The role of the CCR5 Δ32 polymorphism in abdominal aortic aneurysms

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Summary
Background C-C chemokine receptor 5 (CCR5) is involved in the regulation of the inflammatory response. Abdominal aortic aneurysms (AAA) may arise as the result of a chronic inflammatory process which is influenced by genetic predisposition. The CCR5 gene is associated with a 32 base pair deletion (the Δ32 polymorphism). The aim of this study was to investigate the role of the CCR5 Δ32 polymorphism in the development of AAA.

Methods A case-control study was conducted including 285 patients with AAA and 273 control subjects. A blood sample was taken from each individual and DNA was extracted. CCR5 genotype was determined using the polymerase chain reaction (PCR). Flow cytometry was used to investigate the biological activity of the Δ32 polymorphism.

Results There was no significant difference between the AAA and the control group in relation to the Δ32 allele frequency (AAA group 10%, control group = 12%, P = 0.82, chi-squared analysis). Genotype analysis revealed no significant difference between the groups (AAA vs. controls, wild-type homozygotes = 82% vs. 77%, P = 0.33, chi-squared analysis). The polymorphism was shown to be biologically active with the number of Δ32 alleles correlating with cell expression of ccr5 as detected with flow cytometry (P ≤ 0.05).

Conclusion This study demonstrates that the ccr5 Δ32 is a biologically active genetic polymorphism; however, there is no association between this polymorphism and AAA.

Introduction
The incidence of abdominal aortic aneurysm (AAA) has been steadily increasing over recent years, with up to 8% of men over the age of 60 now affected (Singh et al., 2001). Aortic aneurysm has been repeatedly linked with smoking, hypertension, hypercholesterolaemia and male sex (Blanchard et al., 2000), and it is believed that these risk factors play a causal role in aneurysm development. However, as medical management of these comorbidities and antismoking campaigns attempt to address these factors, the incidence of aneurysm continues to rise. This is in contrast to other smoking-related diseases such as ischaemic heart disease, which have seen a fall in incidence over a similar period (Lampe et al., 2005). This has led to uncertainty as to the significance of these factors in aneurysm pathogenesis.

Clifton, in 1977 first reported a possible genetic component of aneurysm disease when he described a family in whom three brothers were all affected by AAA (Clifton, 1977). Since then many familial studies have been conducted in an attempt to establish a genetic link. Several authors have also attempted to characterize the mode of inheritance through segregation studies (Tilson & Seashore, 1984; Powell & Greenhalgh, 1987; Majumder et al., 1991; Verloes et al., 1995; Kuivaniemi et al., 2003).

In recent years, much experimental work has focused on the inflammatory process and significant evidence has emerged in support of an inflammatory component to AAA formation. Examination of the cellular composition of both normal and aneurysmal aortic wall has confirmed increased expression of inflammatory cells: T lymphocytes and macrophages (Ailawadi et al., 2003; Forester et al., 2005). Inflammatory cytokines have been shown to be upregulated in aneurysmal aortic walls compared with normal controls (Juvonen et al., 1997). Specifically interleukin (IL)-1 beta, IL-6 and tumour necrosis factor (TNF) alpha levels were significantly higher in aneurysm patients than healthy controls. TNF alpha and IL-6 have also been shown to be elevated in aneurysmal disease compared with aorto-occlusive disease (AOD), confirming the different underlying processes involved (Sheinberg et al., 2000).

Chemokines are a family of small cytokines, and members of the CC subfamily have two conserved cysteine residues near the amino terminus which define a specific shape. Most CC chemokines induce migration of monocytes and other cell types including natural killer (NK) cells and dendritic cells. Chemokines may contribute to AAA formation through modulation of the immune response.

CC chemokine receptor 5 (CCR5) is a 352 amino acid protein with a calculated molecular mass of 40.6 kDa, which is expressed predominantly on resting T-lymphocytes (memory and effector cells), monocytes and macrophages. CCR5 has several known ligands which act via G-protein...
linked responses; these are macrophage inflammatory protein-1α (MIP-1α), which is also known as CC chemokine ligand 3 (CCL3), MIP-1β or CC chemokine ligand 4 (CCL4) and regulated upon activation, normal T-cell expressed and secreted (RANTES) (CCL5) (Onuffer & Horuk, 2002).

CCR5 has a 32 base pair deletion (Δ32) polymorphism in the promoter region of the gene. This results in a frameshift and premature termination of the protein (Mueller & Strange, 2004). The role of this polymorphism in susceptibility to AAA has been investigated, and a higher incidence of the Δ32 allele was observed in patients with aneurysms as opposed to patients with other vascular pathology (carotid stenosis and peripheral vascular disease) or healthy controls (Ghilardi et al., 2004).

The aim of this study was to investigate the CCR5 Δ32 polymorphism with respect to its biological activity and frequency among a study population with AAA and a control population.

Methods

Cases and controls

A case control study was conducted involving 285 patients with AAA and 273 screened control subjects. Patients were recruited from a variety of clinical settings (screening theatres), and control subjects were recruited from the aneurysm group and the control subjects. The aneurysm group has a higher incidence of ischaemic heart disease, hypertension and hypercholesterolaemia. This is also in keeping with known risk factors for the development of AAA. Patient demographics were largely similar; however, the aneurysm group had a higher median age, a greater proportion of women (all controls were men), and a greater proportion of current smokers. This reflects known risk factors for the development of AAA.

DNA extraction

A 10-mL sample of venous blood was collected from each subject and stored on ice for transportation. Samples were centrifuged for 10 min at 4 °C at 770 g. Buffy coat and plasma samples were extracted, snap frozen and stored at –80 °C. DNA was extracted from buffy coat using a commercial kit (Gentra Flowgen DNA extraction kit, Flowgen Bioscience, Nottingham, UK), and stored at –20 °C.

Genotyping

Cases and controls were genotyped for CCR5 using the polymerase chain reaction (PCR). Primer sequences were purchased from Sigma-Genosys (Sigma-Aldrich House, Haverhill, UK) and were as follows: forward primer: CTG TGT TTG CGT CTC TCC CA; reverse primer: CCT CTT CTC ATG TCG ACA CCG.

The PCR mastermix contained 0.14 μL 50 mM magnesium chloride; 2.5 μL 10 μM dNTP mix; 2.5 μL 10× reaction buffer (containing 100 mM Tris-HCl pH 8.3, 500 mM potassium chloride (KCl), 11 mM magnesium chloride (MgCl₂) and 0.1% gelatin); 2.5 μL 1:10 forward and 2.5 μL 1:10 reverse primer; 2.5 μL template DNA; 0.25 μL Taq DNA polymerase; and 12.11 μL sterile water, giving a final reaction volume of 25 μL.

The PCR conditions were an initial step of 5 min at 95 °C; followed by 30 cycles of 95 °C for 1 min, 57 °C for 1 min and 72 °C for 1 min; and a final extension of 5 min at 72 °C.

The PCR products were run on a 3% agarose gel for 1 h at 60 V and the gels read under ultraviolet light.

Flow cytometry

In order to confirm that the CCR5 Δ32 polymorphism is biologically active, a sample of subjects of known genotype were included in a further set of experiments using flow cytometry to quantify expression of the CCR5 receptor on T lymphocytes. A fresh blood sample was collected and transported to the laboratory at room temperature. Fifty microlitres of whole blood was then incubated with 10 μL of phycoerythrin-labelled CD3 monoclonal antibody and 10 μL of fluorescein isothiocyanate (FITC)-labelled CCR5 monoclonal antibody in the dark at 22 °C for 30 min. The cells were then washed with phosphate-buffered saline and centrifuged. Most of the supernatant was discarded and the cell pellet resuspended. Flow buffer was then added and the samples were analysed using a Becton-Dickinson FACSCalibur Flow Cytometer (BD Biosciences, San Jose, CA, USA).

Statistical analysis

All statistical analyses were performed using the GraphPad Prism® (GraphPad Software, Inc., La Jolla, CA, USA) and SPSS 9.0® (SPSS Inc., Chicago, IL, USA) packages.

Results

Details of the demographic data for each study group are given in Table 1. The two groups were compared using chi-squared analysis to demonstrate any significant differences between the populations recruited.

Patient demographics were largely similar; however, the aneurysm group had a higher median age, a greater proportion of women (all controls were men), and a greater proportion of current smokers. This reflects known risk factors for the development of AAA.

The comorbidities for aneurysm and control subject groups are given in Table 2. This demonstrates some differences in terms of the comorbidities among the aneurysm group and the control subjects. The aneurysm group has a higher incidence of ischaemic heart disease, hypertension and hypercholesterolaemia. This is also in keeping with known risk factors for the development of AAA.

Genotyping results

A summary of the genotyping results is given in Table 3. It can be seen from this that there is no significant difference...
| Study Ref number | Date seen       |
|------------------|----------------|
|                  |                |

| Name             | U             |
|------------------|---------------|
| Age              | Ethnic origin |
|                  |               |

| Sex              | M  | F  |
|------------------|----|----|
|                  |    |    |

| Aneurysm size     |                        |
|-------------------|-------------------------|
|                   |                         |

| Expansion | Date | Size |
|-----------|------|------|
|           |      |      |

| Date of diagnosis |  |
|-------------------|---|
|                   |  |

| Co-morbidity | BP  | Years    | MI  | CABG | Coronary A2 | Angina | 
|--------------|-----|----------|-----|------|------------|--------|
|              |     |          |     |      |            |        |

| PVD  | Bypass | Limb A2 | Amp |
|------|--------|---------|-----|
|      |        |         |     |

| CVA  | Carotid |         |     |
|------|---------|---------|-----|
|      |         |         |     |

| DM  | Insulin | Oral | Diet |
|-----|---------|------|------|
|     |         |      |      |

| COAD | Inhalers | Nebs | Hosp |
|------|----------|------|------|
|      |          |      |      |

| Ca  | Current | <3/12 | >3/12 |
|-----|---------|-------|-------|
|     |         |       |       |

| Chol | Rx | Years |
|------|----|-------|
|      |    |       |

| Other |                        |
|-------|-------------------------|
|       |                         |

| Family History | Y  | N  |                        |
|----------------|----|----|------------------------|
|                |    |    |                        |

| Meds          | Aspirin | Y  | N  |                  |
|---------------|---------|----|----|------------------|
|               | β-blocker | Y  | N  |                 |
|               | Statin  | Y  | N  |                 |
|               | Nitrate | Y  | N  |                 |
|               | Clopidogrel | Y  | N  |                 |
|               | Digoxin | Y  | N  |                 |
|               | Warfarin | Y  | N  |                 |
|               | Diuretic | Y  | N  |                 |
|               | ACEI    | Y  | N  |                 |
|               | Others  |     |    |                 |

| Smoking | Y  | N  | Ex  |
|---------|----|----|-----|
|         | No |     |     |

| Started | Stopped |
|---------|---------|
|         |         |

**Figure 1.** Proforma for data collection. Where BP, hypertension; MI, myocardial infarct; CABG, coronary artery bypass graft; A2, angioplasty; PVD, peripheral vascular disease; CVA, cerebrovascular accident; carotid, carotid endarterectomy; DM, diabetes mellitus; COAD, chronic obstructive airways disease; Ca, malignancy; Chol, hypercholesterolaemia.
observed in the CCR5 genotype or Δ32 allele frequency between the aneurysm group and the control group (using chi squared).

Subgroup analysis of large (> 5 cm) versus small (< 5 cm) aneurysms also revealed no significant effect of the CCR5 genotype or Δ32 allele frequency on size of aneurysm when seen (see Table 4).

In order to examine the effect of the CCR5 Δ32 allele and genotype on aneurysm progression, cases were compared in terms of mean growth rate. This assumes a linear model of aneurysm expansion. Some debate exists regarding the growth pattern of aneurysms, with some authors suggesting an exponential trend (Limet et al., 1991), however to establish a model of growth for this data was beyond the scope of this study. For the purposes of comparison between different genotypic groups therefore, a linear model was assumed.

Serial measurements for each aneurysm were recorded at relevant time points, and the mean growth rate at each time point was calculated by dividing the aneurysm size in centimetres by the number of months since diagnosis. This figure was then converted and reported as centimetre per year. Mean growth rates were compared between groups using a one-way ANOVA test. There were 146 patients with two or more serial measurements for analysis from this series. There were a mean of 6.2

Table 1. Demographic data

| Category                        | Aneurysm group | Control group | P value (Fisher’s exact test) |
|---------------------------------|----------------|---------------|------------------------------|
| Age (median (range))            | 72 (50–89)     | 66 (65–79)    | < 0.0001 (Mann–Whitney U-test) |
| Gender (% male)                 | 263 (92)       | 273 (100)     | 0.007                         |
| Ethnicity (% white)             | 272 (95)       | 267 (96)      | 0.44                          |
| Family history of AAA (%)       | 37 (13)        | 16 (6)        | 0.17                          |
| Current smoker (%)              | 77 (27)        | 37 (14)       | 0.05                          |
| Ex-smoker (%)                   | 172 (60)       | 156 (50)      | 0.46                          |

P ≤ 0.05 taken to represent a significant difference between the two groups.

AAA, abdominal aortic aneurysm.

Table 2. Patient and control subject comorbidity

| Category                        | Aneurysm group | Control group | P value (Fisher’s exact test) |
|---------------------------------|----------------|---------------|------------------------------|
| Ischaemic heart disease (%)     |                |               |                              |
| Angina                          | 34 (12)        | 19 (7)        | 0.33                         |
| Myocardial infarct              | 79 (28)        | 18 (7)        | < 0.01                       |
| Coronary artery bypass graft    | 31 (11)        | 8 (3)         | 0.05                         |
| Angioplasty                     | 13 (5)         | 4 (1)         | < 0.01                       |
| Hypertension (%)                |                |               |                              |
| Insulin dependent               | 9 (3)          | 6 (2)         | 1                            |
| Non-insulin dependent           | 15 (5)         | 18 (7)        | 0.77                         |
| Diet controlled                 | 10 (4)         | 4 (1)         | 0.37                         |
| Hypercholesterolaemia (%)       |                |               |                              |
| Insulin dependent               | 93 (33)        | 47 (17)       | 0.01                         |
| Non-insulin dependent           | 23 (8)         | 5 (2)         | 0.11                         |
| Malignancy (%)                  |                |               |                              |
| Current                         | 21 (7)         | 3 (1)         | 0.07                         |
| During previous 12 months       | 19 (7)         | 10 (4)        | 0.54                         |
| Peripheral vascular disease (%) |                |               |                              |
| Cerebrovascular disease (%)     |                |               |                              |
| Chronic obstructive airways disease (%) |        |               |                              |

P ≤ 0.05 taken to represent a significant difference between the two groups.

Table 3. Summary of genotyping results

| Genotype            | Aneurysm group | Control group | P value | Chi-squared | Degrees of freedom |
|---------------------|----------------|---------------|---------|-------------|-------------------|
| WT/WT               | 208 (82)       | 179 (77)      |         |             |                   |
| WT/Δ32              | 41 (16)        | 50 (21)       | P = 0.33 | 2.24        | 2                 |
| Δ32/Δ32             | 4 (2)          | 4 (2)         |         |             |                   |
| Frequency of Δ32 allele | 49 (10) | 58 (12) | P = 0.82 | 0.05        | 1                 |

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observations per patient at a mean time period of 31 months follow up (Table 5).

In order to address the possibility of an exponential pattern of growth, the natural log of each expansion rate was taken and the statistical testing repeated. Results are given in Table 6. As shown, whether an exponential or a linear growth pattern is assumed, the CCR5 genotype does not seem to significantly influence aneurysm expansion.

**Flow cytometry results**

There were 17 patients included in this set of experiments. In this group of 17, there were six homozygotes for the wild-type gene, six heterozygotes and five homozygotes for the deletion.

Quadrant statistics describe the number of events (cells) which have been detected by fluorescence from both of the antibodies. In this case, cells expressing both CD3 and CCR5 were identified in this way in the right upper quadrant of the dot plot (see Fig. 2). The quadrant statistic represents the number of cells which fell within the right upper quadrant as a proportion of the total number of cells detected. The results for each patient are given in Table 7, demonstrating the percentage of CD3-positive cells which stained positive with FITC-CCR5.

Although these data appear to fit a normal distribution, it has been subjected to non-parametric statistical testing as the standard deviations differ greatly between groups. In view of this, and accounting for the small numbers in each group, a Kruskal–Wallis test has been used with a post-hoc Dunn’s multiple comparison test. The Kruskal–Wallis

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**Table 4. Genotype and aneurysm diameter**

| Genotype     | Large aneurysm (> 5 cm) N = 120 Number (%) | Small aneurysm (< 5 cm) N = 101 Number (%) | Unknown diameter N = 32 Number (%) | Chi-squared | P value | Degree of freedom |
|--------------|-------------------------------------------|-------------------------------------------|-----------------------------------|-------------|---------|------------------|
| WT/WT        | 99 (83)                                   | 81 (80)                                   | 28 (88)                           |             |         |                  |
| WT/Δ32       | 19 (16)                                   | 18 (18)                                   | 4 (13)                            | 1.22        | 0.88    | 4                |
| Δ32/Δ32      | 2 (2)                                     | 2 (2)                                     | 0 (0)                             |             |         |                  |

**Table 5. Comparison of mean expansion rates by genotype**

| Genotype | Median expansion (cm/year) | Range (cm/year) | Sum of squares | Degrees of freedom | P value |
|----------|----------------------------|-----------------|----------------|--------------------|---------|
| WT/WT    | 2.426E-02                  | -0.88–4.56      | 3.441E-02      | 2                  | 0.811   |
| WT/Δ32   | 1.636E-02                  | -0.07–2.64      |                | 2                  |         |
| Δ32/Δ32  | 1.775E-02                  | 0–0.25          |                |                    |         |

**Table 6. Comparison of log mean expansion rates by genotype**

| Genotype | Median expansion (cm/year) | Range (cm/year) | Sum of squares | Degrees of freedom | P value |
|----------|----------------------------|-----------------|----------------|--------------------|---------|
| WT/WT    | -1.13                      | -1.99–0.66      | 0.285          | 2                  | 0.536   |
| WT/Δ32   | -1.06                      | -1.94–0.42      |                |                    |         |
| Δ32/Δ32  | -1.13                      | -1.84–0.6       |                |                    |         |

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*Figure 2.* Example of dot plot from optimization experiments. This dot plot shows on the left hand side, a subgroup of leucocytes (recognizable by their characteristic position in relation to the forward and side scatter scales), which are then isolated and displayed on the right hand plot. The T-lymphocytes are seen on the top half of the grid (identified by their characteristic fluorescence with phycoerythrin-labelled CD3) and those on the top right hand corner represent the CCR5-expressing cells (recognized by their fluorescence with fluorescein isothiocyanate-labelled CCR5).
statistic was found to be 10.21 with a significant P-value of 0.006. When this was subjected to the multiple-comparison test, two of the three comparisons remained significant (Table 8).

Although only two of the subgroup analyses remain significant, there is a clear trend seen towards greater CCR5 expression with increasing numbers of the wild-type allele (see Fig. 3) and in view of the small numbers involved in this arm of the project, this is strong evidence to support the theory that CCR5Δ32 is a biologically active polymorphism.

Discussion

This study investigated the role of the CCR5 Δ32 polymorphism in AAA. It provided convincing evidence that the polymorphism is biologically active and that the number of Δ32 alleles present was inversely proportional to the amount of functional CCR5 receptor expressed on T-lymphocytes. The incidence of the polymorphism did not differ significantly between the study and the control population, which suggests that it is not associated with the development of AAA. Subgroup analysis of small and large aneurysms and growth patterns has also failed to establish any link with the CCR5 Δ32 allele.

These data therefore cannot support the report by Ghilardi (2004) which suggested that the Δ32 polymorphism conferred a genetic predisposition to AAA formation. The polymorphism results in less functional CCR5 receptor and this in turn might be expected to be protective against AAA by downregulation of the inflammatory response, although this study has not found any significant protective effect of the polymorphism. If there were a real association, it could be expected to be reproducible.

Although our study was larger than previous studies (Ghilardi, 2004), the aneurysm and control populations in this study were not identically matched. The study group were older than the controls and also had a higher prevalence of comorbidities such as ischaemic heart disease, hypertension and hypercholesterolaemia. This group also included more women and more current smokers. Although fully matched populations would have been the ‘gold standard’, the large study group size does go some way to offset these discrepancies. As smoking and cardiovascular comorbidity are well known to be associated with AAA, a much longer recruitment process would have been required to eliminate these factors from

| Patient code | Genotype | % CCR5-positive cells |
|--------------|----------|-----------------------|
| BA16         | Δ32/Δ32  | 0.27                  |
| BA22         | WT/Δ32   | 5.25                  |
| BA48         | WT/Δ32   | 6.86                  |
| BA69         | WT/Δ32   | 8.84                  |
| BA78         | WT/Δ32   | 23.84                 |
| BA101        | WT/Δ32   | 12.13                 |
| BA111        | Δ32/Δ32  | 0.07                  |
| BA128        | WT/WT    | 8.53                  |
| BA145        | WT/WT    | 18.87                 |
| BA155        | WT/WT    | 17.03                 |
| BA164        | WT/Δ32   | 19.30                 |
| BA170        | WT/WT    | 45.82                 |
| BA171        | WT/WT    | 10.51                 |
| BA174        | WT/WT    | 10.92                 |
| BA228        | Δ32/Δ32  | 0.36                  |
| BC74         | Δ32/Δ32  | 1.01                  |
| BC78         | Δ32/Δ32  | 0.43                  |

Table 8. Multiple-comparison testing of CCR5 expression by genotype

| Genotype                  | Mean rank difference | P value |
|---------------------------|----------------------|---------|
| WT/WT vs WT/Δ32           | 1.33                 | > 0.05  |
| WT/WT vs Δ32/Δ32          | 9.17                 | < 0.01  |
| WT/Δ32 vs Δ32/Δ32         | 7.83                 | < 0.05  |

Figure 3. CCR5 expression by genotype. Where the lines represent the mean, boxes represent the interquartile range, and error bars give 1 standard deviation of the mean.
the study population, and this would not have been practical in the context of this study.

Although no significant association has been demonstrated between AAA and CCR5 Δ32, there is a large body of evidence to support both a genetic predisposition and an inflammatory process underlying AAA development. CCR5 is one of many chemokine receptors and any one of these could potentially be involved in the genetic component of AAA or modulated to influence aneurysm susceptibility. The inflammatory process is highly complex and it is unlikely that a single nucleotide polymorphism will hold the key to aneurysm susceptibility or prevention; however, further research in this area can only add to current understanding and it is only with this that potential drug management of small aneurysms will become a possibility.

CCR2 is structurally similar to CCR5, and experimental work has recently suggested that it may be directly involved in the pathogenesis of AAA (MacTaggart et al., 2007). In the absence of functional CCR5, it may be that CCR2 is upregulated and ligands can exert their effect via this route. There is a single nucleotide substitution polymorphism in the CCR2 gene (V64I) and it may be that this modulates the inflammatory process to a greater extent than the CCR5 Δ32 polymorphism. This polymorphism has not yet been investigated in humans with respect to AAA formation, but experimental work appears promising.

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