, autoimmune haemolytic anaemia are found in n ormal subjects as well.

A panel of disease specific markers may help to establish a diagnosis and to assess the prognosis in patients with known or suspected systemic autoimmune disease. These new technologies may enable screening for multiple autoantibodies and may further enhance the clinical usefulness of autoantibody testing, making it possible to diagnose autoimmune disease in its earliest stages and to intervene before serious end organ damage occurs(Scofield, 2004).

Chapter 2. Hypothesis and project aims

In spite of the advances in screening and treatments for cancer there continues to be significant mortality from cancer. Randomised controlled trials of screening into some of the commoner cancers like breast, colon, lung, prostate and cervical cancer have shown that early diagnosis has a significant impact on the mortality (Aberle *et al*, 2011; Duggan, 2012; Hardcastle *et al*, 1996; Kitchener *et al*, 2010; Larsson *et al*, 1996; Schroder *et al*, 2012).

Despite advances in screening for each of the above cancers, there remains a significant opportunity to further improve on early detection and reduce cancer specific mortality. To the normal population, diagnosing cancer using a blood test appears a relatively simple and easy approach. The measurement of autoantibodies in the peripheral blood as a means of diagnosising early stage cancer has been reported previously from our institute, including lung and breast cancers(Chapman *et al*, 2007; Chapman *et al*, 2008) . The aim has been to identify raised levels of such antibodies in individuals with cancer and provide preclinical blood tests as an aid to diagnosis that can be used alongside current imaging techniques especially in high risk population. These studies have compared the autoantibody signal in sera derived from both controls with and without cancer (Chapman *et al*, 2007; Chapman *et al*, 2008). These controls are wherever possible matched for risk factors and belong to a high risk group (Chapman *et al*, 2007; Chapman *et al*, 2008). The tumour associated antibody responses in the normal population is largely unknown. The EarlyCDT-Lung^{TM} test (Oncimmune Ltd) measures autoantibodies (AABs) to six cancer-associated antigens (p53, NY-ESO-1, CAGE, GBU4-5,

Annexin1, and SOX2) and have been reported to identify up to 40% of lung cancers regardless of grade or stage of the disease.

We hypothesise that in subjects with no personal history of cancer, antibody response to cancer related antigens alter with demographics (age, gender, and smoking). This thesis aims to investigate the influence of age, gender and smoking on antibody responses to six cancer related antigens, in subjects with no previous personal history of cancer.

As discussed previously, age is the most important risk factor for cancer, and we specifically analysed the AAb response with progressing age. Subjects with family history of cancer are more likely to develop cancers compared to normal population. This being the case, it was our intention to look at the cancer antibody response in a subgroup of individual who were at high risk i.e. smokers and subjects with family history of cancer. Identification of specific autoantibodies is well known to influence the diagnosis of certain autoimmune disease and it was also our intention to investigate if such subjects elicit a response to the panel of TAAs used in our assay.

Factors investigated were

1) Relationship of antibodies to different cancer related antigens with age
2) Influence of gender on AAbs to TAAs in the normal population
3) Influence of smoking on antibody response to TAAs in the Earlt CDT-lung panel
4) Autoantibody response to TAAs in patients with history of autoimmune disease (AI)
5) Autoantibody response to cancer related antigens in subjects with family history (FH) of cancer.

Chapter 3. **Materials and methods**

3.1 Sample size

Graham Healey, the statistician responsible for most of the analyses related to autoantibodies in previous publications from Nottingham was consulted for analyses of sample size and power calculation. The process of sample collection from the healthy population of Nottingham produced a Female:Male ratio of roughly 2:1, with a higher ratio at the older decades (the final overall ratio being 2.6:1). Given that ODs for this type of study tend to have a maximum standard deviation for an antigen of around 0.15 units, and given also that we would like an 80% statistical power to detect a mean OD change of about 0.05 units in any decade, then, based on a standard two-tailed *t*-test, a sample size of 200 females and 100 males is adequate (estimate of power = 76%).

3.2 Inclusion and Exclusion

Subjects with no personal history of cancer from 18 years to 90 years were considered for the study.

Subjects with previous history of hepatitis or HIV were excluded to avoid increased risk of contracting the disease among staff.

3.3 Health questionnaire and consent form

Subjects invited to participate in the study were given written and verbal information. Opportunities to ask questions about the study were also provided. Once an individual decided to participate in the study, they were asked to fill a consent form (Appendix 1) and then a health questionnaire (Appendix 2). A copy of the informed consent was given to the patients, and another copy of the consent and the health questionnaire were stored in a

secure location for record purposes (consent form in the appendix). The information acquired in the questionnaire included age, gender, smoking history, history of benign breast disease, any autoimmune disease, exposure to radiation and family history of cancer. The information collected was entered into a secure database which I later analysed.

3.4 Work force going to community for data collection and blood samples

The study was jointly supported by the University of Nottingham and one of it's spinout companies, Oncimmune. Staffs from both were involved in organizing and managing the project. A project manager organized the events at different locations for optimal blood collection. These included static locations in the city centre, mobile locations using a bus fitted out for blood collection, sports centres, elderly homes, etc). Myself, and / or a fellow doctor were available for explaining the study and consenting the volunteers at all times. I was also involved also in venesection especially in some busy events due to the large number of individual consenting to give blood for our research. At least two registered phlebotomists were available at every site to assist venesection. Two personnel were also available to distribute leaflets in public locations.

Blood sample collection was by standard venipuncture technique under aseptic conditions. All patients were consented and given the choice of providing a minimum of 40 ml or a maximum of 58 ml for future use. All blood samples were allowed to stand at room temperature for one hour and centrifuged at

1000G for 20 minutes. The serum was then removed and aliquoted into anonomised labeled tubes and frozen in 1 ml aliquots at -20 degree prior to use.

3.5 ELISA assays for the detection of autoantibodies to tumour associated antigens in the normal population

In this study we used the EarlyCDT®-Lung, which was first introduced in the clinic in May 2009 to be used as a tool to aid early detection and risk assessment of lung cancer in high-risk patients. It detects the presence of AAbs to a panel of lung TAAs using a proprietary platform technology based on indirect Enzyme Linked Immunosorbent Assay. High precision robotics was used for all liquid handling steps to produce a highly reproducible semi-automated indirect enzyme-linked immunosorbant assay. Purified recombinant antigens were diluted to provide a semi-log titration series for each antigen ranging from 160 to 1.6 nM(Murray *et al*, 2010). Control antigens (BirA and NusA) were included to allow subtraction of signal due to nonspecific binding to bacterial contaminants. Antigen dilutions were passively absorbed to the surface of the microtitre plate wells in high phosphate buffer overnight at room temperature. After washing in phosphate buffered saline containing 0.1% Tween 20 (pH 7.6), microtitre plates were blocked with a gelatine-based blocking buffer. Coated plates were found to be stable for at least 48 hours after coating if washed and stored at 4 degree centigrade in the presence of blocking buffer. Serum samples (diluted 1 in 110 in a blocking buffer) were added to the plates and allowed to incubate at room temperature with shaking

for 90 minutes. After incubation, plates were washed and horseradish peroxidase (HRP)-conjugated rabbit anti-human IgG was added. After a 60-minute incubation, the plates were washed and 3,3',5,5'-Tetramethylbenzidine (TMB) was added. Following 15 minute incubation the optical density (OD) of each well was determined spectrophotometrically at 650 nm. Control plates to which antigen-specific mAbs or an anti-His tag mAb (Novagen) has been added in place of serum were included to validate that the plate coating had been successful and antigen immunoreactivity has been maintained. These plates were probed with rabbit antimouse Ig-HRP.

The assays for the study were performed as semi-automated assays using roborts and I was involved in measurement and running of the assays as part of a team required to run the robots.

3.6 Study design

The 2065 PAS samples were assayed in 11 runs over two distinct time periods between June 2009 and December 2009 as shown in table below. The gender ratio was not constant over these two sets, with the ratio for first period being M:F 1:11.6 and second period 1:0.4 respectively. The reason for this imbalance is that originally the samples had been collected for breast cancer screening, hence the large proportion of females. Later there was more emphasis on lung cancer screening yielding increased numbers of males.

3.7 General analysis strategy

Following the advice from Mr Graham Healey (statistician), the limit of quantitation in this assay is equivalent to OD=0.06 for Annexin 1 and OD=0.12 for all the other five antigens. Hence for the comparisons for each

demographic factor the data values below these limits were set at the limit itself. This reduces the influence of imprecise extreme results on the interpretation.

Table 3.1: Gender by Assay Run

	Gender		
Assay run	Male	Female	Unknown
23/06/09	9	191	
24/06/09	12	187	1
25/06/09	13	184	3
26/06/09	25	170	5
30/06/09	17	182	1
30/06/09	13	137	
01/07/09	15	134	1
10/07/09	8	118	
13/10/09	103	97	
11/12/09	139	70	
16/12/09	212	17	1
All	566	1487	12

Chapter 4. **Results**

Demographics

The study involved 2065 subjects. Male to female ratio was 1: 2.6(566:1487). There were approximately 200 females and 100 males in each decade except at the extremes of age as shown in fig 4.1. The ratio of smoker versus ex-smokers versus non-smoker was 1: 2.4: 3.8 (285:672:1096). There was a progressive decline in the number of current smokers with increasing age as shown in fig 4.2. The proportion of smokers and ex-smokers versus non-smokers remained approximately the same in both genders as shown in Fig 4.3.

One hundred and eighty six patients (9%) had personal history of an autoimmune disease and their distribution by decade and gender are shown in figure 4.4, 4.5 and 4.6. Diabetes mellitus and Rheumatoid arthritis were the commonest among the autoimmune diseases.

Almost half the patients (964) had family history of some form of cancer as depicted on fig 4.7. Proportion of females with family history of cancer (50%) was comparatively higher to that of males (38%) as shown in fig 7.8. Figure 4.9 shows the breakdown of strength of family history in each gender.

Fig 4.1 Gender in each decade

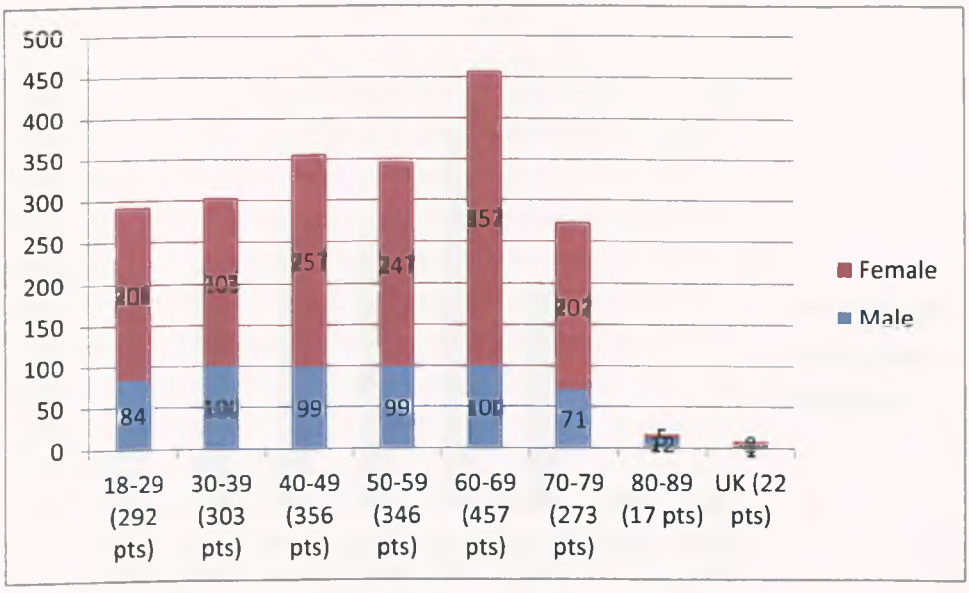


Fig 4.2 Smoking history in each decade

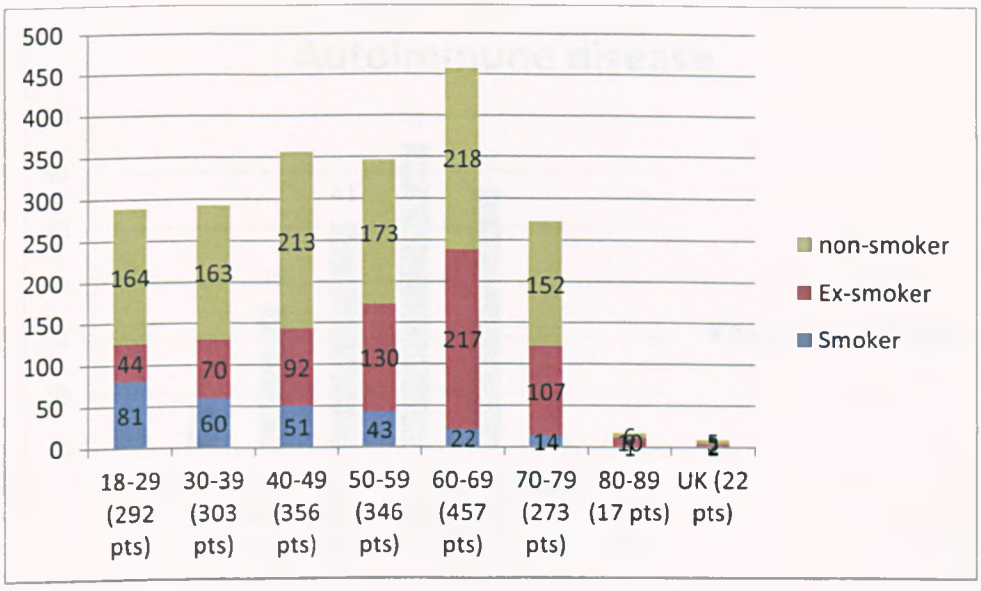


Fig 4.3 Smoking history by Gender

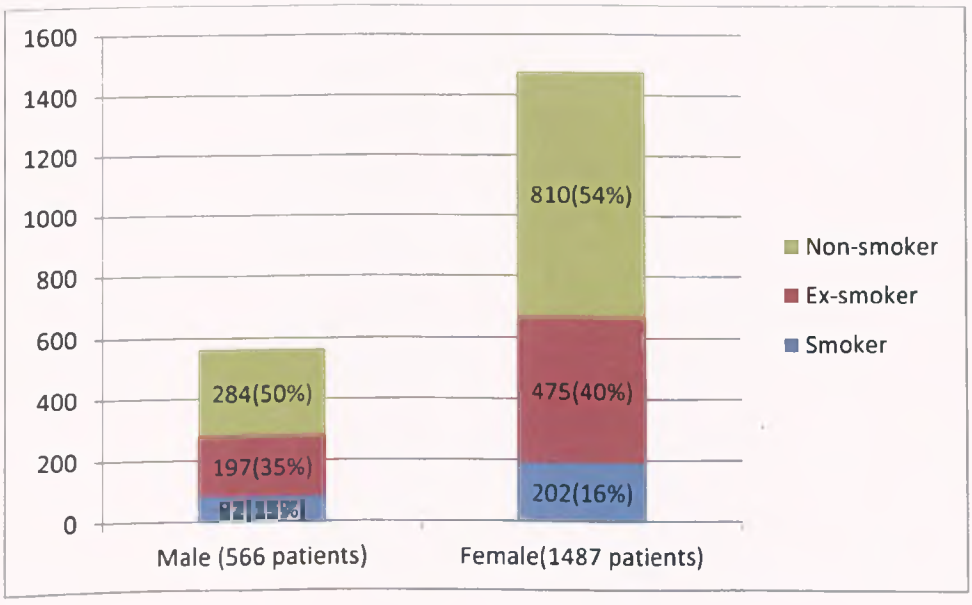


Fig 4.4 Autoimmune disease by decade

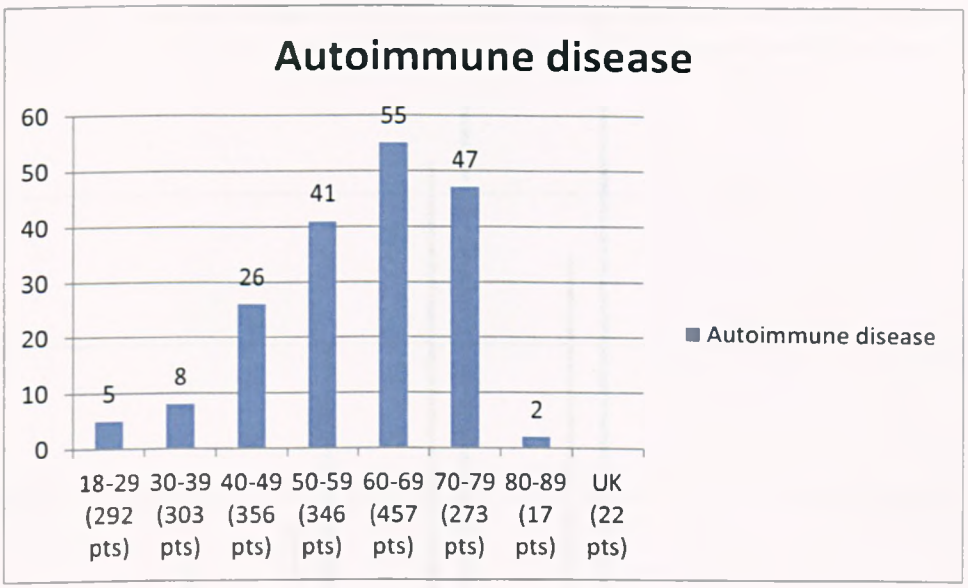


Fig 4.5 Different autoimmune disease by decade

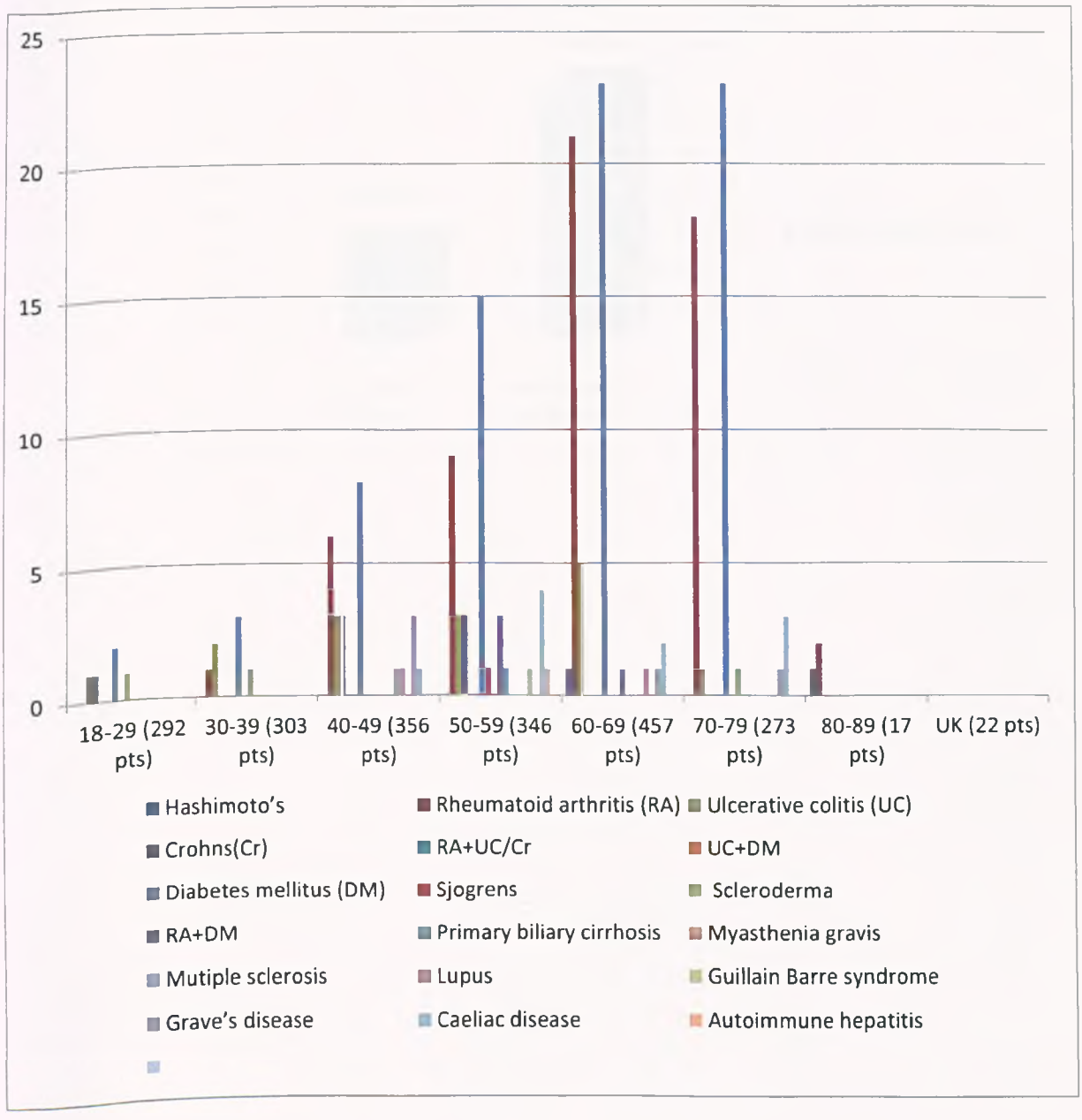


Fig 4.6 Autoimmune disease by gender

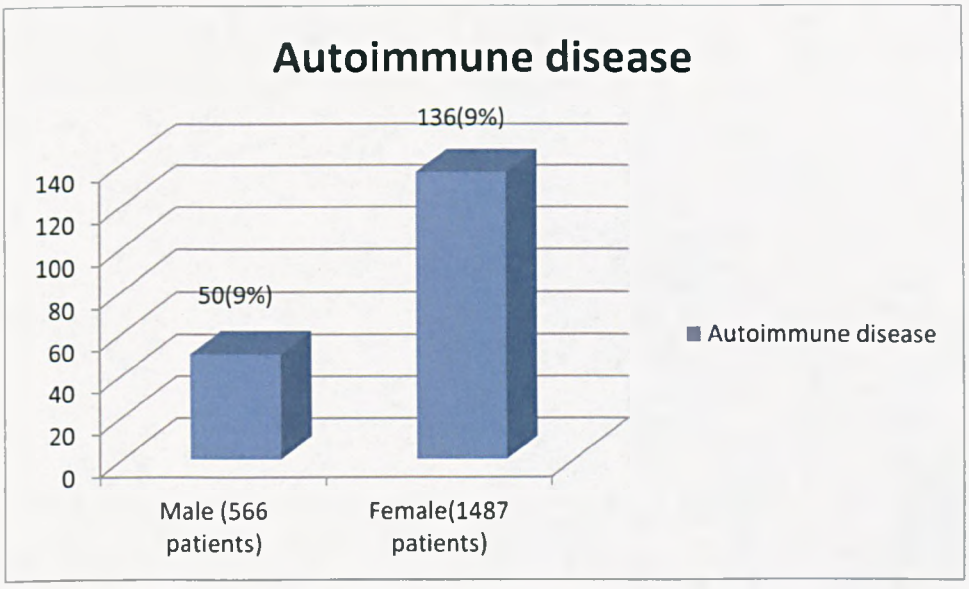


Fig 4.7 Individuals with a reported family history of cancer categorized by degree in each decade

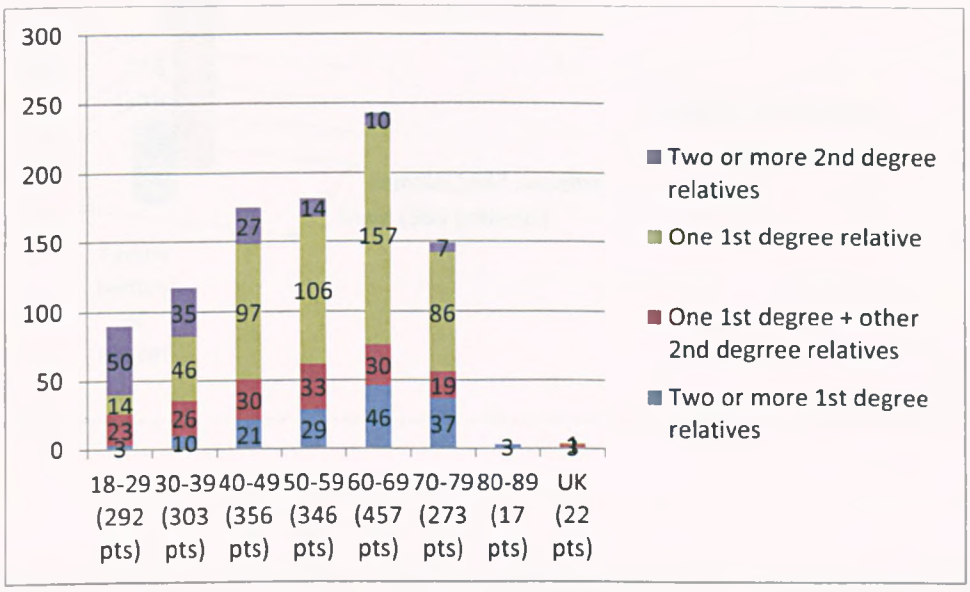


Fig 4.8 Individuals with a reported family history of cancer by gender

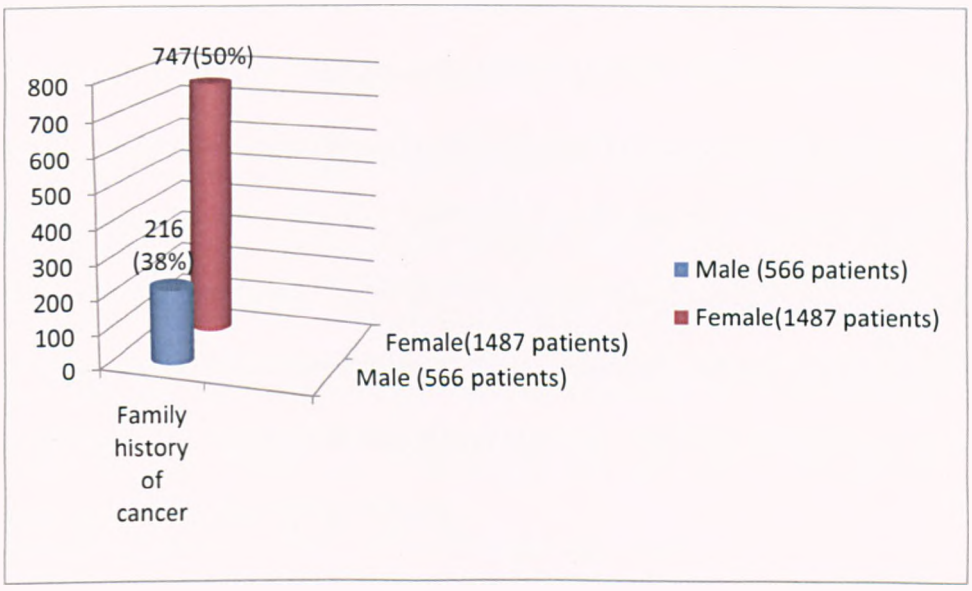
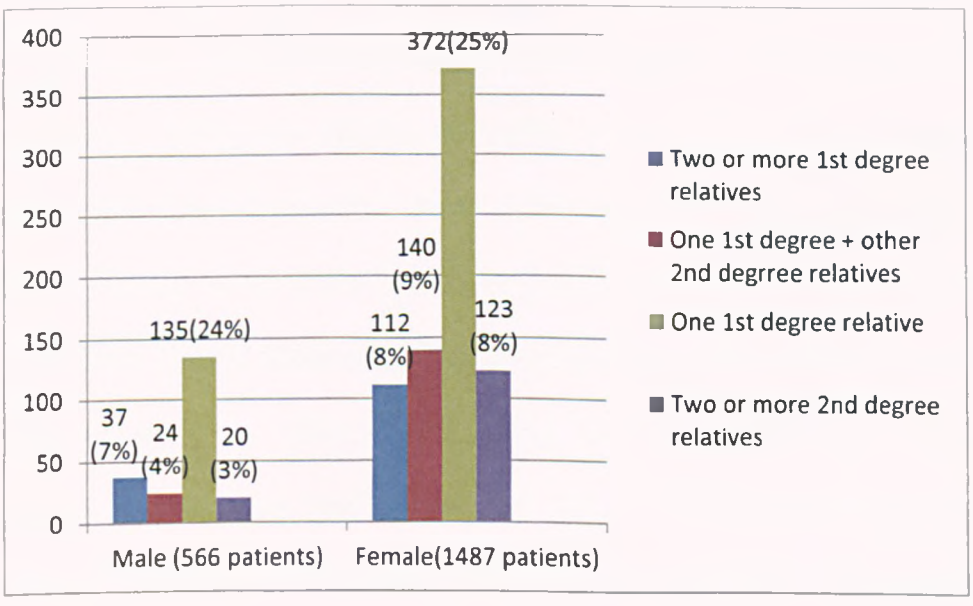


Fig 4.9 Individuals with a reported overall family history of cancer categorized by degree, gender and number



Chapter 5. **Reproducibility**

5.1 Manual assay

Purified recombinant antigens were coated on Flacon 96 wells plates. Each plate had 4 repeats of 3 antigens (p3, c-myc and NY-ESO-1) and VOL thus having 4 identical points on each plate. Plates were coated using electronic pipette in order; 160, 50, 16, 5 and 1.6nM. Sufficient filtered coating buffer for 0nM was also provided. When finished, check was made to see that all wells of 96 microtitre plate were correctly filled. Antigen dilutions were passively adsorbed to the surface of microtitre plate wells overnight at room temperature and used within one day of coating.

After washing in phosphate buffered saline containing 0.1% Tween 20 (pH 7.6), microtitre plates were blocked with a gelatine-based blocking buffer (200 µl/well) using the Tecan PW – 384 plate washer "block" program. Serum samples (diluted manually 1 in 110 in the blocking buffer) were then added to the plates and allowed to incubate at room temperature with shaking for 90 minutes. Following incubation, plates were washed and horseradish peroxidase (HRP)-conjugated rabbit anti-human IgG (Dako, Denmark) was added. Control plates to which antigen-specific monoclonal antibodies (rabbit-anti-mouse Ig-HRP (Dako) had been added in place of serum were included to validate that the plate coating had been successful and antigen immunoreactivity had been maintained. After a 60-minute incubation with shaking, the plates were washed and 3,3',5,5'-Tetramethylbenzidine is added at forty second intervals and incubate on bench for fifteen minutes, before reading. The optical density (OD) of each well was determined spectrophotometrically at 650 nm using the Tecan Infinite Plate Reader. Data

is collected in Megellan and was automatically stored as wsp file and exported to Excel for analysis

The tables 1 and 2 represent reproducibility data of six metastatic breast cancer patients who had their sera analysed using manual assays on two different days. Raw data were analysed using a Microsoft Excel template, and the mean, the standard Deviation (SD) and the coefficient of variation (CV) were calculated. Within run variability less than 15% and in-between run variability between 15% to 25% are acceptable limits.

Table 5.1 Reproducibility CVs and means of 3 antigens involving 3 patients with metastatic breast cancer (C1, C5, C7)

		C1			C5			C7		
	OD	mean	CVe%	CVb%	mean	CVe%	CVb%	mean	CVe%	CVb%
p53	50	0.335	38.07	25.32	0.115	19.98	38.12	0.1915	37.64	7.75
	160	0.2545	68.13	16.39	0.1285	49.02	33.56	0.2825	11.95	29.78
c-myc	50	0.3765	27.13	7.32	0.251	6.89	37.74	0.5535	6.53	22.86
	160	0.5335	41.98	15.24	0.353	12.01	28.44	0.976	2.73	22.31
NY-ESO-1	50	0.4505	18.44	13.65	0.1405	23.04	27.68	0.5905	.341	42.74
	160	0.5815	19.57	11.06	0.215	0.43	36.83	0.843	5.61	37.91

Table 5.2 Reproducibility CVs and means of 3 antigens involving 3 patients with metastatic breast cancer (C8, C9, C11)

		C8			C9			C11		
	OD	mean	CVe%	CVb%	mean	CVe%	CVb%	mean	CVe%	CVb%
p53	50	0.08	27.23	10.60	0.1665	43.90	18.26	0.267	7.08	14.30
	160	0.092	62.27	29.20	0.604	52.34	114.26	0.263	1.21	36.02
c-myc	50	0.1275	4.91	12.75	0.1525	16.48	0.463	0.288	7.69	1.96
	160	0.18	4.75	15.71	0.3935	9.99	82.48	0.3565	2.81	8.52
NY-ESO-1	50	0.1085	38.44	9.77	0.9265	5.19	27.09	0.3885	26.02	7.46
	160	0.1105	50.40	1.91	0.9875	2.19	27.85	0.5115	5.68	20.59

5.2 Semi-automated assay reproducibility

The population antibody study involved 2065 samples assayed in 11 runs and each run on a different day. Approximately 180-200 samples could be run in one day. Each of the 11 runs had 3 same pleural fluid assays (PL-164, PL-186, PL-192) as controls with two repeats per runs in each plate. This allowed a straightforward between-run reproducibility analysis to be carried out, with the two replicates representing the within-run (ie within-plate) variability. For each control, antigen and concentration, between-run (Vb) and within-run (Ve) variance components were estimated using analysis of variance (SAS® Proc Varcomp). Results were expressed as CVs (coefficients of variation) for a single replicate where:

Within-run CV = $100\sqrt{(Ve)}/mean$

Between-run CV = $100\sqrt{(Vb+Ve)}/mean$

For the statistical comparisons, analysis of variance was generally used for OD data of antigen concentration (50nM and 160nM).

Results

Pleural fluid PL164, PL186 and PL192 had 11 runs with 2 repeats per run in each plate. Mean optical density of antibodies to each of 6 antigens in 2 concentrations (50 and 160) was looked into as shown in Table 5.1. The lower the optical density reading, the higher the coefficient of variation. Small

absolute difference can result in large CV's. The lower limit of quantitation for this assay is approximately OD=0.12. For cases where mean OD≥0.12 the within-run CV (CVe) ranged from 3% to 14% and the between-run CV (CVb) from 5% to 26%. Cv's cannot be calculated for negative means. Further detailed summary of CV's by mean OD is given in table 5.2. Figure below shows clearly the reproducibility of the assay whenever the OD mean is above the limit of quantitation (0.12) , with the CVs shooting off to infinity below mean OD = 0.01.

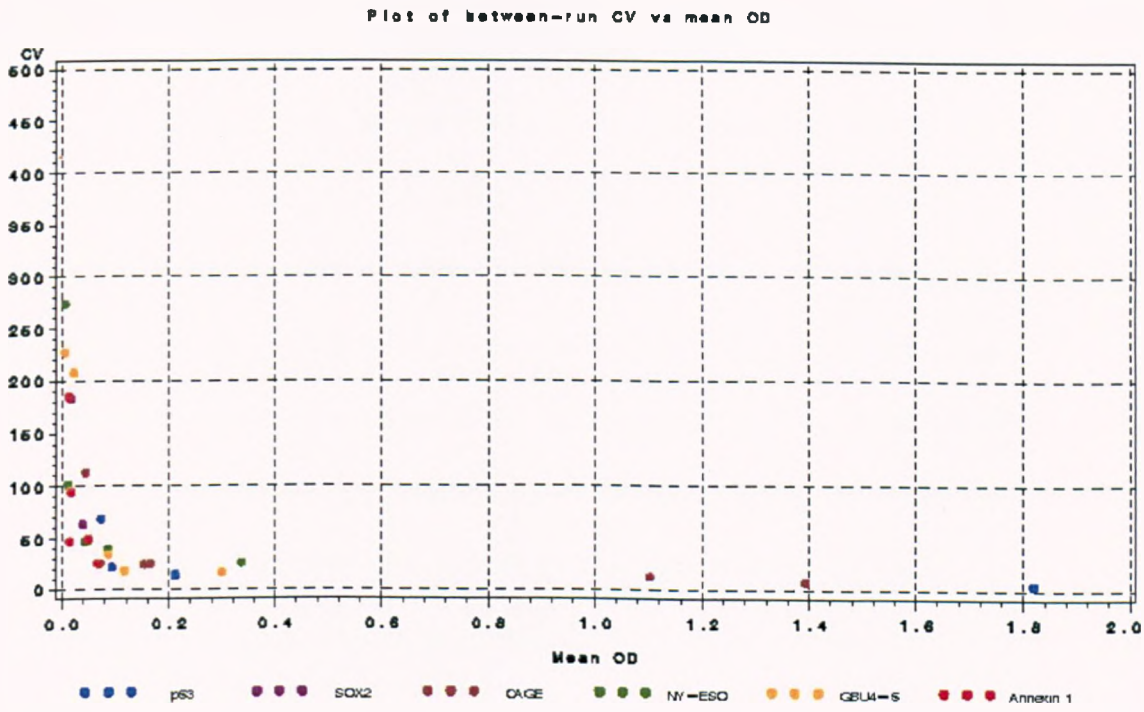
Table5.3- Reproducibility CVs and means by Control and Antigen

		PL-164			PL-186			PL-192		
	OD	mean	CVe%	CVb%	mean	CVe%	CVb%	mean	CVe%	CVb%
p53	50	0.09	15	22	1.82	5	5	0.07	24	69
	160	0.21	12	13	1.82	5	6	0.21	10	15
SOX2	50	-0.03	.	.	0.04	40	47	0.02	88	184
	160	-0.11	.	.	0.04	50	64	-0.03	.	.
CAGE	50	0.07	23	26	1.10	4	13	0.05	27	113
	160	0.17	14	25	1.39	3	8	0.15	14	25
NY-ESO-1	50	0.09	13	40	0.01	275	275	0.00	482	2097
	160	0.34	8	26	0.01	84	102	0.05	29	47
GBU4-5	50	0.09	13	35	0.01	227	227	0.02	45	208
	160	0.30	6	16	0.02	72	96	0.12	14	18
Annexin 1	50	0.02	48	48	0.01	185	185	0.00	509	2238
	160	0.07	18	26	0.02	79	95	0.05	46	50

Table5.4- Summary of CVs by mean OD

	CVe%			CVb%		
Mean OD	0.0-0.1	0.1-0.3	0.3-2.0	0.0-0.1	0.1-0.3	0.3-2.0
p53	15-24	10-12	5	22-69	13-15	5-6
SOX2	40-88			47-184		
CAGE	23-27	14	3-4	26-113	25	8-13
NY-ESO-1	13-482		8	40-2097		26
GBU4-5	13-227	6-14		35-208	16-18	
Annexin 1	18-509			26-2238		
ALL	13-509	6-14	3-8	22-2238	13-25	5-26

Figure 5.1: Between-run CV (CVb) vs OD mean reproducibility of the assay whenever the OD mean is above the limit of quantitation (0.12).



Chapter 6. Relationship of antibodies to different cancer related antigens with Age

To analyse the impact of age on antibodies to cancer related antigens, we classified patients into different decades and analysed if there was a difference between different groups

6.1 Relationship of antibodies to cancer related antigen p53 with age

We used the test for trend, Jonckheere-Terpstra which is a non parametric method for showing trend. It was used to analyse if there was a trend with changing decade. The figure 6.1 shows that there is a progressive increase in the median optical density of antibodies to p53 antigen in all subjects with progressing decades. In Table 6.1, overall median OD of all samples was taken into account. For each decade, it shows the number of subjects with values above and below this median. You see that as the age increases the proportion of subjects with values above this overall median also increases. In short, with progressing decade the proportion of subjects with optical density above the median value also increased.

Fig 6.1 Box and whisker plot showing progressive rise in optical density of antibodies to p53 antigen with progressing decade

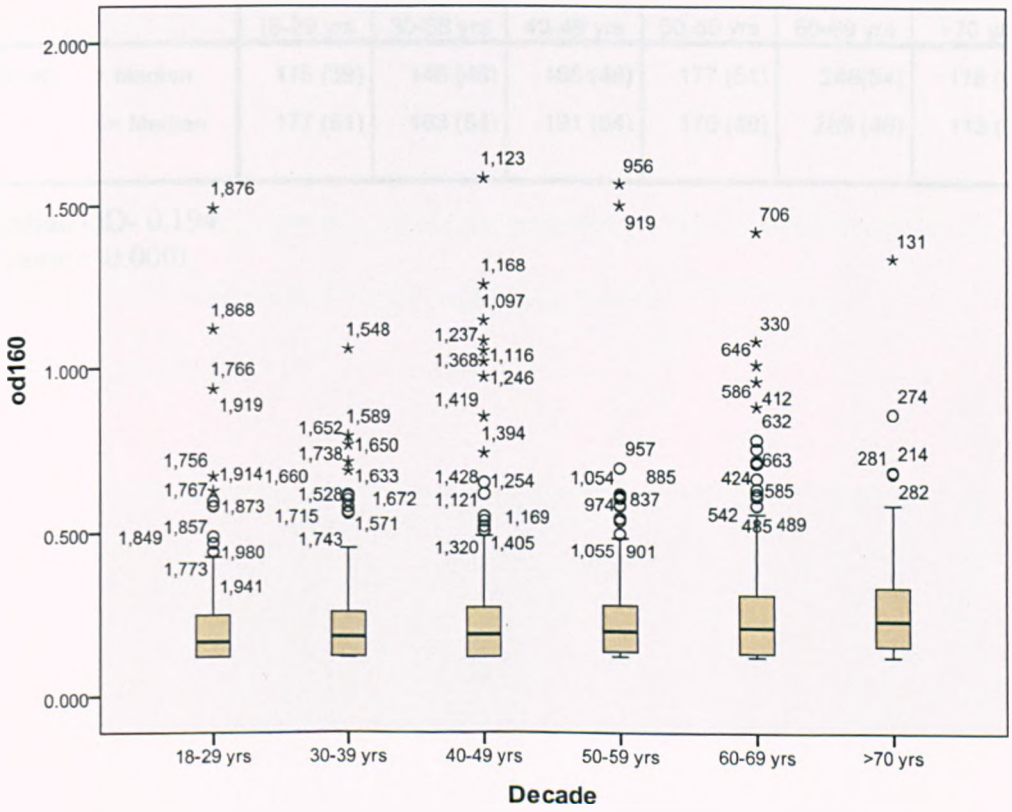


Table 6.1 showing rise in subjects with optical densities of antibodies to p53 antigen above the overall median with progressing decade

		Number of subjects in each decade (%)					
		18-29 yrs	30-39 yrs	40-49 yrs	50-59 yrs	60-69 yrs	>70 yrs
od160	> Median	115 (39)	140 (46)	165 (46)	177 (51)	248(54)	178 (61)
	<= Median	177 (61)	163 (54)	191 (54)	170 (49)	209 (46)	113 (39)

Median OD- 0.194
P value = 0.0001

6.2 Relationship of antibodies to cancer related antigen CAGE with age

Figure 6.2 also shows a progressive increase in the median optical density of antibodies to cage antigens with increasing decade, and the table 6.2 also suggests that as the decade increases the proportion of subjects with optical density above the overall median value also increases.

Fig 6.2 Box and whisker plot showing progressive rise in optical density of antibodies to CAGE antigen with progressing decade

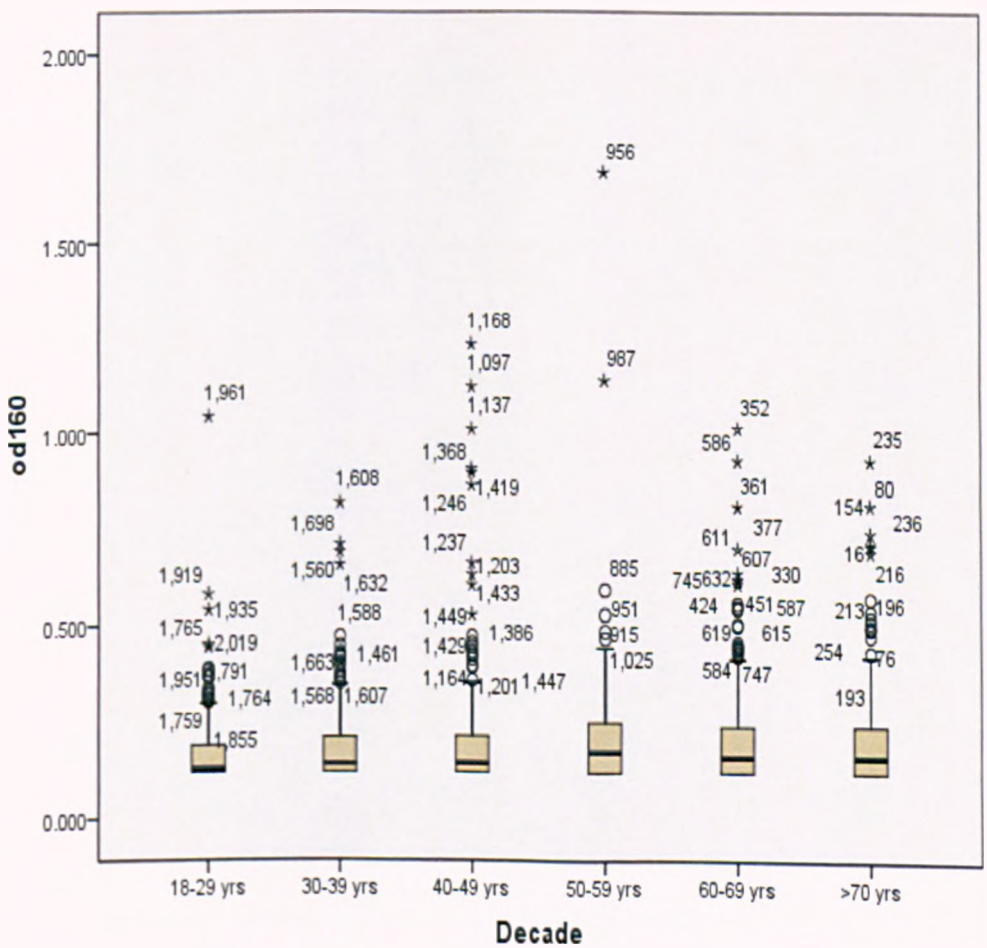


Table 6.2 showing rise in optical densities of antibodies to CAGE antigen above the median with progressing decade

		Number of subjects in each decade (%)					
		18-29 yrs	30-39 yrs	40-49 yrs	50-59 yrs	60-69 yrs	>70 yrs
od160	> Median	114 (39)	142 (47)	168 (47)	199 (57)	240 (53)	159 (55)
	<= Median	178 (61)	161 (53)	188 (53)	148 (43)	217 (47)	132 (45)

Median OD- 0.150

P= 0.0001

6.3 Relationship of antibodies to cancer related antigen NY-ESO-1 with age

Figure 6.3 also shows a trend towards increase in the median optical density of antibodies to NY-ESO-1 antigens with increasing decade, and similarly to previous antigens showing a progressive increase in number of subjects with higher optical density than the median value with progressing decade as shown in table 6.3, although not that pronounced as with previous two antigens.

Fig 6.3 Box and whisker plot showing progressive rise in optical density of antibodies to NY-ESO antigen with progressing decade

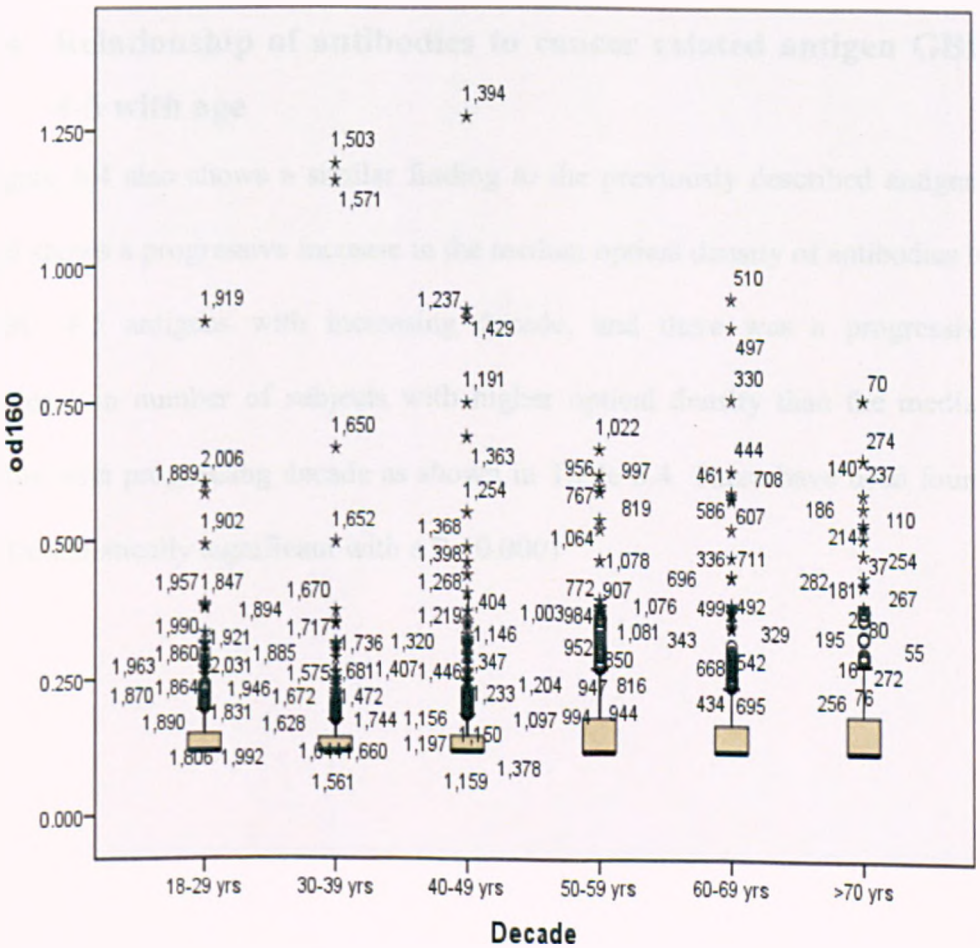


Table 6.3 showing rise in optical densities of antibodies to NY-ESO antigen above the median with progressing decade

		Number of subjects in each decade (%)					
		18-29 yrs	30-39 yrs	40-49 yrs	50-59 yrs	60-69 yrs	>70 yrs
od160	> Median	110 (38)	102 (34)	131 (37)	161 (46%)	193 (42)	136 (47)
	<= Median	182 (62)	201 (66)	225 (63)	186 (54)	264 (48)	155 (53)

Median OD-0.12

P=0.0001

6.4 Relationship of antibodies to cancer related antigen GBU 4-5 with age

Figure 6.4 also shows a similar finding to the previously described antigens and shows a progressive increase in the median optical density of antibodies to GBU 4-5 antigens with increasing decade, and there was a progressive increase in number of subjects with higher optical density than the median value with progressing decade as shown in Table 6.4. These have been found to be statistically significant with a P= 0.0001

Fig 6.4 Box and whisker plot showing progressive rise in optical density of antibodies to GBU 4-5 antigen with progressing decade

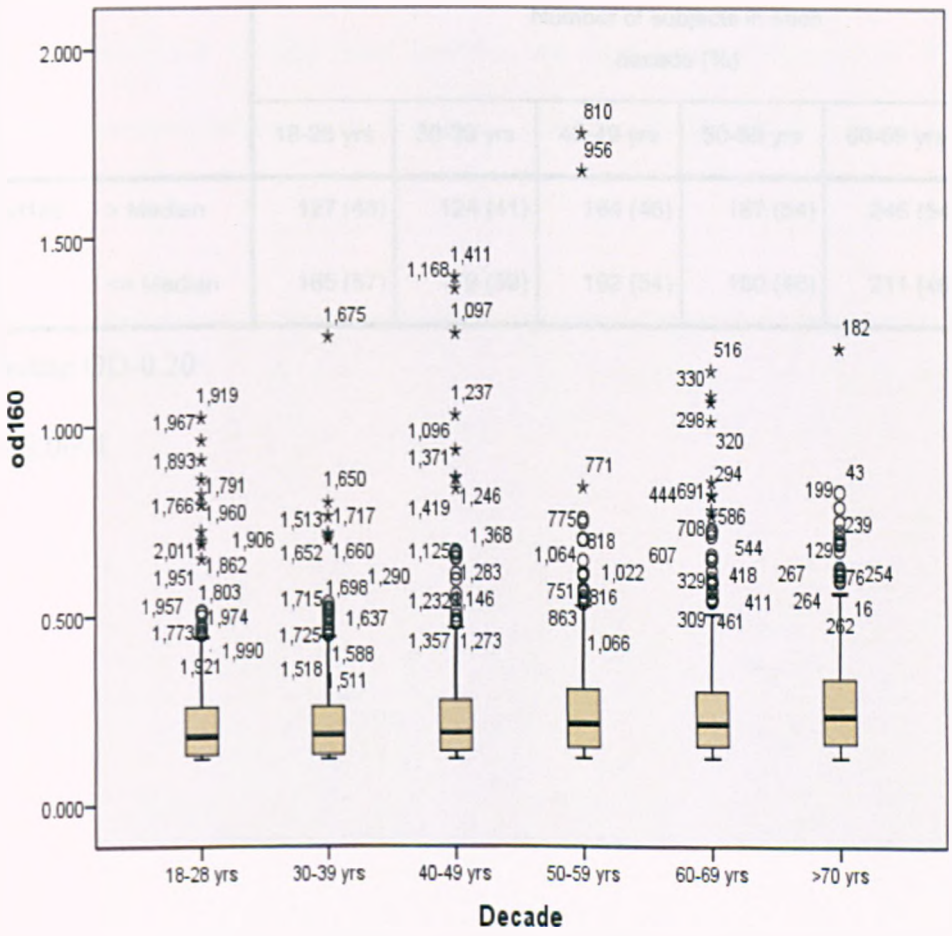


Table 6.4 showing rise in subjects with optical densities of antibodies to GBU 4-5 antigen above the median with progressing decade

		Number of subjects in each decade (%)					
		18-28 yrs	30-39 yrs	40-49 yrs	50-59 yrs	60-69 yrs	>70 yrs
od160	> Median	127 (43)	124 (41)	164 (46)	187 (54)	246 (54)	174 (60)
	<= Median	165 (57)	179 (59)	192 (54)	160 (46)	211 (46)	117 (40)

Median OD-0.20

P= 0.0001

6.5 Relationship of antibodies to cancer related antigen Annexin 1 with age

Both Annexin 1 and Sox-2 did not have any influence with age as shown in respective graphs 6.5 & 6.6 and tables 6.5. & 6.6

Fig 6.5 Box and whisker plot showing no significant difference in mean optical density of antibodies to Annexin 1 antigen with progressing decade

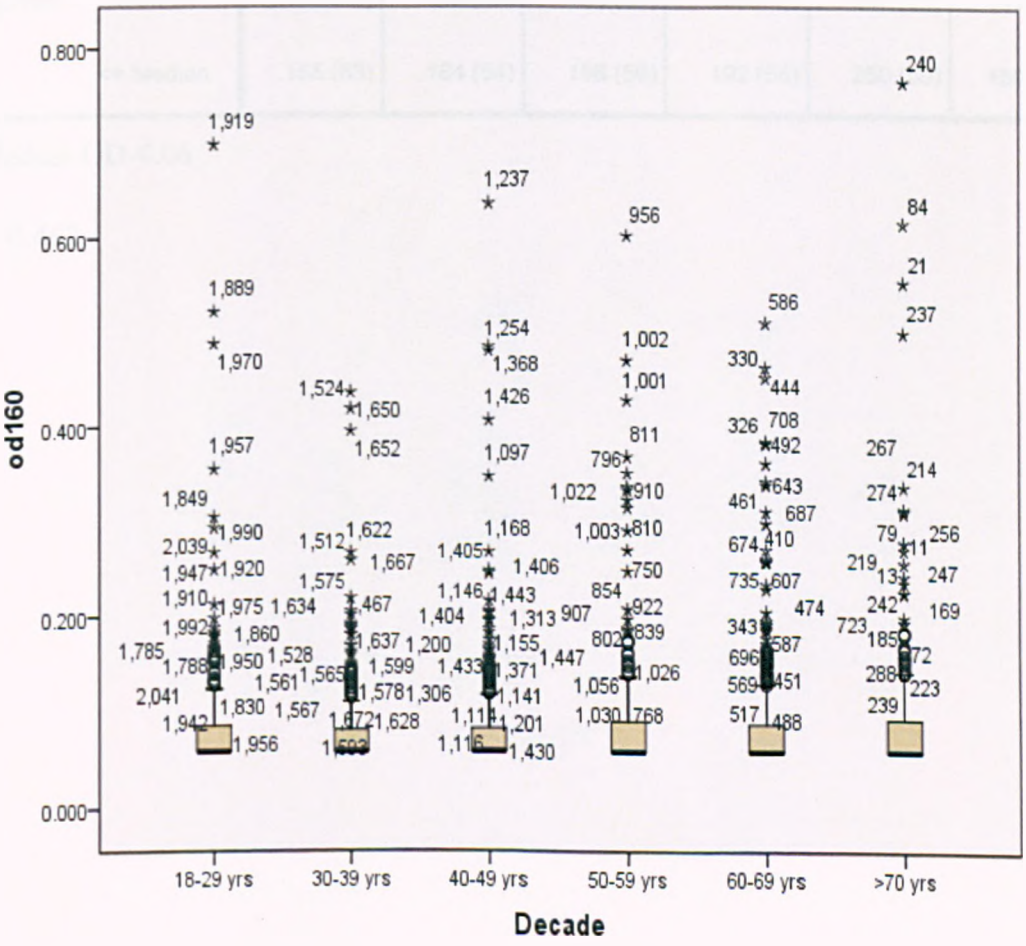


Table 6.5 showing no significant difference in optical densities of antibodies to Annexin 1 antigen above and below the overall median with progressing decade

		Number of subjects in each decade (%)					
		18-29 yrs	30-39 yrs	40-49 yrs	50-59 yrs	60-69 yrs	>70 yrs
od160	> Median	137 (47)	139 (46)	158 (44)	155 (45)	207 (45)	141 (48)
	<= Median	155 (53)	164 (54)	198 (56)	192 (55)	250 (55)	150 (52)

Median OD-0.06

P=0.467

6.6 Relationship of antibodies to cancer related antigen SOX2 with age

Fig 6.6 Box and whisker plot showing no significant difference in mean optical density of antibodies to SOX2 antigen with progressing decade

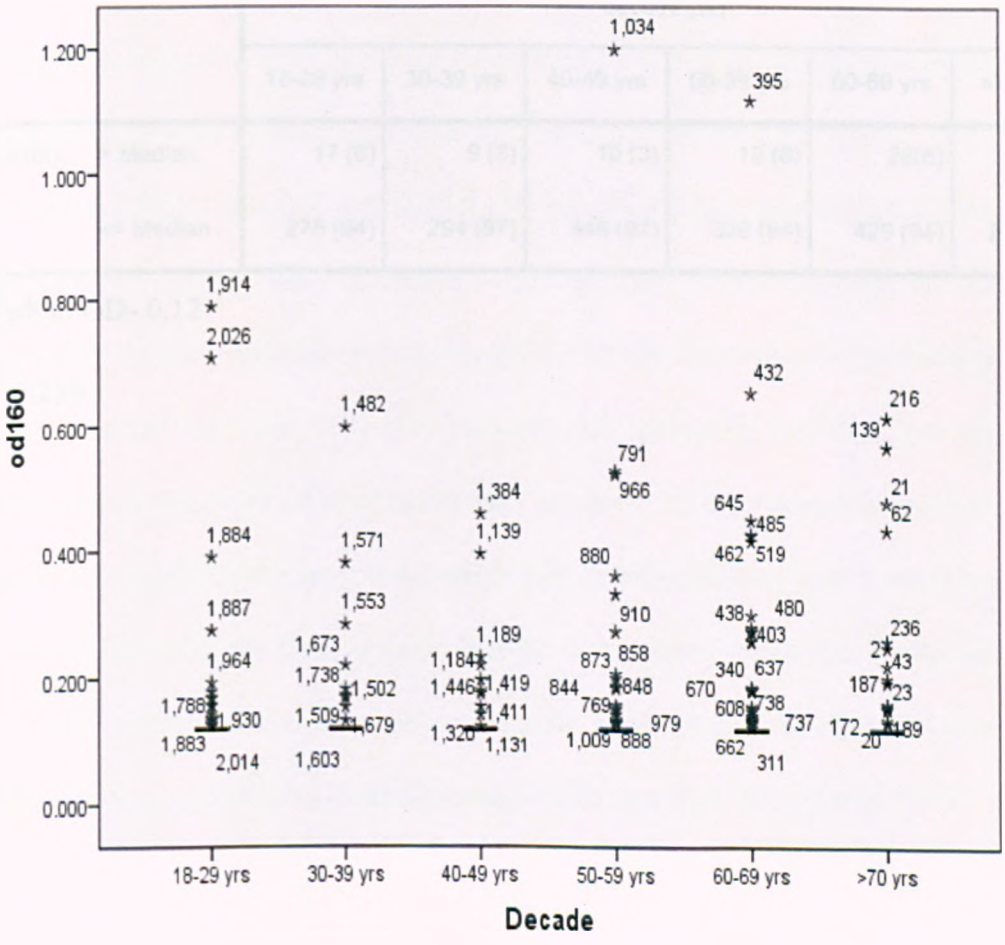


Table 6.6 showing no significant difference in optical densities of antibodies to SOX2 antigen above and below the overall median with progressing decade

		Number of subjects in each decade (%)					
		18-29 yrs	30-39 yrs	40-49 yrs	50-59 yrs	60-69 yrs	>70 yrs
od160	> Median	17 (6)	9 (3)	10 (3)	19 (6)	28(6)	15 (5)
	<= Median	275 (94)	294 (97)	346 (97)	328 (94)	429 (94)	276 (95)

Median OD- 0.12

P=0.230

6.7 Discussion

Our study involved 2065 subjects with approximately equal distribution of females and males in each decade. The study shows that there is a progressive increase in the median optical density for most antibodies to cancer related antigens with progressing decades. The proportion of subjects in each decade of life with optical density values of AAbs above the overall median compared to those below the median increased with each decade for p53, CAGE, NY-ESO-1 and GBU4-5 and it was statistically significant for these antigens. The incidence of cancer also increased with age and increased response of antibodies to these antigens with age could be a reflection of cancer becoming more common with age. It is also possible that increasing age itself has an influence on the level of auto antibodies to some of the tumour associated antigens as could be the case with benign autoimmune disease and it could be a true reflection of the level in each decade. It has been shown that incidence of benign autoimmune disease increases with age(Morganti *et al*, 2005) and levels of immunoglobulins G also increases with age(Rink & Seyfarth, 1997).

We have seen that older age groups have higher autoantibody response to cancer related antigens. There are no studies in the present literature which have followed up these older age groups with higher autoantibody response to see if they develop cancer. This would be a future project to follow-up these selected group of subjects with higher OD values.

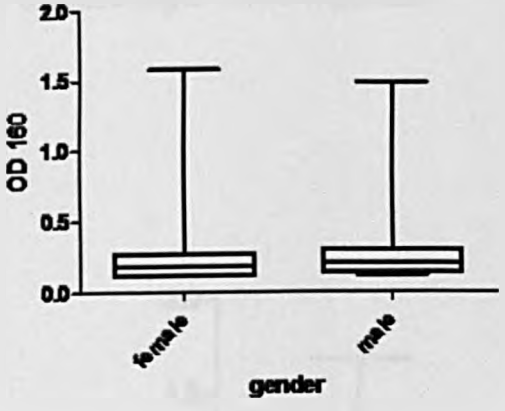
With regard to Annesxin 1 and SOX-2, the autoantibody response did not alter with age. It is probable that the response against these antigens (Annexin 1 and SOX2) is truly cancer specific and age has little influence on its levels.

Chapter 7. Influence of gender on auto antibodies to cancer related antigens in a normal population

7.1 Autoantibody response to p53 antigen between genders

Between females and males there was no difference in autoantibody response to p53 antigen as shown in figure 7.1. Unpaired t-test comparing males and females shows that there is no difference in the mean optical densities between males and females with regard to p53 antigen (P=0.06).

Fig 7.1 Showing no significant difference in mean optical densities of antibodies to p53 antigen between genders



7.2 Autoantibody response to CAGE antigen between genders

When we looked into the autoantibody response to CAGE, the mean optical density was significantly higher in males with a P value of 0.0001 as shown in figure 7.2. To eliminate the effects of smoking and age, 1:1 matching was done for sex, smoking history and age (+/- 3 years). There were 528 subjects in each group. Even after matching, the analysis showed a statistically significant higher mean optical density in males (Mann-Whitney P=0.0001) as shown in figure7.3.

Fig 7.2 Showing significant difference in mean optical densities of antibodies to CAGE antigen between genders

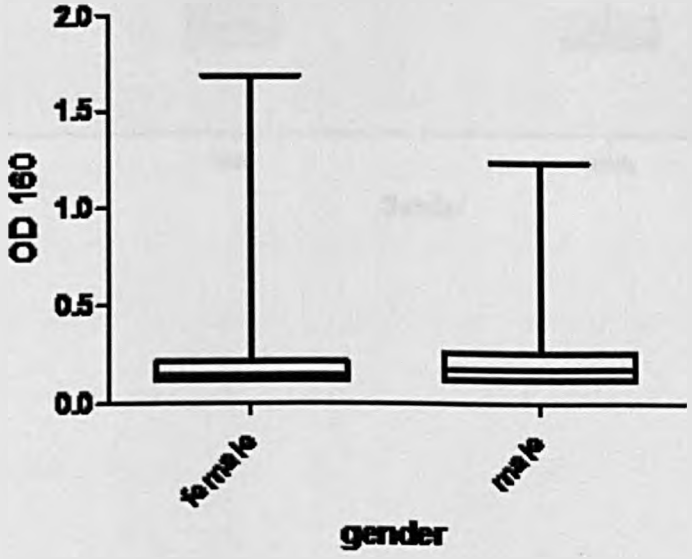


Fig 7.3 Showing significant difference in mean optical densities of antibodies to CAGE antigen between genders after 1:1 matching for age, gender and smoking.

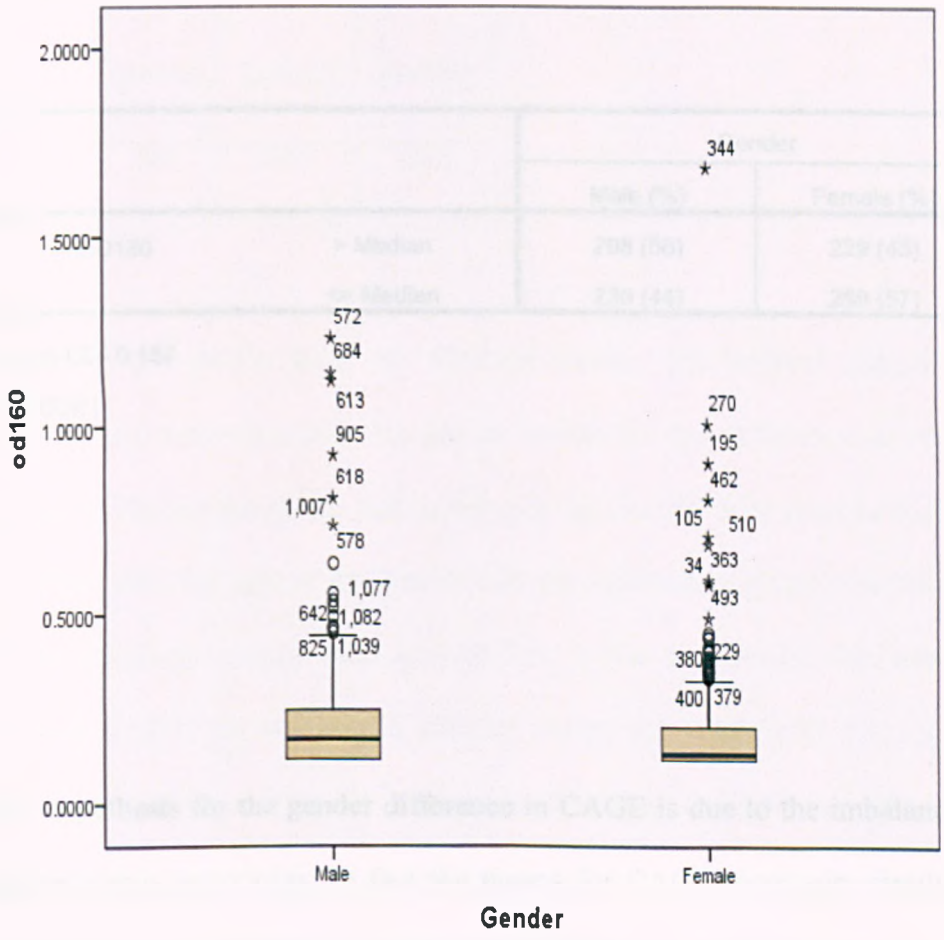


Table 7.1 Showing significant difference in mean optical densities above and below the overall median of antibodies to CAGE antigen between genders after 1:1 matching for age, gender and smoking.

		Gender	
		Male (%)	Female (%)
OD160	> Median	298 (56)	229 (43)
	<= Median	230 (44)	299 (57)

Median OD- 0.157

P=0.0001

One hypothesis for the gender difference in CAGE is due to the imbalance in gender across assay runs. In fact the means for CAGE were very similar in period A as shown in table 7.2, but in period B there was an increase in males with a difference in mean optical densities between genders. This could suggest that the difference for CAGE is not a universal effect but may only apply to certain sub-groups.

Table7.2- OD mean for gender by assay period

Period	Male	Female	Total
A	0.184 (n=112)	0.186 (n=1303)	0.186 (n=1415)
B	0.220 (n=454)	0.183 (n=184)	0.209 (n=639)
	566	1487	2065

Following the advice from Mr Graham Healey (statistician) analysis of variance was used to adjust the gender means for the imbalance in gender frequency and means across individual runs for CAGE. This specific analysis was done with the help of Mr Healey and the explanation given is as follows. In the statistical analysis module SAS/Stat, in the commercial data analysis software SAS®, the analysis of variance command Proc GLM was applied with and without a term for assay run. Omitting the run term gives the unadjusted gender comparison between ignoring run. Including the run term removes the between-run variation from the error variance and also corrects the gender means for the deviation of the individual run means from the overall average over runs. It can be seen in the table below that after adjusting for the imbalance in gender frequency and means for individual runs for CAGE, it has largely removed the gender difference, suggesting that the observed gender difference for CAGE might be a technical artefact.

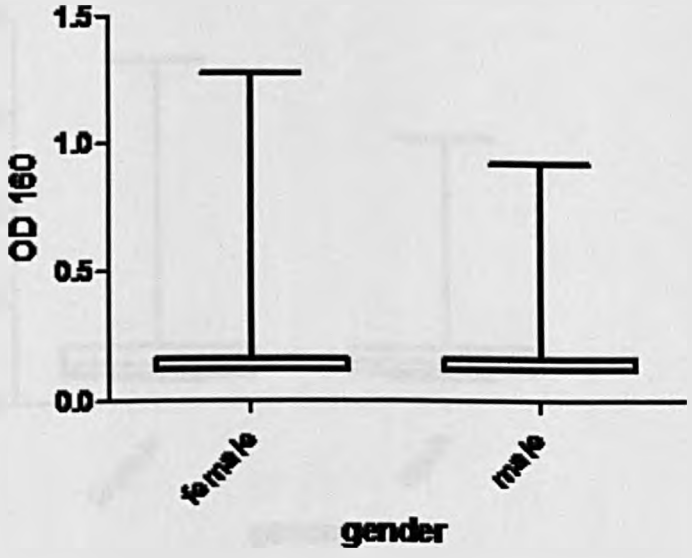
Table7.3 OD means for Cage, unadjusted and adjusted for run differences

	Unadjusted			Adjusted		
	Male	Female	*p*-value	Male	Female	*p*-value
Cage	0.213	0.186	<0.0001	0.195	0.190	0.56

7.3 Autoantibody response to NY-ESO-1 antigen between two 2 genders

Unpaired t-test comparing males and females shows that there is no difference in the mean optical densities between males and females with regard to antibodies to NY-ESO antigen (P=0.138). Figure 7.4 shows between females and males there was no difference in autoantibody response to NY-ESO-1 antigen.

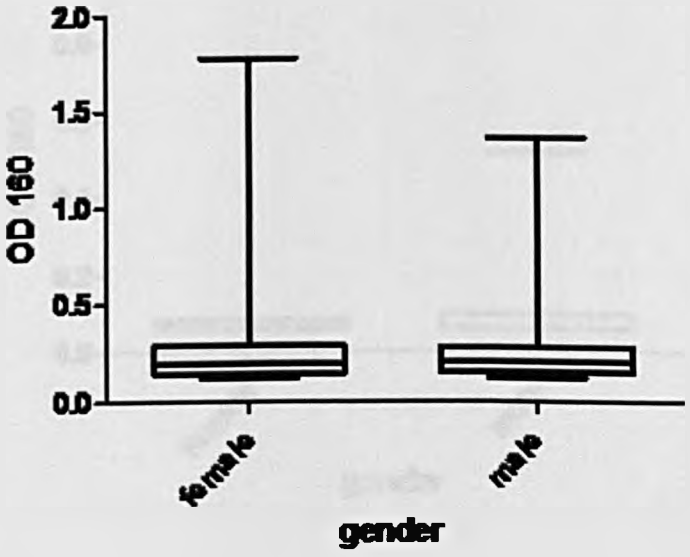
Fig 7.4 showing no difference between the mean optical densities of antibodies to NY-ESO antigen between genders



7.4 Autoantibody response to GBU 4-5 antigen between genders

Unpaired t-test comparing males and females shows that there is no difference in the mean optical densities between males and females with regard to GBU 4-5 antigen (P=0.721). Figure 7.5 shows between females and males there was no difference in autoantibody response to GBU 4-5 antigen.

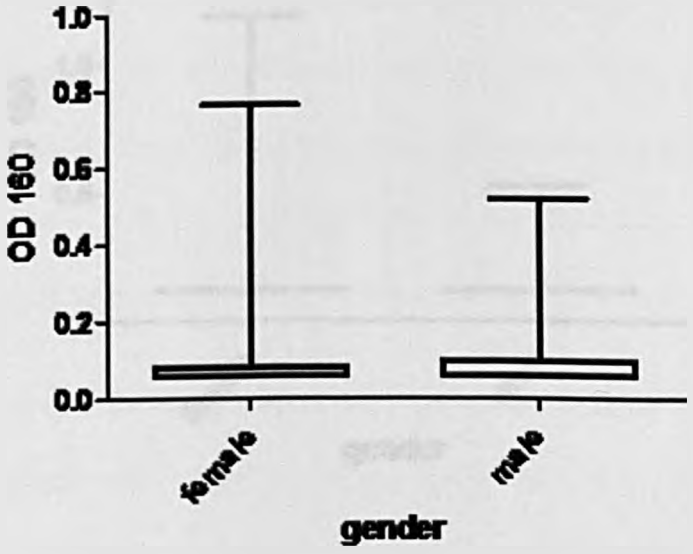
Fig 7.5 showing no difference between the mean optical densities of antibodies to GBU 4-5 antigen between genders



7.5 Autoantibody response to Annexin-1 antigen between genders

Figure 7.6 shows no difference between the mean optical densities of antibodies to Annexin 1 antigen between genders. Unpaired t-test comparing males and females shows that there is no difference in the mean optical densities between males and females with regard to Annexin 1 antigen (P=0.412).

Fig 7.6 showing no difference between the mean optical densities of antibodies to Annexin 1 antigen between genders



7.6 Autoantibody response to SOX2 antigen between genders

Figure 7.7 shows no difference between the mean optical densities of antibodies to SOX2 antigen between genders. Unpaired t-test comparing males and females shows that there is no difference in the mean optical densities between males and females with regard to SOX 2 antigen (P=0.196).

Fig 7.7 showing no difference between the mean optical densities of antibodies to SOX-2 antigen between genders

7.7 *Discussion*

Male to female ratio of subjects in our study was 1: 2.6(566:1487). There were approximately 200 females and 100 males in each decade except at the extremes of age. When we looked into the autoantibody response, except for CAGE, there was no significant difference in mean optical densities between males and females. As for CAGE there was significant difference between the groups even after eliminating the effects of smoking and age by 1:1 matching for sex, smoking history and age (+/- 3 years). Over 500 subjects in each gender were matched for age (+/- 3 years) and smoking history. However, this also showed a significant difference for autoantibody response to CAGE antigen. CAGE is a cancer testes antigen and in normal individuals its expression is largely restricted to testes(Alsheimer *et al*, 2005). It is possible that this antigen levels are inherently elevated in males, and with genetic changes with age they are no longer recognised as self and thus producing an elevated antibody response. However, if the above hypothesis is true, this should be the case with NY-ESO as well as it is also a cancer testes antigen.

The tests for males and female were run no differently as they were all mixed together and subgroup analysis being done after all the runs have been completed. There is the possibility of variation between runs on different days, although this should apply to all antigens with no exception to CAGE AAb response. It is probable that when making so many exploratory analyses, some of the P values less than 0.05 will turn out to be spurious. Since there is no really good biological hypothesis to explain this result, it might be worthwhile repeating this on a different sample set if possible.

Chapter 8. Influence of smoking on antibody response to cancer related antigens

8.1 Autoantibody response to p53 antigen in smokers ex-smokers and non-smokers

Autoantibody response to cancer related antigens p53 was low in smokers as shown in fig 8.1. The mean autoantibody response was more in the ex-smoker group compared to the other two groups. Kruskal-Wallis test was used to see if there was any difference between the groups and the test was found to be statistically significant with a P value less than 0.0001.

To eliminate the possible effect of age and gender, matched analysis was again done to see if there is a difference in the three smoking history groups and the trend remained the same despite matching as shown in figure 8.2. and was statistically significant with a p value equal to 0.0001. Median optical densities in the Ex-smoker group was significantly higher as shown in table 8.1.

Fig 8.1 Showing higher autoantibody response to p53 antigen in ex-smokers compared to the other two groups.

Fig 8.2 Autoantibody response to p53 antigen in smoking groups matched for age

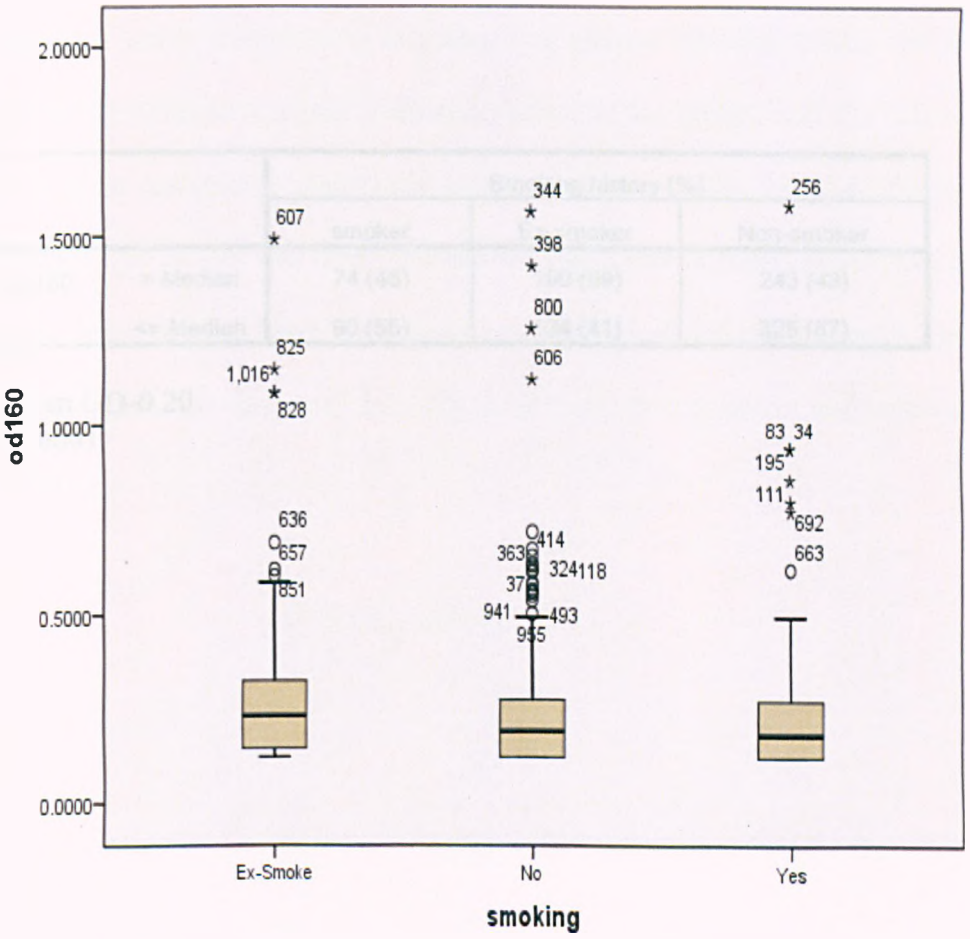


Table 8.1 showing number of subjects above and below the median optical density of antibody to P53 antigen in different age matched and sex matched smoking groups

		Smoking history (%)		
		smoker	Ex-smoker	Non-smoker
od160	> Median	74 (45)	190 (59)	243 (43)
	<= Median	90 (55)	134 (41)	325 (57)

Median OD-0.20
P=0.0001

8.2 Autoantibody response to CAGE antigen in smokers ex-smokers and non-smokers

Autoantibody response to cancer related antigens CAGE was also low in smokers as shown in figure 8.3. The autoantibody response was more in the ex-smoker group compared to the other two groups. Kruskal-Wallis test was used to see if there was any difference between the groups and the test was found to be statistically significant with a P value < 0.004. Matched analysis was again done, and the study showed no significant difference between the groups when subjects were age and sex matched ($P= 0.058$) as shown in figure 8.4. Table 8.2 also shows the subjects in different groups above and below the median.

Fig 8.3 Showing higher autoantibody response to CAGE antigen in ex-smokers compared to the other two groups.

Fig 8.4 showing autoantibody response to cage in smoking groups matched for age and gender

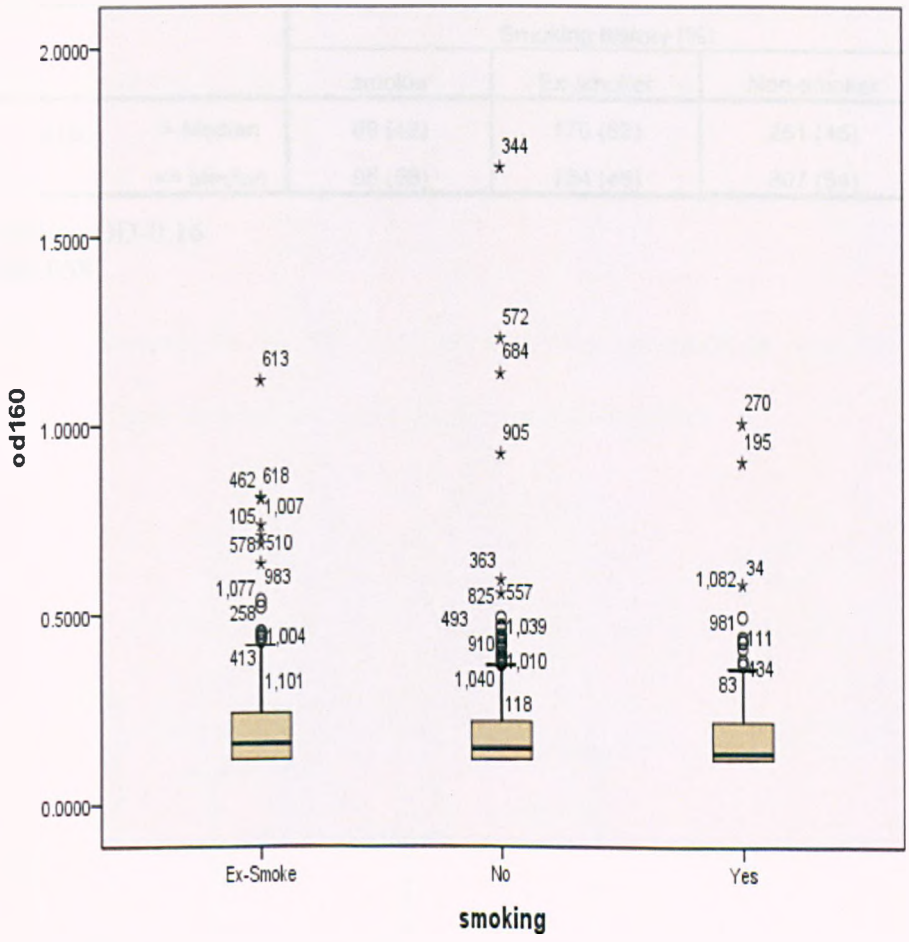


Table 8.2 Showing number of subjects above and below the median optical density of antibody to CAGE antigen in different age matched and sex matched smoking groups

		Smoking history (%)		
		smoker	Ex-smoker	Non-smoker
od160	> Median	69 (42)	170 (52)	261 (46)
	<= Median	95 (58)	154 (48)	307 (54)

Median OD-0.16
P=0.058

8.3 Autoantibody response to NY-ESO-1 antigen in smokers, ex-smokers and non-smokers

Autoantibody response to cancer related antigens NY-ESO-1 was universal in all the three groups. Kruskal-Wallis test used to make comparison between groups and it did not show any statistical difference between the groups (P value = 0.05) as shown in figure 8.5. Age matched comparisons were also made as shown in fig 8.6 and this also did not reach statistical significance (p=0.12).

Fig 8.5 Showing no significant difference in the autoantibody response to NY-ESO-1 antigen in smokers, ex-smokers and non-smokers

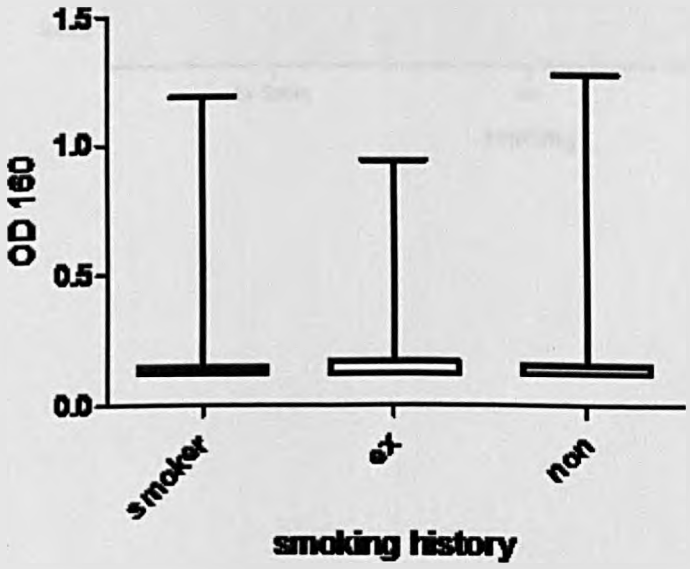


Figure 8.6 Autoantibody response to NY-ESO-1 in smoking groups matched for age

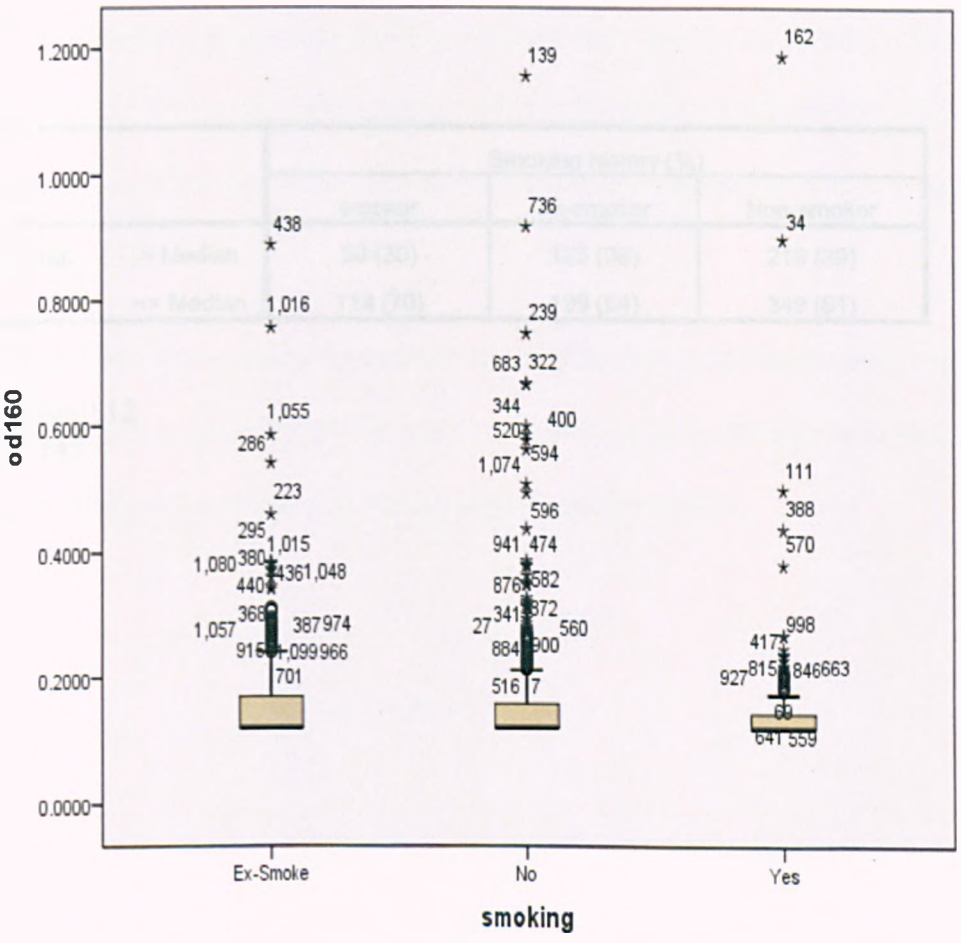


Table 8.3 showing number of subjects above and below the median optical density of antibody to NY-ESO antigen in different age matched and sex matched smoking groups

		Smoking history (%)		
		smoker	Ex-smoker	Non-smoker
od160	> Median	50 (30)	125 (36)	219 (39)
	<= Median	114 (70)	199 (64)	349 (61)

Median-0.12
P=0.145

8.4 Autoantibody response to GBU4-5 antigen in smokers, ex-smokers, and non-smokers

As with the first 2 antigens, autoantibody response to cancer related antigen GBU 4-5 was also low in smokers. The autoantibody response was also more in the ex-smoker group compared to the other two groups as shown in figure 8.7. Kruskal-Wallis test was used to see if there was any difference between the groups and the test was found to be statistically significant with a P value less than 0.0007.

Age matched analysis was again done to see if there is a difference in the three smoking history groups and the trend remained the same despite matching as shown in fig. 8.8 and was statistically significant (P=0.001).

Fig 8.7 Showing higher autoantibody response to GBU 4-5 antigen in ex-smokers compared to the other two groups.

Fig 8.8 Autoantibody response to GBU 4-5 antigen in smoking groups matched for age

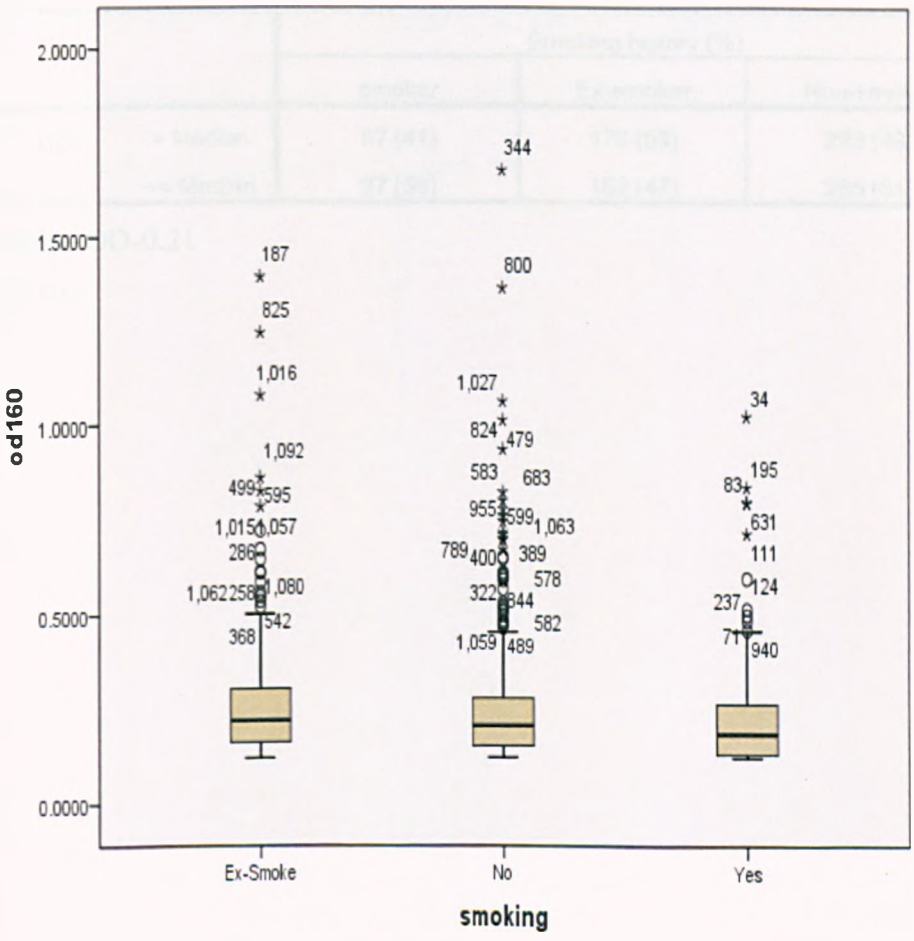


Table 8.4 showing number of subjects above and below the median optical density of antibody to GBU 4-5 antigen in different age matched and sex matched smoking groups

		Smoking history (%)		
		smoker	Ex-smoker	Non-smoker
od160	> Median	67 (41)	172 (53)	283 (49)
	<= Median	97 (59)	152 (47)	285 (51)

Median OD-0.21

P=0.037

8.5 Autoantibody response to Annexin 1 antigen in smokers, ex-smokers, and non-smokers

The autoantibody response to cancer related antigen Annexin 1 was low across the all three groups. Kruskal-Wallis test however showed a significant difference between groups (P=0.004) as shown in figure 8.9. Age and sex matched analysis also maintained the difference between the three groups with a P value of 0.003 as shown in figure 8.10

Fig 8.9 Showing low autoantibody response to Annexin 1 antigen across all three groups.

Fig 8.10 Autoantibody response to Annexin 1 antigen in smoking groups matched for age

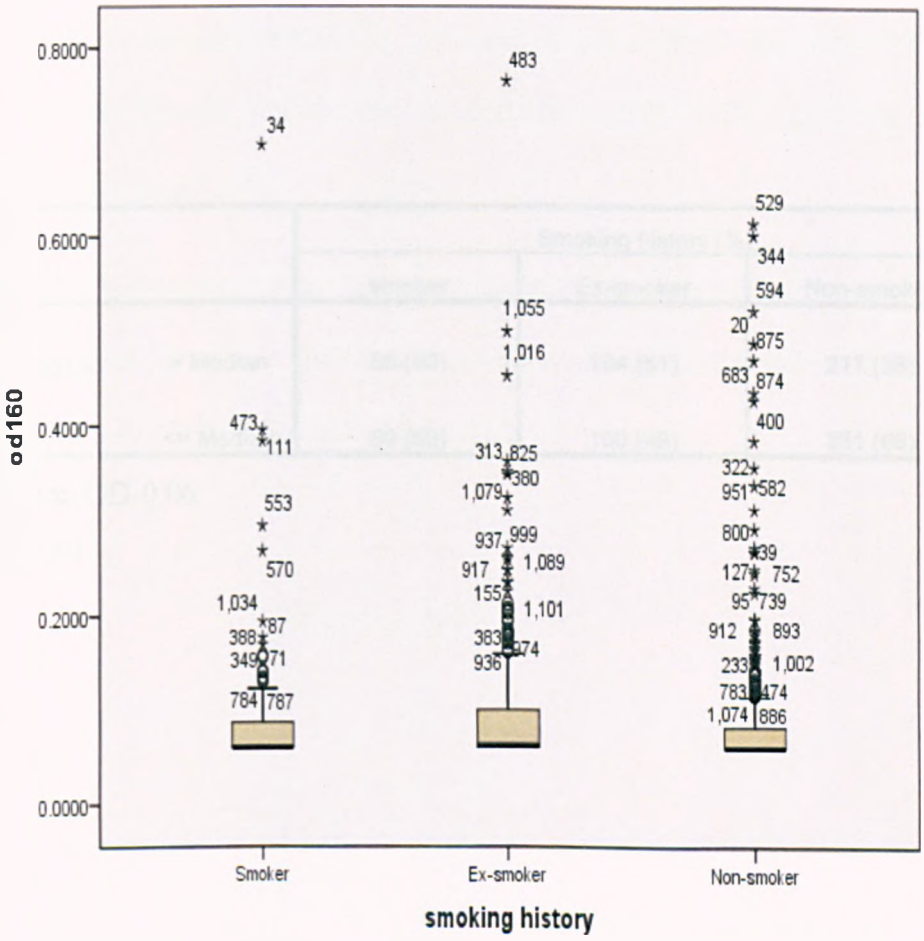


Table 8.5 showing number of subjects above and below the median optical density of antibody to Annexin 1 antigen in different age matched and sex matched smoking groups

		Smoking history (%)		
		smoker	Ex-smoker	Non-smoker
od160	> Median	65 (40)	164 (51)	217 (38)
	<= Median	99 (60)	160 (49)	351 (68)

Median OD-0.06

P=0.001

8.6 Autoantibody response to SOX-2 antigen in smokers, ex-smokers, and non-smokers

Autoantibody response to cancer related antigens SOX 2 was also low across the all three groups with the least response in the smoking group as shown in figure 8.11. Kruskal-Wallis test was used to see if there was any difference between the groups, and the test was found to be statistically significant with a P value less than 0.042

Age matched analysis was again done to see if there is a difference in the three smoking history groups and this did not show any difference between the three groups (p=0.308) as shown in figure 8.12.

Fig 8.11 Showing low autoantibody response to SOX-2 antigen across all three groups.

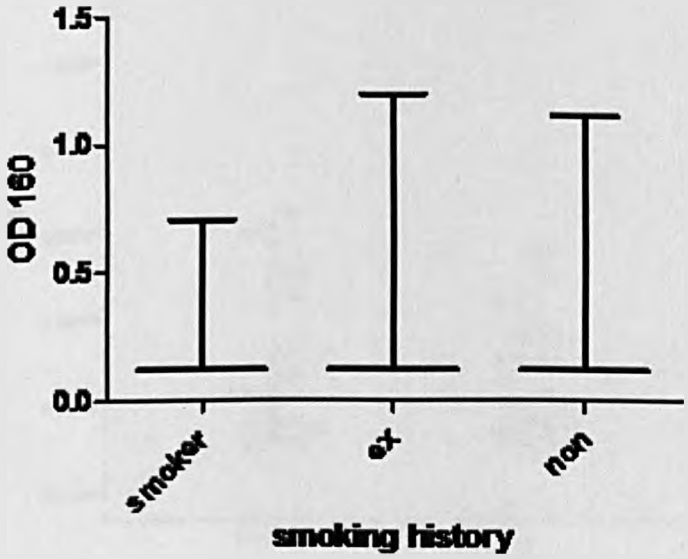


Fig 8.12 Autoantibody response to SOX-2 antigen in smoking groups matched for age

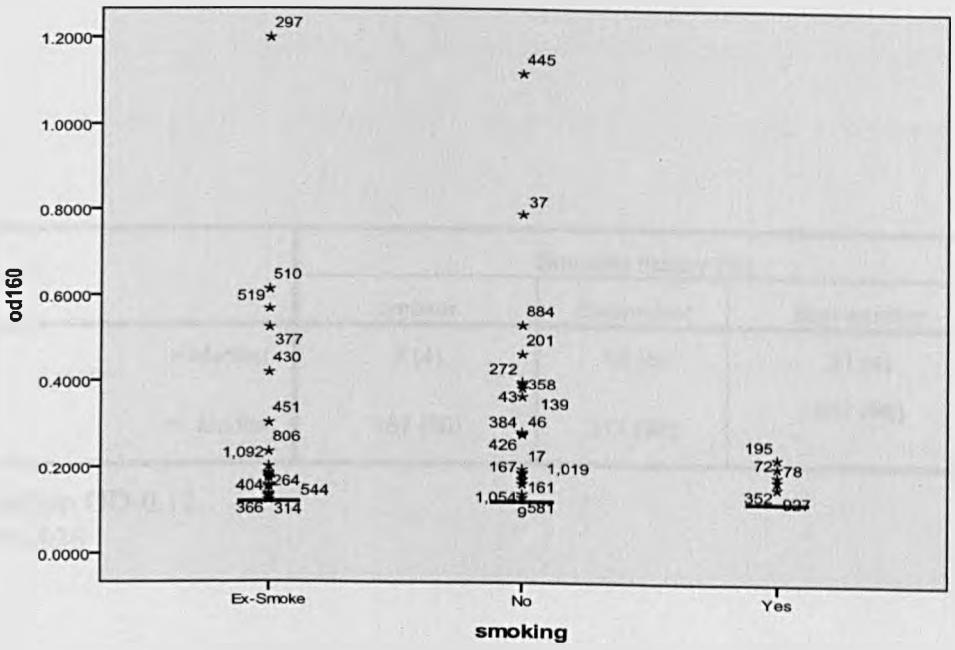


Table 8.6 Showing number of subjects above and below the median optical density of antibody to SOX-2 antigen in different age matched and sex matched smoking groups

		Smoking history (%)		
		smoker	Ex-smoker	Non-smoker
od160	> Median	7 (4)	13 (4)	21 (4)
	<= Median	157 (96)	311 (96)	547 (96)

Median OD-0.12
P=0.936

8.7 Discussion

Autoantibody response to p53, CAGE, GBU 4-5 and Annexin 1 and SOX2 were high in ex-smokers compared to smokers and non smokers and this reached statistical significance when comparisons between the mean optical densities between the groups were made. When age matched analysis were done, the differences were statistically significant for p53, GBU 4-5 and Annexin1.

The autoantibody response was comparatively low in smokers compared to ex-smokers and it is probable that smoking produces a suppression of immune response (Arnson *et al*, 2010; Subramanyam, 2011; Wu *et al*, 2011) leading to their relatively low response. The rise in autoantibody response was more in the ex-smoker group compared to the other two groups in all the antigens except for NY-ESO 1, suggesting the possibility of rebound effect when smoking is stopped. Why this was not the case with NY-ESO 1 is not clear. To explore further the "rebound" hypothesis which proposes that on cessation after a period of smoking, AAb levels rise to a higher level than during periods of smoking or non-smoking, the year of quitting for ex-smokers were extracted from the database. Any association between AAb levels and time lapse since quitting might provide support to this hypothesis. The table below summarises the results by decades of year quitting and very little difference was seen for most antigens back to 1970, but decades before that there was an observed increase in the mean AAb level for all antigens except SOX2.

Table8.7 - Mean OD by year of smoking cessation for Ex-smokers

Year quit	n	p53	SOX2	Cage	NY-ESO-1	GBU4-5	Annexin 1
1950-69	47	0.288	0.127	0.238	0.177	0.287	0.116
1970-79	124	0.237	0.127	0.178	0.156	0.250	0.084
1980-89	151	0.252	0.133	0.197	0.159	0.246	0.086
1990-99	104	0.251	0.130	0.221	0.162	0.239	0.092
2000-09	209	0.246	0.130	0.199	0.159	0.254	0.086
All	635	0.250	0.130	0.201	0.160	0.251	0.089
p-val (KW)		0.08	0.60	<0.001	0.46	0.49	0.04
p-val (JT)							

However, the difference was not statistically significant. As you can see many of the ex-smokes had stopped smoking a number of years prior to the blood sample date and thus it is not clear whether this rebound effect is a true phenomenon. Further work would be required to establish such an effect.

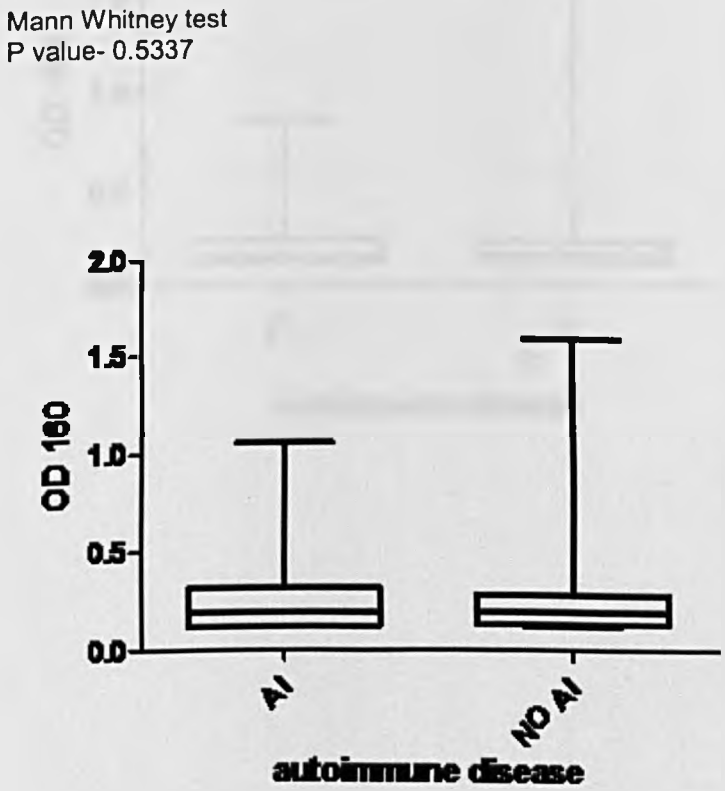
P53 is one of the well known antigens which has been linked to cancers closely associated with smoking. However previous studies have shown that although antibodies to p53 antigen is frequently elevated in patients with cancers which are closely linked to smoking, it has failed to show significantly elevated levels in smokers without cancer(Dilek Dincol, 2001; Sangrajrang *et al*, 2003)

Chapter 9. Autoantibody response to cancer related antigens in patients with history of autoimmune disease

9.1 Autoantibody response to p53 in those patients with and without autoimmune disease

Nine percent of the subjects had personal history of autoimmune disease. Comparisons were made between those with autoimmune disease and those without the disease. The study did not show any statistically significant difference in response to all six cancer related antigens when comparisons were made with those with and without personal history of autoimmune disease as shown in respective figures.

Fig 9.1 showing no difference in antibody response to p53 antigen between the two groups

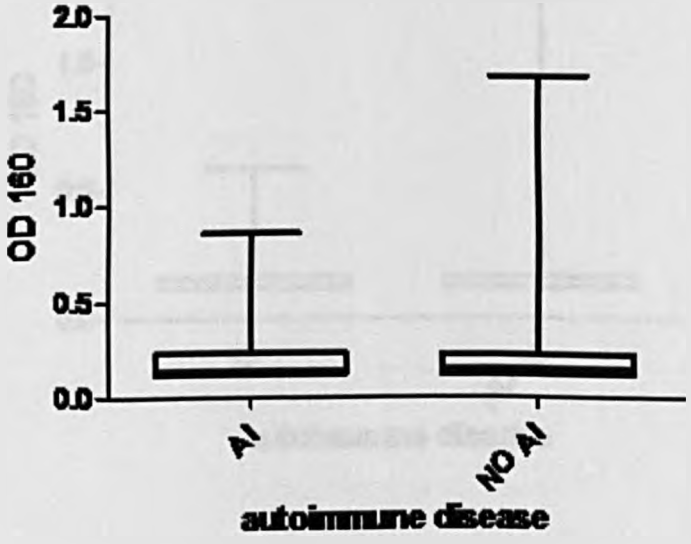


9.2 Autoantibody response to CAGE in those patients with and without autoimmune disease

Fig 9.2 showing no difference in antibody response to CAGE antigen between the two groups

Mann Whitney test

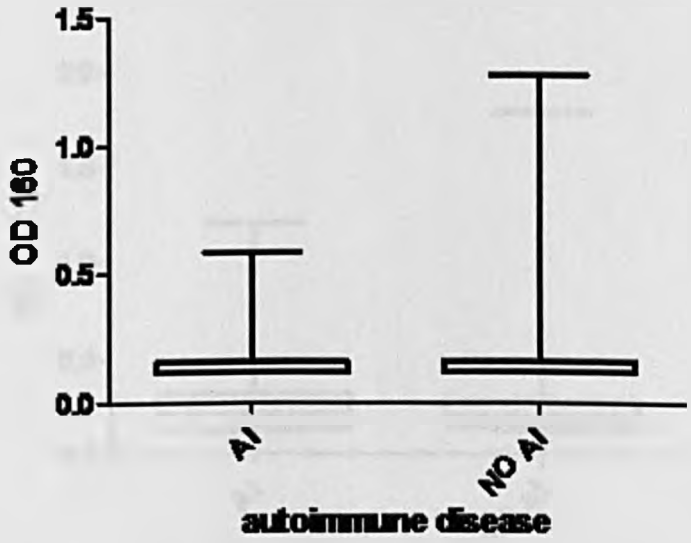
P value- 0.784



9.3 Autoantibody response to NY-ESO-1 in those patients with and without autoimmune disease

Fig 9.3 showing no difference in antibody response to NY-ESO antigen between the two groups

Mann Whitney test
P value 0.5705

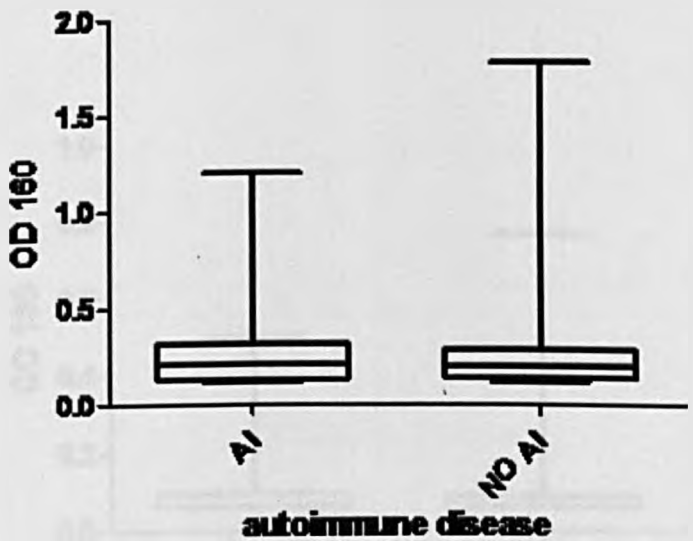


9.4 Autoantibody response to GBU 4-5 in those patients with and without autoimmune disease

Fig 9.4 showing no difference in antibody response to GBU 4-5 antigen between the two groups

Mann Whitney test

P value- 0.7638

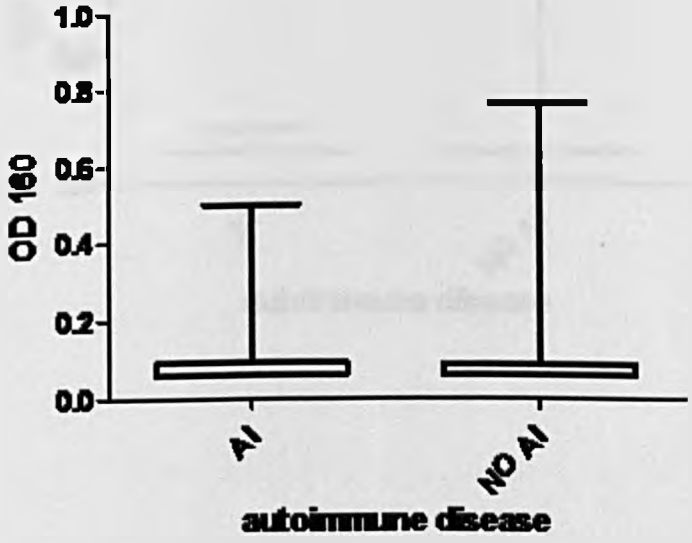


9.5 Autoantibody response to Annexin-1 in those patients with and without autoimmune disease

Fig 9.5 showing no difference in antibody response to Annexin 1 antigen between the two groups

Mann Whitney test

P value- 0.1152

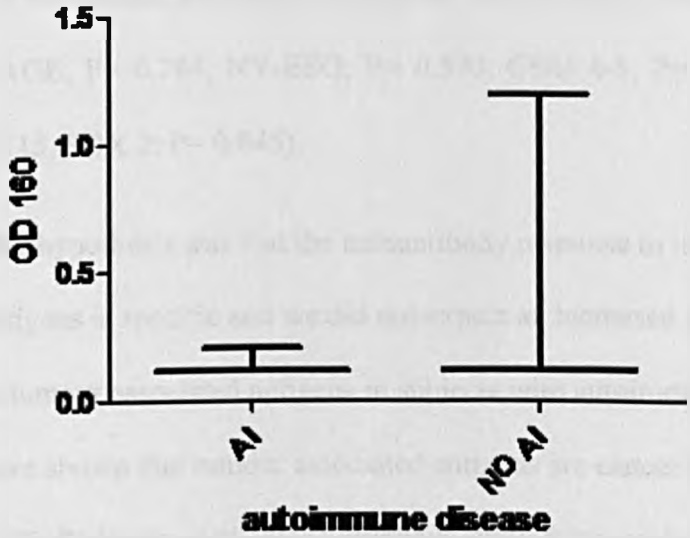


9.6 Autoantibody response to SOX2 in those patients with and without autoimmune disease

Fig 9.6 showing no difference in antibody response to SOX2 antigen between the two groups

Mann Whitney test

P value- 0.6454



9.7 Discussion

Autoimmune diseases (AI) are prevalent in our society and some of them have links to cancer (Aggarwal *et al*, 2012; Orozco *et al*, 2012; Segal *et al*, 1985). However, it is not known if the autoantibody response to TAAs in subjects with AI differs to that of the normal population.

In our study, 186 subjects (9%) had personal history of an AI. Diabetes mellitus and Rheumatoid arthritis were the commonest among the AI.

Mean optical densities of all six cancer related antigens were analysed between the two groups and this did not show any statistical difference (p53; P= 0.533, CAGE; P= 0.784, NY-ESO; P= 0.570, GBU 4-5; P= 0.763, Annexin 1; P= 0.115, SOX 2; P= 0.645).

Our hypothesis was that the autoantibody response to tumour associated antigens is specific and we did not expect an increased autoantibody response to tumour associated antigens in subjects with autoimmune disease. Studies have shown that tumour associated antigens are cancer specific (Lubin *et al*, 1995; Pedersen *et al*, 2011). Previous study from our institute has looked into the specificity of the human immune response against the six recombinant tumour-associated antigens measured in the *Early*CDT™-Lung test(A Murray, 2011). The six recombinant antigens (p53, SOX2, CAGE, NY-ESO-1, GBU4-5 and Annexin 1) were analysed by SDS-PAGE and Western Blotting. The blots were probed both with antigen specific monoclonal antibodies and positive sera. Enzyme Linked Immunosorbant Assays (ELISA) were performed in which positive sera were pre-incubated with a range of different proteins before being allowed to react with the test antigens in order to show

antigen-specific inhibition of binding of these sera. Inhibition ELISAs using positive sera showed that binding to each antigen could be inhibited by the antigen itself but not by bovine serum albumin, VOL (a recombinant antigen produced and purified in the same way as the *Early*CDT™-Lung antigens or by one of the other cancer antigens in the panel.

Our present results also showed no significant increased response to cancer related antigens in these subjects with autoimmune disease compared to those without the disease. The implication of this result would be that, patients with autoimmune disease could be tested for autoantibodies to tumour associated antigens and the results should be as meaningful as to someone without autoimmune disease.

Chapter 10. Autoantibody response to cancer related antigens in subjects with family history of cancer

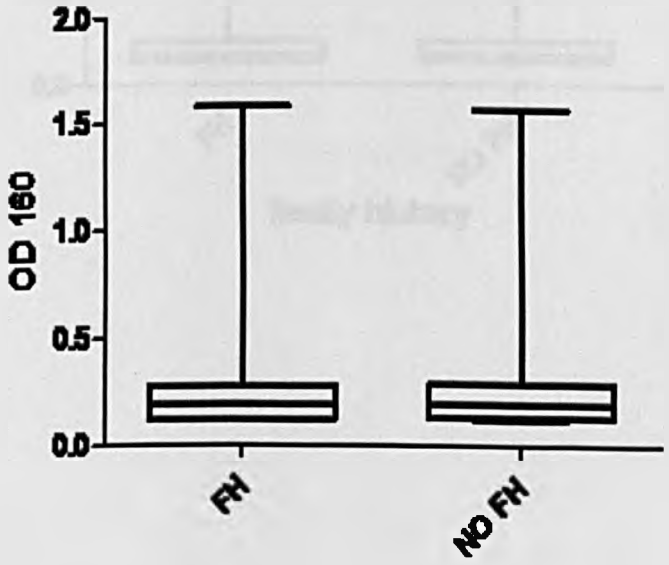
10.1 Autoantibody response to p53 antigen in those subjects with and without family history of cancer

Approximately 50% of subjects had a relative with history of cancer. Comparisons were made between those with and those without family history of cancer. The study did not show any statistically significant difference in response to all six cancer related antigens when comparisons were made with those with and without family history of cancer as shown in respective figures.

Fig 10.1 showing no difference in antibody response to p53 antigen between the two groups

Mann Whitney test

P value- 0.4741

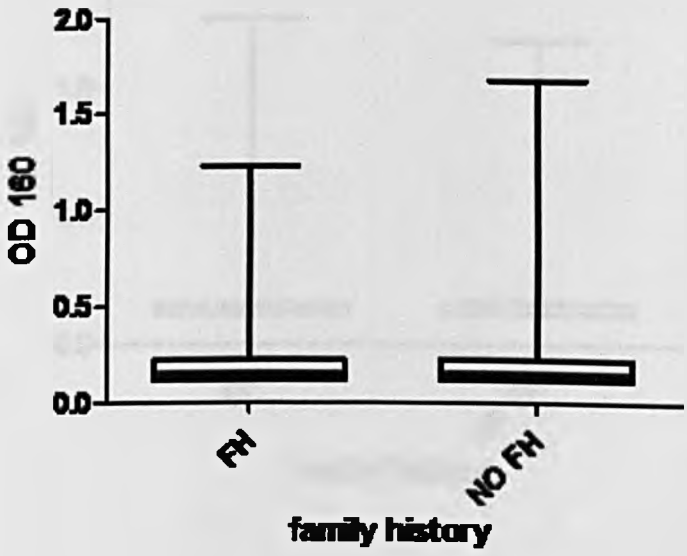


10.2 Autoantibody response to CAGE in those subjects with and without family history of cancer

Fig 10.2 showing no difference in antibody response to CAGE antigen between the two groups

Mann Whitney test

P value- 0.9814

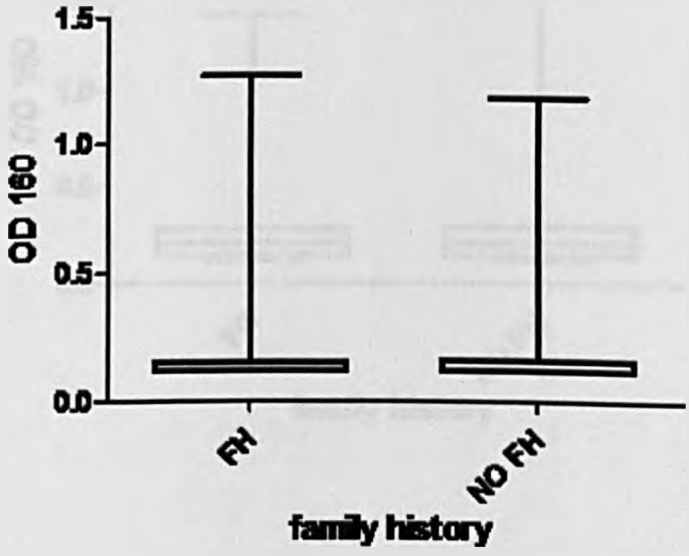


10.3 Autoantibody response to NY-ESO-1 in those subjects with and without family history of cancer

Fig 10.3 showing no difference in antibody response to NY-ESO-1 antigen between the two groups

Mann Whitney test

P value- 0.1047

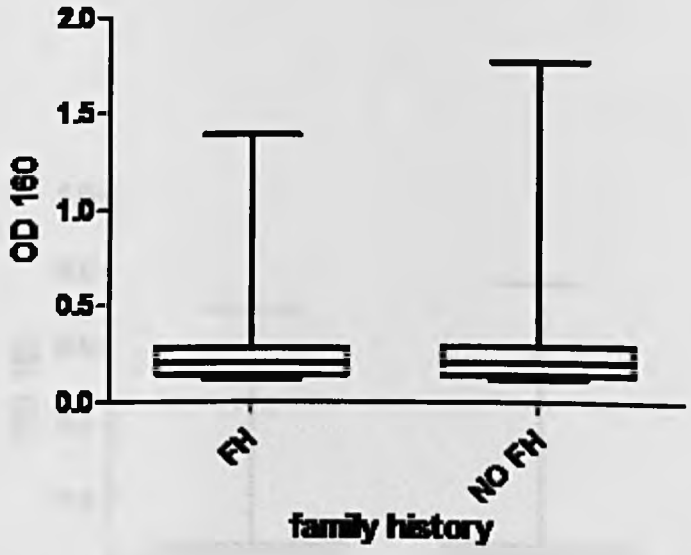


10.4 Autoantibody response to GBU 4-5 in those subjects with and without family history of cancer

Fig 10.4 showing no difference in antibody response to GBU 4-5 antigens between the two groups

Mann Whitney test

P value- 0.4213



10.5 Autoantibody response to Annexin 1 in those subjects with and without family history of cancer

Fig 10.5 showing no difference in antibody response to Annexin 1 antigen between the two groups

Mann Whitney test

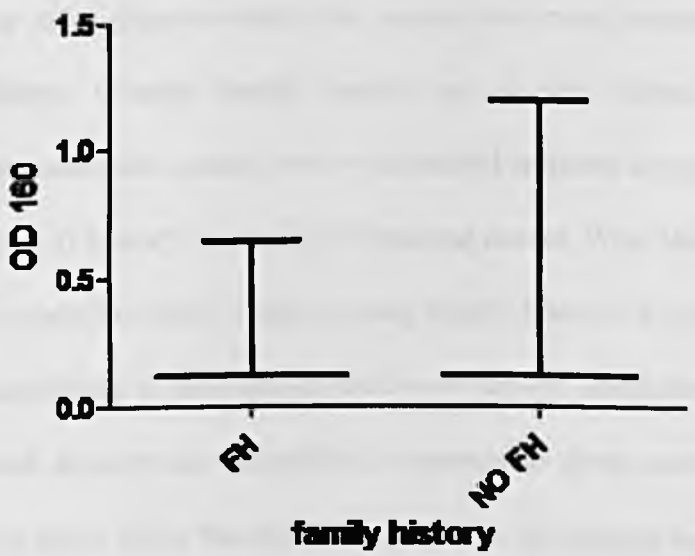
P value- 0.4684

10.6 Autoantibody response to SOX2 antigen in those subjects with and without family history of cancer

Fig 10.6 showing no difference in antibody response OX-2 antigen between the two groups

Mann Whitney test

P value- 0.7192



10.7 Discussion

Family history is one of the strong risk factors for many of the common cancers. Approximately one in two of our subjects in our study had first or second degree relative with cancer. When comparisons were made with regard to antibody response in the two groups with and without family history of cancer, it failed to reach statistical significance. Mean optical densities of antibodies to all six cancer related antigens were analysed between the two groups and this did not show any statistical difference (p53; P= 0.4741, CAGE; P= 0.9814, NY-ESO; P= 0.1047, GBU 4-5; P= 0.4213, Annexin 1; P= 0.4684, SOX2; P= 0.7192).

In our study we looked into subjects across all decades of life and not simply the older decades where the cancers are more common, as we wanted to address whether family history per se was linked to the presence of autoantibodies against tumour associated antigens as opposed to someone with a family history is actually developing cancer. What these data show is that on a population basis, simply having family history of cancer doesn't make you more likely to have autoantibodies to tumour associated antigens. If a subject show an increased autoantibody response to these tumour associated antigens, it is more likely that this is due to them developing cancer themselves rather than being linked to their family history. This is in line with the hypothesis that these elevated autoantibodies reflect the induction of carcinogenesis. However, in subjects with specific mutation like BRCA1 or 2, you may get antibodies directly linked to the mutation. There is no data available at present to suggest that individuals with these mutations make antibodies to a protein associated with genetic mutation, and is probably because they see these proteins as self.

In these individuals with high risk of developing cancer, you may have to look for autoantibodies to other tumour associated antigens.

Chapter 11. Conclusion and future direction

Our work has added new findings in the field of autoantibodies i.e. their prevalence in normal population and in association with various demographic risk factors. My thesis is different from other published work in that, the population we targeted were community based and not subjects attending the hospital i.e. patients. Previous studies on autoantibodies have limitations due to their selection of controls or the lack of such controls. Often studies use limited number of normal individuals without cancer as controls which may not be representative of the normal population as a whole. The controls in some studies are often younger and relatively healthy and some studies report using 'blood donors' as controls and all these subject groups do not represent a normal community population.

This unique community based work looks into the spectrum of autoantibody response in the normal population and looks into different demographic subgroups within the community. In our study there was a progressive increase in the median optical density of antibodies to tumour associated antigens with progressing decades. The proportion of subjects in each decade of life with optical density values above the overall median compared to those below the median increased with progressing decade for p53, CAGE, NY-ESO-1 and GBU4-5 and it was statistically significant for these antigens. The incidence of cancer also increases with age and increased response of antibodies to cancer related antigens with age could be a reflection of cancer becoming more common with aging.

General autoantibody levels increases with age (Aho, 1980; Moscardi *et al*, 1997). It has been shown that incidence of benign autoimmune disease increases with age(Morganti *et al*, 2005) and levels of immunoglobulins G,M

and A also increases with age(Rink & Seyfarth, 1997). Although there are no previous studies on autoantibodies to cancer related antigens with reference to age, it is possible that as with benign autoimmune disease there is a true rise in antibodies to cancer specific antigens with age. If this rise is a true reflection of antibody response with age, this could be an important factor when setting the cut off for autoantibody tests in the future. Further follow-up of these subjects is warranted to research this further, and as consent for this has been already taken from these subjects for the above, it could be undertaken in the future.

Autoantibody response to TAA's was similar in both genders with all the antigens except CAGE showed no statistical difference between the two genders. To eliminate the effects of other possible factors like age and smoking, over 500 subjects in each gender were matched for age (+/- 3 years) and smoking history. However, this also showed a significant difference for autoantibody response to CAGE antigen. CAGE is a cancer testes antigen and in normal individuals its expression is largely restricted to testes(Alsheimer *et al*, 2005). Antigen levels could be inherently elevated in males, and with genetic changes with age, they are no longer recognised as self and thus producing an elevated antibody response. With the limitation of available data, this would require confirmation of another normal dataset. Another possibility we have explored for the gender difference in CAGE is the imbalance in gender across assay runs. The PAS samples were assayed in two distinct time periods (period A and period B) and the gender ratio was not constant over these two sets, with the ratio for first period being M:F 1:11.6 and second

period 1:0.4 respectively. The means of OD for CAGE were very similar in period A but in period B there was an increase in males with an obvious difference in mean optical densities. This could suggest that the difference for Cage is not a consistent effect. Furthermore, when statistical adjustments were used to eliminative the effect of run difference, there was no statistically difference between the means between two genders for CAGE. Overall therefore the available evidence is that there is no difference in autoantibody response to cancer related antigens with gender which is what we anticipated in the beginning. This is further interesting given the fact that there is increase in antibody response to benign autoimmune disease in females.

Autoantibody response to all cancer related antigens were consistently low in smokers. It is well known that smoking can produce a suppression of immune response (Lu *et al*, 2007; Robbins *et al*, 2004). This could explain the relatively low antibody response in smokers. The rise in autoantibody response was more in the ex-smoker group compared to the other two groups with all the antigens except NY-ESO1 suggesting the possibility of rebound effect when smoking is stopped. To analyse further the rebound phenomenon of rise in AAB level after cessation following a period of smoking, the year of quitting for ex-smokers was extracted from the database. There was very little difference for most antigens back to 1970, but decades before that there was an observed increase in the mean AAb level for all antigens except SOX2. We do not have sufficient data to analyse how long the rebound phenomenon might last and there is no available literature at present to support this finding.

Nine percent of the subjects had personal history of autoimmune disease. These subjects did not show any significant response to these cancer related antigens compared to those subjects without personal history of autoimmune disease. This data supports the previous report from our institute that the immune response to these cancer specific antigens which is measured by detections of IgG antibodies is antigen specific. A significant proportion of subjects who are screened for cancer would have autoimmune disease, and our study clearly shows that autoantibody levels to these cancer antigens are not altered in patients with autoimmune disease and their measurement to detect early cancer should reflect as for the rest of the population.

Almost half the subjects either had first or other close relative with cancer. The autoantibody responses to cancer antigens were no different from those without family history of cancer. We looked into subjects across all decades of life and did not focus on simply the older decades where the cancers are more prevalent. This is because we wanted to address whether family history per se was linked to the presence of autoantibodies against tumour associated antigens as opposed to someone with a family history is actually developing cancer. These data show that on a population basis, just having family history of cancer doesn't make you more likely to have autoantibodies to tumour associated antigens. If a subject show an increased autoantibody response to these tumour associated antigens, it is more likely that this is due to them developing cancer themselves rather than being linked to their family history and warrant further diagnostic tests. However in subjects with specific mutation like BRCA1 or 2 this may not be the case, as you may get antibodies

directly linked to the mutation. Mutation could lead to abnormal production of protein which could be recognised by our immune system as foreign. However, at what stage of the genetic change these proteins become immunogenic and why some proteins are immune privileged rendering them not to be identified by the immune system is not clear. Although various theories have been proposed(Kazarian & Laird-Offringa, 2011) including increased level of expression or altered localization and post-translational modifications for the immunogenicity of some antigens, it is clear that further research is required in this field.

Further research arising from this thesis would be to focus on following the individuals in this population based study to find out how many develop lung cancer and whether or not the autoantibody response matches in these individuals. This could also give us an idea as to the lead time from the tumour marker rise to the clinical presentation of lung cancer. This is beyond the scope set out for this thesis.

Further research should be also possible with blood samples collected in this study to identify new antibodies which could improve the accuracy of the present screening panel to aid in the diagnosis of lung cancer. Further research should also focus on the development of other panel against other common cancers given that these individuals are now being tracked on the cancer registry.

Chapter 12. **References and appendix**

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Appendix 1- Consent form

The University of Nottingham

HEALTHY VOLUNTEER CONSENT FORM

Population study of autoantibodies in peripheral blood

The University of Nottingham & Oncimmune Ltd

The patient should complete the whole of this sheet herself. **Please cross out as necessary**

Have you read and understood the patient information sheet? YES / NO

Have you had an opportunity to ask questions and discuss the reason for requesting a sample of your blood? YES / NO

Have all the questions been answered satisfactorily? YES / NO

Have you received enough information about the reasons for requesting these samples? YES / NO

Who have you spoken to? Prof/Dr/Mrs/Mr .....

Do you understand that you are free to refuse to allow your blood to be taken and used in the ways described in the information to patients sheet without having to give a reason? YES / NO

Do you confirm that you have not been a subject in any other research study in the last three months which involved: taking a drug; having any invasive procedure (eg blood >50ml, endoscopy) or exposure to ionising radiation. YES / NO

Do you agree to?

- *40mls (8 teaspoonfuls) / *100mls (20 teaspoonfuls) being taken? YES / NO
  (*delete whichever you do not wish to give
- being contacted on one further occasion in the next 18 months? YES / NO

Do you agree to allow your samples to be used?

- for research, development and production of blood tests as described YES / NO
- for any future such use as may be decided by the investigators YES / NO

Do you understand that any information provided in the questionnaire will be kept confidential YES / NO

Faculty of Medicine & Health Sciences
06/03/08

However relevant information may be made available in confidence to:

- regulatory bodies
- commercial companies

interested in any research findings or any tests or therapies produced by the University of Nottingham and/or Oncimmune YES / NO

Do you understand and agree that research obtained involving your blood may be published although you as an individual will not be identifiable? YES / NO

Do you agree to researchers accessing local and national cancer databases in future to find out if you have or have not developed cancer at a future time point? YES / NO

Do you agree to allow the researchers to pass on a sample of your blood in future to other researchers? -

- within the European Union YES / NO
- outside the European Union YES / NO

Do you understand you are relinquishing all right, title and interest in such material to the University of Nottingham and/or Oncimmune and signing away the rights to any discovery? YES / NO

Do you agree to this? YES / NO

Signature (patient) ..... Date.....

Name (in block capitals) .....

I have explained the study and the above person has indicated their willingness to take part.

Signature (Consenter) ..... Date .....

Name (in block capitals) .....

Faculty of Medicine & Health Sciences
06/03/08

Appendix 2- Health questionnaire

The University of Nottingham

Population Study Questionnaire

Volunteer Information **Date:** / /

Name: (First/MI/Last)	**Sex:** M ○ F ○	**Date of Birth:** (mm/dd/yyyy) / /
Address:		
Current Occupation:		

Smoking History

Are you a smoker? Currently ○ Previously ○ Never ○
Current Smokers *How long have you smoked for?* On average how many cigarettes do you smoke each day
Previous Smokers When did you stop smoking? How many years did you smoke for? On average, how many cigarettes did you smoke each day?

Past Medical History

Do you have benign breast disease? Yes ○ No ○			
If yes, please specify			
Do you currently have any of the following benign Autoimmune Disorders?			
Rheumatoid arthritis	Yes ○ No ○	Lupus	Yes ○ No ○
Scleroderma (skin)	Yes ○ No ○	Sogjerns syndrome	Yes ○ No ○
Wegener's granulomatotis	Yes ○ No ○	Diabetes mellitus	Yes ○ No ○
Hashimoto's thyroiditis	Yes ○ No ○	Graves' disease	Yes ○ No ○
Coeliac disease	Yes ○ No ○	Crohn's disease	Yes ○ No ○
Ulcerative colitis	Yes ○ No ○	Multiple sclerosis	Yes ○ No ○
Guillain-Barre syndrome	Yes ○ No ○	Addison's disease	Yes ○ No ○
Primary bilary sclerosis	Yes ○ No ○	Sclerosing cholantitis	Yes ○ No ○
Autoimmune hepatitis	Yes ○ No ○	Goodpasture's syndrome	Yes ○ No ○

Exposure to Radiation

Have you ever been exposed to radiation?	Yes ○ No ○
If yes, what was the reason for the exposure?	
How long was the exposure for?	

Personal History of Cancer

Do you have any personal history of cancer?	Yes ○ No ○
If yes, what type of cancer was diagnosed?	
What date was this diagnosed (or when were you informed)?	
What treatment did you have?	
Have you had a recurrence of this cancer at any time?	Yes ○ No ○

Family History of Cancer

Do you have any family history of cancer?		Yes ○ No ○ If yes please specify:
Family Member	**Type of cancer**	**Age diagnosed**