A Sub-study of the ASCOT Trial

An intensive phenotyping study to enable the future examination of genetic influences on hypertension-associated cardiovascular disease

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Introduction

In this study, detailed cardiovascular phenotypic data will be collected from a subset of patients recruited into the Anglo-Scandinavian Cardiac Outcomes Trial (ASCOT). One thousand patients will have left ventricular mass, common carotid intima-media thickness, retinal microvascular geometry, and urinary markers of vascular damage and thrombosis measured after 1 year of ASCOT therapy (baseline assessment), and repeated after a further 2 years (follow-up assessment).

The collection and referencing of these data will allow future testing of the hypothesis that there are as yet unrecognised genetic influences (deleterious and/or protective) on the development of hypertension-associated cardiovascular disease (HACVD). Genetic analyses are likely to include analyses by the candidate gene approach and genome-wide scanning. We plan to compare:

- the genetics of subjects with severe HACVD at baseline and those with little HACVD at baseline, and
- the genetics of subjects with the most rapid progression of HACVD during follow-up and those with least progression.

Additionally the collected data will allow the comparison of the effects of a beta-blocker ± diuretic regimen with the effects of a calcium antagonist ± angiotensin-converting-enzyme inhibitor regimen on regression of HACVD; the examination for any interaction between genes and antihypertensive therapy on the progression or regression of HACVD; and the further assessment of the prognostic value of regression of HACVD.

Background

Hypertensive disease is a complex polygenic, multifactorial disorder resulting from interaction of several genes with each other and with the environment. In addition to genes with direct influence on blood pressure, hypertensive patients may also carry genes with influence on cardiovascular risk factors such as hyperlipidaemia or hyperhomocysteinaemia, as well as genes affecting end-organ damage or contributing to the causation of acute cardiovascular events. It is well established that the presence of target organ damage, such as left ventricular hypertrophy and carotid wall thickening, in both normotensive and hypertensive patients predicts future cardiovascular events. We hypothesise that there are as yet unrecognised genetic influences (deleterious and/or protective) on the development of hypertension-associated organ damage.

We will test this hypothesis in a cohort of 1000 patients in the Anglo-Scandinavian Cardiac Outcomes Trial (ASCOT). ASCOT is a randomised...
trial which will compare the long-term effects of a standard antihypertensive regimen (beta-blocker ± diuretic) with a more contemporary regimen (calcium antagonist ± angiotensin-converting enzyme (ACE) inhibitor), on non-fatal myocardial infarction and fatal coronary heart disease (CHD) in a cohort of hypertensive patients at high risk of cardiovascular events, followed for an average of 5 years. In addition ASCOT will allow the comparison of the effects of atorvastatin vs placebo, on non-fatal myocardial infarction and fatal CHD, among patients with a total cholesterol &lt; 6.5 mmol/l.

The potential for genetic analyses of human disease is currently rapidly expanding. Within a few years, expressed sequence tags representing almost all sequences expressed in humans will be determined, and their genomic positions defined. The discovery of most of the variants in the human genome that contribute to the genetic diversity of the human population will result in the construction of dense polymorphic maps. However the rapid growth of expressed sequence tag and single nucleotide polymorphism databases will be worthless without reference to accurate phenotypic data. The main ASCOT study will provide an initial sound platform of standardised clinical measurements from a large sample of hypertensive patients. Further detailed cardiovascular phenotypic data will be collected from 1000 patients recruited into ASCOT at one of two trial centres (St Mary's Hospital, London, and Beaumont Hospital, Dublin). By careful secondary analysis of clinical and biochemical risk factors, it will be possible to correct for co-variates which otherwise would be likely to mask genetic effects. This will be essential in teasing apart the likely significance and mechanism of action of genotypic variants in the development of organ damage.

Objectives

We aim to establish a detailed cardiovascular phenotypic database within subjects recruited to the ASCOT study. One thousand patients will have a range of assessments of cardiovascular structure and function, measured after 1 year of ASCOT therapy, and repeated after a further 2 years. This will enable future examination for genetic influences (deleterious and/or protective) on severity of HACVD. Through repetition of cardiovascular phenotyping after 2 years, we will be able to examine for genetic influences and gene-therapy interactions on the rate of progression or regression of HACVD in these hypertensive patients.

Additional objectives of this study include; the determination of any differential effects of the different treatment strategies within ASCOT on HACVD; and the further clarification of the predictive strength of structural and biochemical measurements of cardiovascular pathology as indicators of risk in populations of hypertensive patients.

Study methods

This sub-study has been approved by local ethics committees, and is being conducted in accordance with informed consent regulations, and according to Good Clinical Practice for Trials in Medicinal Products in the European Community.

Subjects

All patients, fulfilling the entry criteria for the main ASCOT trial, and willing to donate a blood sample for use in an anonymised genetic database, are eligible for this sub-study. A total of 1000 patients will be recruited, 500 patients from each of two ASCOT regional centres, St Mary’s Hospital, London, and Beaumont Hospital, Dublin.

Genotyping

Blood samples collected into EDTA (20 ml) will be used for DNA extraction. Lymphocytes will be extracted from citrated blood and frozen for subsequent immortalisation. Both DNA and lymphocyte samples will be anonymised through a re-labelling process. Prior to any genotyping, all codes linking the samples being analysed to patient identifying data will be destroyed.

The precise genetic analyses that will be utilised will depend upon the considered scientific importance of particular genes and variants within genes at the time of the genetic analysis, and also the cost and availability of technology to be used. It is likely that some or possibly all of the approaches below will be performed.

- Known polymorphisms: We will genotype known alleles in genes thought to confer cardiovascular disease risk, not only to assess their role in this population but also to allow tests of interactions between these and novel variants. These known polymorphisms will include variants in genes possibly influencing hypertension (ACE); thrombosis (fibrinogen common pro-coagulant variant, platelet receptor PLA2 variant); homocysteine metabolism (MTHFR TT genotype) and atherogenesis (CETP variant).
- Candidate genes: Candidate genes influencing vascular tone, inflammation, lipids, thrombosis, and growth will be screened for biallelic markers and other polymorphisms (Table 1).
- Genome wide scan: Polymorphisms of candidate genes is restrictive, as the sequences and/or functions of most genes are incompletely characterised. An alternative approach could be to perform a genome-wide screen for markers related to disease and use these to identify genes of interest. Potentially, a genome wide scan of 20000 biallelic markers or more could be performed.
Table 1 Some of the candidate genes, with influence on vascular tone and growth, inflammation, lipids, and thrombosis, that may be screened for biallelic markers and other polymorphisms

<table>
<thead>
<tr>
<th>Vascular tone</th>
<th>Inflammation</th>
<th>Lipids</th>
<th>Thrombosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Angiotensinogen</td>
<td>TNF</td>
<td>APO-E</td>
<td>Fibrinogen</td>
</tr>
<tr>
<td>Angiotensin</td>
<td>IL-1</td>
<td>Lp(a)</td>
<td>Prothrombin</td>
</tr>
<tr>
<td>ACE</td>
<td>HOX-1</td>
<td>Lp lipase</td>
<td>Factor VIIa</td>
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<tr>
<td>ATR</td>
<td>COX-1</td>
<td>CETP</td>
<td>Factor V</td>
</tr>
<tr>
<td>Renin</td>
<td>COX-2</td>
<td></td>
<td>GP Ib/IIa</td>
</tr>
<tr>
<td>Endothelin</td>
<td>MTHFR</td>
<td></td>
<td>GP Ib</td>
</tr>
<tr>
<td>Nitric oxide synthase</td>
<td></td>
<td></td>
<td>PAI-1</td>
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Phenotyping

Patients from St Mary’s and Beaumont Hospitals participating in this sub-study will have the following additional procedures performed after 1 and 3 years of ASCOT therapy. The principle end points being measured by each examination are indicated in italics.

- Echocardiography, left ventricular mass-index, (LVM);
  M-mode left ventricular measurements of septal (IVS) and posterior wall (LVPW) thicknesses and left ventricular internal diameter (LVID) will be made at end-diastole from the short-axis parasternal view using ASE convention. Three consecutive cycles will be measured and averaged, and left ventricular mass (LVM) calculated using the formula:

  \[
  LVM = 0.8 \times [(IVS + LVID + LVPW)^3 - LVID^3] + 0.6 \text{ g.}
  \]

  Mass will be normalised for body surface area.

- Carotid ultrasound, common carotid intima-media thickness (IMT) and prevalence of plaque;
  High resolution ultrasound imaging of the left common carotid artery and bulb will be performed using a HDI 5000 scanner (Advanced Technologies Laboratories, WA, USA). The left common carotid artery and bulb will be screened to detect any plaques (focal thickening of the intima-media layers of >1.3 mm or 50% greater than surrounding IMT). Diastolic images of each detected plaque, in longitudinal and transverse sections so as to display maximum thickness, will be saved. For the determination of common carotid IMT, diastolic longitudinal images from each of three projections (posterior oblique, lateral and anterior oblique), of the distal common carotid focused on the far wall will be utilized (Figure 1). All images will be measured using a purpose designed user-directed image analysis program.

- Fundal photography, arterial length/diameter ratio (L:D ratio), bifurcation angle (\(\omega\)) and junction exponents (\(x\));
  After pupil dilatation, high resolution digitised images of the superior temporal and nasal quadrants of the fundus of the right eye will be acquired using a digital camera attached to a fundus camera. Network quantification will be performed, off-line, using a purpose-designed software program. Retinal arterial network architecture will be summarised in terms of average junction exponent (\(x\)), bifurcation angle (\(\omega\)) and arteriolar segment length/diameter ratio (L:D ratio) (Figure 2). These measures of network geometry reflect a range of important intact network properties, such as power costs, efficiency, shear stresses and vessel density.

- Urine sampling, urinary markers of vascular damage and thrombosis (2,3-dinor-6-keto-PGF1, 11-dehydro thromboxane B2, 8-Epi-PGF2);
  75 ml urine samples will be collected and stored at -20°C until analysed. Samples will be extracted and further purified using solid phase C18 columns and thin layer chromatography. Analysis will be by negative ion, chemical ionisation, gas chromatography/mass spectrometry using a Finn...
gan INCOS-XL in tandem with a Varian 3400 gas chromatograph.\textsuperscript{53}

Twenty-four hour ambulatory blood pressure monitoring, using a validated device,\textsuperscript{34} will be performed at randomisation and annually thereafter for the duration of the study. After 1 year and after 3 years of ASCOT therapy a 30-ml blood sample will be taken and serum will be stored for subsequent measurement of further known cardiovascular risk factors such as homocysteine, insulin, fibrinogen, and lipoprotein(a). These additional clinical and biochemical measures will allow correction for covariates which otherwise would be likely to mask genetic influences on organ damage.

**Sample size and statistical methods**

We intend to compare the genotypes of those subjects with HACVD in the highest tertile, with the genotypes of those with HACVD in the lowest tertile, and also the genotypes of the tertile with the greatest progression of HACVD, with those of the tertile with least progression. Sample size calculations assumed testing for 500 candidate genes, a frequency of 30% for the higher risk genotype in the lowest tertile, and a significance threshold of 0.2 for each test. Allowing 80% power to detect a true effect, an odds ratio of 2.0 requires 316 in each group. Hence, to allow comparison of the genetics in subjects with HACVD in the highest and lowest tertiles by the candidate gene approach, a total of 950 subjects are required.

Analysis of variance will be used to explore for effects of treatment, age, sex, severity of hypertension, and lipid levels on rates of progression/regression of HACVD. Life tables analysis (cumulative event free survival) will be used to assess whether regression of HACVD is associated with protection against morbidity and mortality.

**Discussion**

The measurement of left ventricular mass index, carotid plaque prevalence and intima-media thickness, microvascular geometry and metabolites of prostaglandins and isoprostanes, will provide a relatively comprehensive in vivo assessment of cardiovascular structure and function, from the heart to the smallest arterioles. Phenotypic assessment will occur on two occasions. Baseline measures, taken after 1 year of ASCOT therapy, are likely to be dominated by the particular stage of the hypertensive disease—the duration and severity of BP elevation—and previous antihypertensive treatment. By contrast, the change in phenotypic measures, occurring over a period of 2 years, whilst all subjects are receiving standardised treatments, is likely to provide a better indication of whether a patient has a rapidly developing or more benign form of target organ disease.

Left ventricular hypertrophy and carotid wall thickening have both been shown to be strongly associated with hypertension,\textsuperscript{38-36} to regress with antihypertensive therapy\textsuperscript{39-42} or statin therapy,\textsuperscript{43,44} and to be pressure independent risk factors for cardiovascular morbidity and mortality.\textsuperscript{19,21}

The blood vessels lying on the surface of the retina present a readily accessible microvascular tree in man in vivo. Divergence from optimal geometric pattern in vascular networks have been described with old age, and in patients with hypertension and atherosclerosis.\textsuperscript{45-47} The impact of therapy on retinal microvascular architecture, and the prognostic value of retinal microvascular alterations are currently unknown.

Prostaglandins are products of the oxidation of arachidonic acid catalysed by the enzyme cyclooxygenase. The products formed have widespread biological effects and are involved in many processes relevant to vascular disease including inflammation, thrombosis and vasoconstriction. Increased generation of both prostacyclin and platelet derived thromboxane have been observed in a variety of vascular pathologies.\textsuperscript{48-51} An alternative metabolic pathway has been described whereby free radicals directly attack arachidonic acid resulting in an endoperoxide structure that spontaneously isomerises to prostaglandin-like molecules, the so called isoprostanes. Isoprostanes are both vasoconstrictor and mitogenic.\textsuperscript{52} In addition they have been found to activate a PGF2 receptor linked to cardiomyocyte hypertrophy.\textsuperscript{53} Excretion of isoprostanes, such as 8-Epi-PGF2\textsubscript{a}, is increased in patients with atherosclerosis,\textsuperscript{54,55} and high local concentrations have been detected in plaques.\textsuperscript{56} Statin therapy-induced reductions in cholesterol has been accompanied by falls in urinary thromboxane metabolite excretion\textsuperscript{57} and isoprostanes.\textsuperscript{58} Hence assays of prostaglandin metabolites and isoprostanes will provide a biochemical index of vascular disease and of free-radical induced cell injury.

Less than 40% of the observed variation in left ventricular mass can be attributed to BP level. It appears likely that both genetic and neurohormonal factors may also influence cardiac size.\textsuperscript{5} In 1990, Rigat first described an insertion/deletion polymorphism in the gene encoding for the ACE.\textsuperscript{59} Deletion homozygotes have relatively high plasma ACE concentrations and it was postulated that these might be more likely to develop left ventricular hypertrophy (LVH). Studies addressing this hypothesis, although initially promising,\textsuperscript{6,7} have subsequently proved disappointing.\textsuperscript{9,10} There appears to be no association between variants of the angiotensinogen gene and different patterns of hypertensive LVH.\textsuperscript{11} A tandem repeat DNA polymorphism of endothelial nitric oxide synthase gene has been reported to protect against LVH in Japanese hypertensive patients.\textsuperscript{42}

Candidate gene studies exploring the associations of genotype with large vessel atherosclerosis have in the main yielded negative results; angiotensinogen
and ACE genotype;\textsuperscript{13} endothelial nitric oxide synthase;\textsuperscript{14} beta-fibrinogen;\textsuperscript{15} and apolipoprotein E.\textsuperscript{16} However a report from a population in Northern Italy has described greater common carotid IMT among subjects with the ACE DD genotype.\textsuperscript{17}

We anticipate that the accurate and extensive phenotyping of this large cohort of high risk hypertensive patients, and the use of the newest genetic technologies, will allow the detection of previously unrecognised genetic influences on the development of HACVD. The prospective nature of this ASCOT sub-study will also allow assessment of the predictive strength of the phenotypic indices and the consequences of treatment-induced changes.

References

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