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Population-based study of acute- and long-term care costs after stroke in patients with AF

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Abstract

Background—New treatments for atrial fibrillation (AF) patients have been shown to be effective at reducing subsequent vascular event recurrence. However, there are few data on stroke costs in AF patients to allow the cost-effectiveness of these treatments to be assessed.

Aims—Using data from a population-based study, we assessed the acute and long-term costs of stroke in AF patients.

Methods—Healthcare costs one year before and 5-years after stroke were obtained from a large population-based study (Oxford Vascular study). Costs were assessed for the 3-months post-stroke (acute period), and annually thereafter (post-acute period). Annual post-acute costs were compared to annual baseline costs. Based on patients' living arrangements, costs of institutionalisation after the event were included.

Results—A total of 191 strokes occurred in 153 patients with known prior AF. Mean healthcare costs after stroke were £10,413 (S.D. 15,105) in the acute phase, with annual post-acute healthcare costs non-significantly smaller than those incurred before the event (£2,400 vs. £3,356, respectively; p=0.198). However, for the 136 strokes surviving past the 90-day acute-period, costs were non-significantly higher than those incurred in the year before the event (£3,370 vs. £2,566, respectively; p=0.333). After stroke, 25 (13%) patients were newly admitted into long-term

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warden, nursing or residential care, resulting in annual costs of £6,880 (S.D. 15,600) averaged across the 136 stroke cases surviving past the acute-period.

Conclusions—Although annual post-acute phase hospital and primary healthcare costs in stroke patients with prior AF were not significantly different to those incurred before the stroke, long-term nursing/residential care costs were substantial.

Introduction

Stroke was estimated to cost the UK health care system £5 billion in 2004 (1). Case-fatality is about 20% (2), with 50% of survivors being disabled one year after stroke (3). Outcome is particularly adverse in the 20% of patients who have stroke with a history of atrial fibrillation (AF) (4–6), in whom acute healthcare costs are increased (5, 6). Standard care for AF patients is treatment with vitamin K antagonists (VKA) such as warfarin (7, 8). However, results from recent randomised controlled trials of direct thrombin inhibitors and factor Xa inhibitors have found these new treatments to be as or more effective than warfarin at reducing thromboembolic events despite having a lower risk of intracranial bleeding and no greater risk of gastrointestinal bleeding than warfarin (9, 10). These new agents could therefore improve the effectiveness of stroke prevention in patients with AF, but cost-effectiveness must also be taken into account (11). Estimates of cost-effectiveness are often based on decision-analytic models that use estimates of the cost of outcomes prevented and of effectiveness from randomised controlled trials (12).

For cost-effectiveness results to be valid and reliable, model inputs, such as costs and outcomes, must be derived from appropriate sources using adequate methodologies. A recent systematic review of studies of the costs of stroke found relatively few studies in which costs of stroke in patients with AF had been assessed and no studies of costs beyond the acute stage (13). We aimed therefore to determine the acute and longer-term healthcare costs of stroke in patients with a history of AF, including the costs of long-term institutionalisation, using data from a population-based study (Oxford Vascular Study, OXVASC). It is generally considered that population-based studies with full case ascertainment are the most accurate sources of information on disease incidence, mortality, and outcome (14).

Methods

The methods of OXVASC have already been described (15). Briefly, the population comprises over 91,000 patients registered with 63 family doctors in 9 Oxfordshire general practices. Patients in whom stroke was suspected were ascertained and considered for study inclusion. Although study recruitment is ongoing, only stroke patients recruited in the first 5 years of the study (1 April 2002 to 31 March 2007) were included in the analysis in order to allow sufficient follow-up to estimate longer-term costs reliably.

Ascertainment was by prospective daily searches for acute events and retrospective searches of hospital and primary care administrative records (15). Suspected stroke patients were assessed urgently by a study clinician. Informed consent was sought, and assessments of neurological impairment, history and timing of presentation, medical and social history, and

risk factors were performed. The current study was confined to those patients in whom a diagnosis of AF (paroxysmal or permanent) had been made prior to the stroke, irrespective of any antithrombotic treatment. Using information on patients' age and history of stroke, transient ischaemic attack (TIA), hypertension, congestive heart failure and diabetes, CHADS₂ scores were calculated and used to estimate the risk of stroke (16). Surviving patients were followed-up at 1, 6, 12, 24 and 60 months after the event. At follow-up, amongst other outcomes, patients' living arrangements and handicap were assessed, with the latter being measured using the modified Rankin Score (mRS) (17).

Healthcare resource use

Patients were followed-up from the time of event until death or end of follow-up at 5 years. For patients not reaching the 5-year follow-up, follow-up finished on 30th January 2010. Our methods of identification of resource use in the acute phase after stroke have been reported previously [6]. In addition, patients' computerised hospital and primary care records were reviewed, with resources consumed within the 12 months prior to the event also being obtained. Hospital resource use evaluated included accident & emergency (A&E) visits, emergency transport, outpatient care visits, day cases and hospitalisations, including community hospitals. Primary care resource use included home, surgery and telephone consultations with nurses or general practitioners (GP).

Hospital records contained information on the date of outpatient visits, specialty and whether it was a new or follow-up visit. Visits for diagnostic investigations were also obtained. For each spell in hospital, the date of admission and discharge was recorded, including dates of transfers between wards, and their medical specialty. Hospitalisations were defined as spells in which the patient was admitted for at least one night. Length of stay was calculated as the number of days between admission and discharge. A day case was recorded when the date of discharge was the same as that of admission.

As part of the study, patients were asked about their living arrangements at follow-up and at initial ascertainment. Living arrangements were categorised as own home, relatives'/friends' home, warden housing and long-term residential or nursing home care. Resource use was priced using unit costs, with all costs being valued in 2008/09 UK pounds sterling (£). When unit costs were obtained from sources detailing costs before 2008/09, costs were updated using the Hospital and Community Health Services pay and price inflation index (18). Outpatient visits, outpatient investigations and A&E visits were priced using unit costs derived from NHS reference costs (19). For each hospital ward, the unit costs per day case/day in hospital were derived from NHS reference costs and trust financial returns (19, 20). Only for the cost of stroke unit care was the unit cost derived from published studies (6, 21). Unit costs of emergency transport and primary care visits were obtained from a UK compendium on the costs of health and social care, which also provided information on the costs of warden housing and long-term nursing home and residential home care.

Statistical analyses

Patients with AF may have multiple strokes (22). Consequently, assessing the mean cost per patient could potentially include the costs of more than one cerebrovascular event. To better

assess the individual cost of the stroke, the unit of analysis in this study was the number of events rather than the number of patients. Therefore, for those patients in the analysis suffering a subsequent stroke, follow-up for the initial event ended at the time of the subsequent event, with the subsequent event being followed-up as a new event. In addition, follow-up was also ended for those patients suffering a major coronary event, such as a myocardial infarction (MI). However, when strokes occurred in short succession of each other it was difficult to separate the costs between the initial event and the subsequent one. As a result, strokes occurring up to 90 days post-first stroke were indexed to the initial event.

Healthcare resource use and costs were estimated for the first 90 days after stroke (acute period) and then for the period past this acute period until end of follow-up (post-acute period). Costs were stratified in this way as published evidence shows that average healthcare costs after stroke are highest in the months immediately after the event, with mean healthcare costs quickly tailing off (23, 24). However, stroke onset is associated with old age and generally occurs in patients with other comorbidities (15). Such patients are therefore likely to consume substantial healthcare resources even if they had not suffered the event. Healthcare resource use and costs incurred during the 12 months before the stroke (baseline period) were therefore compared with the costs incurred in the post-acute period after the stroke for all those cases surviving past the acute-period. For patients suffering subsequent events, the baseline period used was the 12 months before the initial index stroke. No comparisons were made between baseline and acute period resource use and costs, as evidence suggests that, shortly after the event, the majority of costs incurred can be directly attributable to the stroke (6).

For all periods (i.e. before the event, acute and post-acute), emergency and outpatient care visits, day cases and hospitalisations were treated as count variables, and reported as number of visits/hospitalisations per patient per follow-up year, and reported alongside their standard error (S.E.). Rate differences between the baseline and post-acute periods were estimated and reported alongside 95% confidence intervals (CI), with statistical differences evaluated assuming a Poisson distribution (25). Total days in hospital and costs for the baseline, acute and post-acute period were also assessed. Days in hospital and costs were reported as means together with their standard deviation (S.D.). To compare the days in hospital and costs during the year before the event with the days in hospital and costs incurred during the post-acute period, the latter were annualised by dividing patients' follow-up days in hospital and costs over the number of years of patient follow-up. Mean differences between the two time-periods were reported alongside 95% CI, and statistical differences in mean estimates were evaluated using a Student's two-sided paired t-test. Statistical significance was set at $p < 0.050$.

The costs of long-term accommodation in warden housing and nursing or residential care were also estimated. For patients already in long-term care at the time of the stroke, costs of long-term care were not included, as these were considered to be unrelated to it. For patients moving into long-term care between follow-ups, date of institutionalisation was assumed as the mid-point between the two follow-ups, or the date of discharge from hospital if patients were still in hospital at that time. Days in warden housing or nursing/residential care home were estimated as the difference between the dates of follow-up and institutionalisation,

which were then multiplied by the respective unit cost. Days in long-term care and long-term care costs were annualised using the same methods as for post-acute healthcare costs.

Mean total costs were also reported by event severity. Severity was defined as handicap at 6 months, with patients reporting mRS scores lower than 3 being classed as non-disabled, scores of 3 or 4 classed as moderately disabled, and those reporting scores of 5 classed as totally dependent. Patients dying within 30 days of the event were classed as case-fatalities. For those patients dying after 30 days but before 6 months, stroke severity was defined by mRS at one month.

Mean total costs were also reported by the estimated risk of suffering a stroke, as defined by the CHADS₂ score. Risk of stroke was defined as low for CHADS₂ scores of 0, moderate for scores of 1 and 2, and high for scores between 3 and 6 (16).

Results

Patient sample

Between April 2002 and March 2007, 729 patients suffered a stroke as their index event in OXVASC. Of these, 153 (21%) patients had a history of AF and form the basis for this analysis. Patients already had significant comorbidity prior to their index event, with 32 (21%) having a history of MI, 96 (63%) having hypertension and 30 (21%) being disabled (Table 1).

The 153 patients included in the analysis had a total of 191 strokes during the study period. Of these, 162 (85%) were ischaemic, 17 (9%) were haemorrhagic strokes and 12 (6%) were of unknown type. Due to high mortality rates during the first 90 days after haemorrhagic and unknown strokes, for the analyses of post-acute costs, all strokes were combined together. Mean follow-up for each event was 731 (S.D. 725) days. For the 191 events, end of follow-up occurred due to death in 101 (53%), a subsequent stroke or MI in 41 (21%), and reaching 5-year follow-up in 24 (13%), with a further 25 (13%) not reaching 5-year follow-up.

Of the 191 strokes, 40 (21%) were fatal within 30 days, 55 by 90 days (acute period) and 65 by 6 months (Table 2). Of the 25 strokes dying between 90 days and 6 months, 2 (8%) were classed as non-disabling strokes based on 1-month mRS, 15 (60%) were classed as moderately-disabling, and 8 (32%) as totally-disabling. For the 126 strokes not resulting in death at 6 months, 64 (51%) events were non-disabling based on 6-month mRS, 52 (41%) were classed as moderately disabling, and 10 (8%) as totally-disabling. Event severity by stroke subtype is reported in Table 2.

Healthcare resource use and costs

Resource use rates by healthcare category, including days in hospital, are reported in Supplemental-table 1. For the 191 strokes included in the analysis, the mean hospitalisation rate during the acute period was 1.30 per patient per 90-day follow-up, with mean days in hospital for the first 90 days after stroke being 72 (S.D. 34). Post-actively, for the 136 strokes surviving the post-acute phase, the average hospitalisation rate was 0.41 per patient per follow-up year, compared with 0.48 before the stroke (mean difference -0.06, 95% CI: -0.07

to -0.05). Although patients spent a higher number of days in hospital post-acutely than in the year before the event (7 vs. 5 days), this difference was not statistically significant (mean difference: 2 days; 95% CI: -3 to 6; $p=0.529$).

For the 191 strokes included in the analysis, the mean total cost incurred during the 3-month acute phase was £10,413 (S.D. 15,105) (Table 3). The majority of these costs were due to hospitalisations (£9,841), accounting for 95% of all healthcare costs. Costs after stroke rose sharply with event severity, with mean costs per non-disabling stroke of £3,945 (S.D. 7,558) compared with £17,406 (S.D. 18,417) and £25,279 (S.D. 16,397) for moderately- and totally-disabling strokes respectively. The mean costs for case-fatal strokes were lower at £2,681 (S.D. 2,661), due to the short interval between event and death (mean of 9 days, S.D. 9). Table 3 also presents acute healthcare costs stratified by stroke subtype.

At baseline, mean annual costs were £1,753 (S.D. 2,946), £3,339 (S.D. 8,603) and £3,304 (S.D. 9,474) before the non-, moderately-, and totally-disabling stroke, respectively. A total of 55 stroke patients did not survive past the acute phase. In the year before the stroke these patients incurred higher annual average costs than those surviving past the acute phase (£5,308 vs. £2,566), although this difference did not reach statistical significance ($p=0.072$).

For the 136 strokes surviving past the acute-period, regardless of event severity, annual post-acute costs were non-significantly higher than those incurred in the year before the event (Table 4). Only for GP and emergency care was there a significant difference in costs between the two periods. Although more severe strokes incurred higher costs than less severe ones post-acutely (non-disabling £2,135 (S.D. 3,675) per follow-up year, moderately disabling £4,165 (S.D. 7,668), and totally disabling £6,324 (S.D. 14,898) more severe stroke patients were also incurring higher costs at baseline, mainly due to higher levels of baseline disability and co-morbidities.

Due to 55 patients dying during the first 90 days past the stroke (i.e. acute phase), annual average post-acute costs were £2,400 (S.D. 6,223) if estimated over the 191 strokes included in the analysis. This was non-significantly lower than the average annual costs, £3,356 (S.D. 7,952), incurred before the event by the same patients (mean difference: -£956; 95% CI: -£2,393 to £480; $p=0.198$).

Long-term nursing and residential care

Of the 191 strokes included in the analysis, 11 (6%) occurred whilst the patient was already living in long-term care accommodation (3 warden housing and 8 nursing home care). Of the 136 stroke cases surviving past the acute-period, 25 (18%) strokes resulted in new long-term care institutionalisation (2 in warden housing, 7 in residential care and 16 in nursing home care). Long-term care institutionalisation varied considerably by event severity. Of the 66 non-disabling strokes, 4 (6%) were institutionalised, compared with 15 out of the 58 (26%) moderately disabling strokes, and 50% for totally-disabling strokes (6 out of the 12 totally disabling strokes). On average, newly institutionalised stroke patients spent 365 (S.D. n/a), 313 (S.D. 66) and 267 (S.D. 132) days in warden housing, nursing home care and residential care respectively, per follow-up year after the stroke.

Averaged across all stroke cases surviving the acute-period (n=136) new institutionalisation after stroke resulted in an average annual stay in long-term care of 56 (S.D. 124) days. This resulted in average annual costs of £6,880 (S.D. 15,600) post-stroke. As with healthcare costs, costs of long-term care increased considerably with event severity. For the 66 non-disabling strokes, average costs of long-term care were £942 (S.D. 3,765) for each follow-up year after stroke, compared with £10,646 (S.D. 19,068) for moderately disabling strokes (n=58), and £21,335 (S.D. 22,463) for totally-disabling strokes (n=12).

Health and social care costs were not closely related to pre-morbid CHADS₂ score (Supplemental-table 2).

Discussion

Previous studies have shown that average costs of stroke in patients with AF are higher than those without the condition. In a hospital-based study, Diring et al. (1999) found that the main predictors of costs included neurological impairment, heparin treatment, presence of AF, female sex, and presence of ischemic heart disease (5). Luengo-Fernandez et al. (2006) found that the acute care costs of stroke were significantly higher in patients with AF, but the association disappeared after controlling for event severity (6). Both these studies, however, only assessed costs during the acute-care phase and did not estimate long-term costs.

Our study aimed to assess acute and long-term healthcare costs after stroke in patients with AF. In addition, the costs of long-term care institutionalisation were also considered. Our results show that, in patients with AF, average acute-care costs of stroke were £10,413, with costs increasing considerably with event severity. After the acute-phase, stroke patients incurred annual healthcare costs of £3,370, with these costs also varying considerably by event severity.

Since most AF-related stroke occurs in relatively elderly patients, many of whom suffer from previous co-morbidities, it is likely that substantial healthcare resources would be used irrespective of stroke onset. Therefore, in an attempt to distinguish between costs incurred as a direct result of the stroke and those due to other co-morbidities, annual post-acute healthcare costs were compared to those incurred during the year before the stroke. We found that, overall, annual post-acute healthcare costs were non-significantly higher than those incurred before the event, with only GP and emergency care costs being significantly higher than those at baseline. There was some evidence that those stroke patients not surviving past the acute phase incurred higher costs than those who survived past this period. This would therefore suggest that patients not surviving their stroke tended to be in poorer health than survivors, hence the increased costs incurred before stroke onset.

Healthcare services are not, however, the only services that stroke patients utilise. Long-term care institutionalisation forms a considerable proportion of costs incurred after stroke (13). After controlling for patients already living in warden, nursing or residential care homes, we found that over 10% of patients surviving past the acute stroke phase were newly admitted into long-term care, resulting in annual costs of £6,880 after stroke averaged across all patients. This increase in cost also reflects the fact that majority of the AF-related ischaemic

strokes in our study (158/175) were cardioembolic in aetiology (Trial of Org 10172 in Acute Stroke Treatment – TOAST – classification) which are known to be more severe and incur higher mean costs of acute and long term care (26).

The limitations of this study should be highlighted. Firstly, not all strokes included in the study were followed-up until death or 5 years post-stroke. A total of 66 (35%) cases were censored, the majority of which was due to follow-up being stopped when the patient suffered either a subsequent stroke or MI (n=41, 62%). Secondly, not all health and social care costs relevant to stroke care were included in the analysis. As with many studies assessing the long-term costs after stroke (13), potentially considerable costs, such as long-term support of patients living in their own houses (e.g. paid carers and home adaptations) were not included in the analysis. In our analysis, 50% of totally-disabling strokes and 74% of moderately-disabling strokes were discharged from hospital into the community. As a result, a sizeable proportion of these patients would require paid social care. Due to the difficulties in assessing the amount and levels of paid care required by these patients, we were unable to obtain this information. In addition, healthcare costs such as those of medications or private hospital care were also omitted from the analysis. Thirdly, due to the small numbers of haemorrhagic strokes it was difficult to make any meaningful statistical comparisons between stroke subtypes. This was particularly the case when estimating long-term care costs as the sample size was further diminished due to case-fatality. Fourthly, our study had a low rate of premorbid anticoagulation use in patients with prior AF. The majority (61%) of our patients who were not on warfarin did not have a clearly documented reason in their medical notes. We were unable therefore to reliably estimate the proportion of costs related to stroke occurring in patients with AF that was unavoidable. However, the low rate of pre-morbid anticoagulant use in our study is consistent with several other recently published population-based studies (27–29), suggesting that our findings are generalisable. Finally, our study only considered the health and social care costs associated with stroke. No attempt was made to assess indirect costs, such as those associated with relatives or friends providing unpaid care to stroke patients, which have shown to place a considerable economic burden on society (1).

We believe that our results will be helpful to analysts requiring reliable stroke costs as part of their analyses to estimate the cost-effectiveness of new drugs to prevent stroke in patients with AF. Using a population-based study, our results highlight the considerable costs incurred by stroke patients with AF shortly after stroke. Although annual post-acute phase hospital and primary healthcare costs in stroke patients with prior AF were not significantly different to those incurred before the stroke, long-term nursing/residential care costs were substantial.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Table 1

Baseline characteristics of study sample

	Study sample (n=153)
Age (years), mean (S.D)	80 (10)
Males	81 (53)
Previous disability *	30 (21)
Previous MI	32 (21)
Previous angina	36 (24)
Hypertension	96 (63)
Previous stroke	40 (26)
Diabetes	8 (5)
Pre-morbid warfarin use	30 (20)
Smoking status †:	
Never	71 (47)
Ex-smoker	73 (48)
Current	8 (5)
CHADS₂ score, median (IQR) ‡	3 (2 to 4)
0 – Low risk	8 (4)
1 to 2 – Moderate risk	67 (36)
3 to 6 – High risk	111 (60)

All data expressed as n (%), except where specified

* 12 missing

† 1 missing

‡ 5 missing

Table 2

Event severity by stroke subtype

	All strokes (n=191)	Ischaemic (n=162)	Haemorrhagic (n=17)	Unknown (n=12)
Non-disabling	66 (35%)	59 (36%)	5 (29%)	2 (17%)
<i>Dying within 90 days*</i>	0	0	0	0
<i>Dying within six months*</i>	2 (3%)	2 (3%)	0	0
Moderately disabling	67 (35%)	60 (37%)	3 (18%)	4 (33%)
<i>Dying within 90 days*</i>	9 (13%)	8 (13%)	0	1 (25%)
<i>Dying within six months*</i>	15 (22%)	14 (23%)	0	1 (25%)
Totally-disabling	18 (9%)	17 (10%)	1 (6%)	0
<i>Dying within 90 days*</i>	6 (33%)	6 (35%)	0	0
<i>Dying within six months*</i>	8 (44%)	8 (47%)	0	0
Case-fatal	40 (21%)	26 (16%)	8 (47%)	6 (50%)

* As a percentage of non-disabling, moderately disabling or totally disabling strokes

Table 3

Mean acute healthcare costs by severity and subtype for 191 strokes

	All strokes Mean £ (S.D.)	Non-disabling Mean £ (S.D.)	Moderately-disabling Mean £ (S.D.)	Totally-disabling Mean £ (S.D.)	Case-fatal Mean £ (S.D.)
Nurse visits	£22 (44)	£40 (52)	£19 (36)	£2 (6)	£8 (41)
GP visits	£100 (109)	£115 (65)	£133 (146)	£48 (62)	£43 (83)
Emergency care	£211 (198)	£142 (176)	£242 (218)	£272 (173)	£244 (182)
Outpatient visits	£191 (213)	£338 (223)	£185 (182)	£93 (101)	£2 (15)
Day cases	£48 (£216)	£76 (290)	£38 (168)	£93 (287)	0
Hospitalisations	£9,841 (15,073)	£3,234 (7,498)	£16,791 (18,421)	£24,771 (16,238)	£2,382 (2,681)
Total costs	£10,413 (15,105)	£3,945 (7,558)	£17,406 (18,417)	£25,279 (16,396)	£2,680 (2,661)
<i>Ischaemic stroke</i>	<i>£10,844 (15,733)</i>	<i>£3,401 (7,659)</i>	<i>£17,743 (18,986)</i>	<i>£24,234 (16,272)</i>	<i>£3,059 (2,808)</i>
<i>Haemorrhage</i>	<i>£10,683 (12,885)</i>	<i>£9,903 (4,510)</i>	<i>£25,442 (9,635)</i>	<i>£43,036 (n/a)</i>	<i>£1,592 (1,886)</i>
<i>Unknown stroke</i>	<i>£4,206 (5,650)</i>	<i>£5,117 (5,639)</i>	<i>£6,326 (8,972)</i>	<i>n/a</i>	<i>£2,489 (2,848)</i>

Table 4

Annual post-acute and baseline healthcare costs for 136 strokes surviving past the acute period

	Baseline Mean £ (S.D.)	Post-acute Mean £ (S.D.)	Difference Mean £ (95% CI)
Nurse visits	£90 (196)	£110 (190)	£19 (-28 to 66)
GP visits	£231 (198)	£330 (401)	£98 (27 to 169) *
Emergency care	£35 (73)	£133 (258)	£99 (56 to 141) †
Outpatient visits	£256 (446)	£264 (356)	£8 (-71 to 88)
Day cases	£187 (532)	£229 (1,144)	£42 (-158 to 242)
Hospitalisations	£1,767 (6,193)	£2,305 (6,512)	£537 (-972 to 2,047)
Total costs	£2,566 (6,586)	£3,370 (7,156)	£804 (-832 to 2,440)
<i>Non-disabling (n=66)</i>	<i>£1,753 (2,946)</i>	<i>£2,135 (3,675)</i>	<i>£382 (-781 to 1,546)</i>
<i>Moderately-disabling (n=58)</i>	<i>£3,339 (8,603)</i>	<i>£4,165 (7,668)</i>	<i>£825 (-2,133 to 3,783)</i>
<i>Totally-disabling (n=12)</i>	<i>£3,304 (9,474)</i>	<i>£6,324 (14,898)</i>	<i>£3,019 (-8,841 to 14,880)</i>

* Significant at p<0.050

† Significant at p<0.001