Characterisation of ischaemic stroke
in diet-induced obese rats

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List of abbreviations

ACA - anterior cerebral artery
ADC - apparent diffusion co-efficient
ANOVA - analysis of variance
ASPA - animals scientific procedure act
ATP - adenosine triphosphate
BA - basilar artery
BMI - body mass index
CBF - cerebral blood flow
CCA - common carotid artery
CRP - C-reactive protein
CT - computer tomography
DIO - diet-induced obesity
DIO-R - diet-induced obesity resistant
DWI - diffusion weighted imaging
ECA - external carotid artery
EPI - echo planar imaging
FA - flip angle
FOV - field of view
H&E - haematoxylin and eosin
HFD - high fat diet
ICA - internal carotid artery
IL - interleukin
MCA - middle cerebral artery
MCAo - middle cerebral artery occlusion
MCP-1 - monocyte chemotactic protein-1
MRI - magnetic resonance imaging
OPA - occipital artery
PB - phosphate buffer
PBS - phosphate buffered saline
PCA - posterior cerebral artery
PFA - paraformaldehyde
PPA - pterygopalatine artery
PWI - perfusion weighted imaging
ROS - reactive oxygen species
ST - slice thickness
STA - superficial temporal artery
TIA - transient ischaemic attack
TNFα - tumour necrosis factor-α
tPA - tissue plasminogen activator
TE - echo time
TR - time to repeat
Abstract

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Stroke is a leading cause of death and disability. Obesity predisposes the risk of ischaemic stroke and is associated with poorer stroke prognosis. The mechanisms by which obesity increases ischaemic damage are not well understood. However, co-morbidities that share common features with obesity have been shown to exacerbate stroke damage due to deficits in cerebral blood flow (CBF) after stroke. This suggests a similar mechanism may exist in obesity.

The long term aim of work stemming from this project will be to investigate whether obesity exacerbates ischaemic stroke damage due to deficits in cerebral perfusion. Therefore, the current MPhil project aims to establish and characterise a rat model of diet-induced obesity (DIO) and assess the effect of the diet model on ischaemic stroke.

Male Wistar rats were fed either a control or high fat diet (HFD) for 12 weeks then stroke was induced by 60 min middle cerebral artery occlusion (MCAo) followed by 4, 24 or 48 h reperfusion. Lesion volumes were analysed histologically or by diffusion weighted magnetic resonance imaging.

HFD rats were divided into diet induced obese (DIO) and DIO-resistant (DIO-R) groups dependent on their weight gain relative to controls. Defined this way, DIO rats had greater body mass and body fat content than controls. Unexpectedly, stroke surgery failed to produce lesions in many animals. The poor stroke surgery success rate resulted in very low animal numbers meaning definitive conclusions regarding the effect of obesity on stroke could not be drawn. However, preliminary data from one cohort of rats suggests that DIO rats may experience poorer outcome from ischaemic stroke based on lesion volume and behavioural scores.

We conclude that the animal models developed in the current study require further work to enable future studies into the effect of obesity on CBF following stroke. Understanding the relationship between obesity and enhanced ischaemic damage remains an important avenue of research which will aid future development of treatments for this subtype of stroke patients.
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1. Introduction

1.1.1 Stroke: A major health issue

Stroke is a leading cause of death and disability; A fifth of strokes are fatal and over half of surviving patients report loss of independence due to ongoing disability (Stroke Association, 2013). It is estimated that stroke affects 152,000 people per year in the UK and is accountable for 7-10% of all deaths, making it the fourth biggest killer (Townsend et al., 2012). Three month mortality is 17%, with a quarter of survivors living in a care facility at this time point (Jeng et al., 2008).

With over a million stroke survivors in the UK (Townsend et al., 2012), consideration of disability in regards to quality of life is important. Additionally, disability from stroke holds a heavy socioeconomic burden on the country as a quarter of stroke patients are under retirement age and long term care is necessary for many survivors. Less than half of surviving patients make a complete recovery, the remainder are left with disabilities which vary in nature, severity and duration. For example, over a third of survivors will have lasting arm paralysis, a third suffer from aphasia and a fifth develop dementia within six months (Stroke Association, 2013). One in ten previously independent patients will be discharged to a residential care facility following stroke (Stroke Association, 2013). Even patients who recover successfully back to independent living may be affected more subtly, for example by changes in personality or mood which can affect quality of life long term (Kim et al., 2005).

1.1.2 Subtypes of stroke

There are two main subtypes of stroke: haemorrhagic and ischaemic. Haemorrhagic stroke arises from the spontaneous rupture of a cerebral vessel while ischaemic stroke occurs when a blood clot becomes lodged within a cerebral vessel preventing adequate blood flow to a region of the brain. In the latter form, persistent hypoxia and glucose starvation of the neural tissue (ischaemia), due to cerebral blood flow (CBF) restriction, results in cell death and tissue infarction (Stroke Association, 2013).
1.1.3 *Ischaemic stroke*

Ischaemic stroke is the predominant form of the disease, accounting for 85% of incidents. The location of clot formation is used to further categorise stroke type. Thrombotic strokes, accounting for 35% of all strokes, are due to the formation of a clot within a large vessel of the brain, commonly where there is already partial occlusion of the vessel by an atheroma in patients with atherosclerosis (fig. 1). Embolic strokes, comprising 30% of strokes, arise from clots formed elsewhere in the body, commonly in the heart, which travel to the brain to cause obstruction of cerebral blood flow (fig. 1). The third category of ischaemic stroke, lacunar stroke, causes a fifth of strokes and arises from the blockage of small cerebral arteries supplying the deep nuclei of the brain such as the thalamus (Zorowitz et al., 2004).

*Figure 1: Ischaemic stroke.* Coronal view of human brain depicting ischaemic stroke caused by either an embolic or thrombotic occlusion of the middle cerebral artery (MCA). Also detailed are the cerebral arteries commonly involved in ischaemic stroke. Figure adapted from NIH (2011) and The Internet Stroke Centre (2013).
In ischaemic stroke, obstruction usually occurs in vessels extending from the Circle of Willis including the middle cerebral artery (MCA), internal carotid artery (ICA), basilar artery (BA) and the anterior and posterior cerebral arteries (ACA and PCA; fig. 1). In particular, the MCA is the vessel most commonly affected by ischaemic stroke (Balaban et al., 2011). Damage to the brain territory supplied by the occluded vessel determines the nature of the initial symptoms and on-going disability. For example, the MCA supplies the motor areas of the brain thus initial contralateral limb paralysis and long-term ataxia are associated with MCA occlusion (Zorowitz et al., 2004). Occlusion of the proximal cerebral arteries, which includes the MCA, ICA and BA, tends to result in major strokes which incur a higher risk of death, disability and longer hospitalisation in comparison to more minor stroke (Cipriano et al., 2009).

1.1.4 Transient ischaemic attack

In addition to the incidence of stroke in the UK, almost 50,000 people experience a primary transient ischaemic attack (TIA) each year (Stroke Association, 2013). A TIA, or mini-stroke, is defined as a temporary disruption to neurological processes due to cerebral ischemia which does not lead to infarction (Easton et al., 2009). TIAs are highly predictive of stroke, especially thrombotic stroke (Zorowitz et al., 2004) with 5% of cases progressing to a full stroke within seven days (Stroke Association, 2013), thus timely medical intervention is vital in TIA patients. Treatment of TIA often includes anti-platelet therapy (aspirin or clopidogrel) to minimise clot formation potential. Patient-specific regimes may also be implemented to combat underlying stroke risk factors, for example anti-hypertensive therapy (perindropril) for those with high blood pressure or statins for patients with high cholesterol levels (Rothwell et al., 2007).

1.1.5 Diagnosis and treatment of ischaemic stroke

Stroke patients often present with weakness in one side of their body, issues with producing or understanding speech and various other neurological deficits (Zorowitz et al., 2004). As many of the presenting symptoms are common between stroke subtypes, brain scans are required for an accurate diagnosis. Computer tomography (CT) or magnetic resonance imaging (MRI) is used as standard to confirm the diagnosis and distinguish the type of stroke. CT or MRI angiogram is used to locate the occlusion site, while unenhanced CT or diffusion weighted MRI is
sensitive to infarcted tissue. Haemorrhage can be identified by susceptibility-weighted MRI and areas of compromised blood flow detected by perfusion weighted scans (Kurz et al., 2013).

Currently the only licensed treatment for ischaemic stroke is thrombolysis by intravenous injection of tissue plasminogen activator (tPA; alteplase). This treatment aims to reopen the occluded vessel and restore circulation to the ischaemic area in an effort to prevent the progression of compromised tissue to infarction (Kurz et al., 2013). However, it is estimated that only 2-5% of ischaemic stroke patients currently receive tPA, as the therapeutic time window from time of symptom onset (4.5 h) is very short (Kim et al., 2014).

Identifying and developing potential new therapies for ischaemic stroke is an expanding field. Current efforts focus on means of clot removal with new anti-thrombotic drugs (Kraft et al., 2012) or with endovascular devices (Singh et al., 2013), minimising neuronal damage with anti-inflammatory drugs (Otwell et al., 2010) and promoting neural regeneration after stroke damage with the use of stem cells (Banerjee et al., 2012).

The use of new generation mechanical thrombectomy devices is particularly promising. A recent systematic review across five clinical studies found that mechanical thrombectomy had a more beneficial effect than tPA treatment alone on three month functional outcome, although a greater improvement in mortality rates was not seen (Lambrinos et al., 2016).

1.2 Pathophysiology of stroke

1.2.1 The initial insult in ischaemic stroke

In ischaemic stroke, arterial occlusion reduces CBF in the territory of the occluded artery. In the ischaemic injury “core”, CBF falls below a critical limit at which it is incapable of supplying the neural tissue with adequate oxygen and glucose (ischaemia) to maintain electrical or metabolic function (del Zoppo et al., 2011). Within minutes the core succumbs to necrosis and becomes irreparably infarcted. For cells in the core, metabolic arrest quickly results in membrane depolarisation due to depletion of the adenosine triphosphate (ATP) reserves required to maintain membrane polarisation via ion-pumps. Neuronal depolarisation causes the release
of glutamate which builds up to toxic levels in the extracellular space due to arrested function of neurotransmitter scavenging systems. So called “excitotoxicity” ensues as over-activation of glutamate receptors causes mass calcium influx in adjacent neurons which in turn release glutamate, and thus excitotoxicity spreads from the core into the surrounding tissue. High intracellular calcium initiates various intracellular signalling cascades associated with cell death, including the synthesis of reactive oxygen species (ROS), which contribute to mitochondrial failure, and expression of proteolytic enzymes, which initiate degradation of the cellular structures (Dirnagl et al., 1999).

1.2.2 The penumbra, hypoperfusion and ischaemic stroke

The tissue bordering the infarct core, which is at risk of becoming infarcted, is known as the penumbra. Here, the CBF rate is between the critical metabolic threshold (10 mL/100 g/min) and the critical electrical threshold (22 mL/100 g/min; del Zoppo et al. 2011) meaning electrical activity is arrested but adenosine ATP stores and membrane polarisation are not affected (Fisher, 2006). Tissue in the penumbra is at risk of damage over the coming minutes to hours (Dirnagl et al., 1999). However, this tissue could potentially be rescued if adequate CBF were reintroduced and damage mechanisms halted in a timely manner (Fisher, 2006).

It should be noted that in comparison to normal CBF, which is 50 mL/100 g/min (Palomares and Cipolla, 2011), the margin between the two critical thresholds is narrow. Therefore, factors affecting blood flow are critical in determining whether penumbral perfusion will drop below the critical metabolic threshold. Additionally, these factors may contribute to poor perfusion following re-canalisation, termed the no-reflow phenomenon (del Zoppo et al., 1991).

There are several aspects of stroke pathology which can affect CBF and therefore could play a critical role in determining the fate of penumbral tissue. These include, the change in expression of vasoactive mediators, such as endothelin-1 (Itoh and Suzuki, 2012; Lo et al., 2005), the narrowing of vessels due to adhesion of immune cells to the vessel wall (del Zoppo et al., 1991) and the compression of vessels by astrocyte end-feet swelling and local oedema (Lo et al., 2005).
1.3 Obesity and ischaemic stroke

1.3.1 Risk factors for stroke

Age, ethnicity and gender are all non-modifiable characteristics which affect the likelihood of stroke (Appelros et al., 2009). Age is strongly correlated with increased risk of stroke occurrence and with poor prognosis in terms of both morbidity and mortality (Ankolekar et al., 2012). Black people and people of South Asian origins are at greater risk of stroke compared to those from Middle Eastern or white European backgrounds (Ankolekar et al., 2012).

Risk factors classified as modifiable, indicate that early prophylactic intervention could reduce the incidence of stroke. Hypertension is the primary risk factor for stroke and contributes to the development of half of all cases (Lawes et al., 2008). Hypertension, defined as blood pressure >140/90 mmHg, affects nearly a third of the UK population, of which only a quarter are effectively managing their blood pressure with medical intervention (Townsend et al., 2012). Atrial fibrillation is the leading cause of embolic stroke (Zorowitz et al., 2004) and is causal in 12,500 strokes annually in the UK (Stroke Association, 2013). Treatment with anticoagulants such as warfarin greatly reduces the risk of atrial fibrillation causing a stroke. However, a recent study of nearly 40,000 arterial fibrillation patients reported less than half were receiving anti-thrombotic treatment (Björck et al., 2013).

Life style risk factors include smoking of cigarettes, which is the causal factor for one in ten fatal strokes, excessive alcohol consumption, which triples the risk of stroke, and lack of physical exercise, which carries a relative risk of 1.5 for stroke (Stroke Association, 2013).

Obesity is a key risk factor for stroke (Strazzullo et al., 2010), along with many other factors relating to diet and metabolism, including diabetes (Stroke Association, 2013) and high cholesterol (Townsend et al., 2012), as discussed below.

1.3.2 Obesity

Obesity is defined as an excess accumulation of adiposity (fat), usually accredited to over-nutrition and sedentary lifestyle. Healthy and unhealthy weights are categorised based on the body mass index (BMI) which is calculated by the division of weight (kg) by height squared (m²). A BMI of 18.5-24.9 kg/m² is considered
healthy while individuals with BMIs exceeding 25 kg/m$^2$ and 30 kg/m$^2$ are classed as overweight and obese, respectively (World Health Organization 2003). The average adult BMI in the UK was estimated at 27 kg/m$^2$ in 2008, indicating the UK as an overweight nation. In England alone, approximately 37% of the population are overweight while 26% are obese. Excess weight in childhood is also prevalent in England, with approximately 27% of children aged 5-17 years having a BMI greater than 25 kg/m$^2$ (Nichols et al., 2012). The BMI method has been criticised for being too simplified as it cannot account for the distribution of fat which is important in terms of health risk. Indeed, central adiposity is much more associated with ill health than even fat distribution. Thus waist to hip ratios can also be used to classify obesity. Waist to hip ratios greater than 0.85 and 0.9 are considered unhealthy in women and men, respectively (Alberti and Zimmet, 1998).

1.3.4 Metabolic Syndrome

Obesity can contribute to a state of disease known as metabolic syndrome. Individuals with metabolic syndrome typically have diabetes mellitus, or are indicated as pre-diabetic due to insensitivity to insulin or impaired glucose tolerance. Additionally, patients may also be obese, hypertensive or test positive for excess blood levels of lipids (hyperlipidemia) or high levels of albumin in the urine (Alberti and Zimmet, 1998). As of 2010 in England, over one in twenty adults were diagnosed with diabetes and nearly 60% have high cholesterol (Townsend et al., 2012). These statistics, combined with previously mentioned high levels of obesity and hypertension, reflect the high numbers of people affected by metabolic syndrome in the UK.

1.3.5 Effect of obesity on stroke risk and prognosis

Being overweight or obese is linked with a number of disease states, including an increased risk of cardiovascular disease, diabetes and cancer (World Health Organization 2003) and is associated with higher rates of total mortality (Engeland et al., 2003). In the UK, the NHS spends approximately £3 billion on health issues related to obesity annually and nearly 200,000 deaths each year are due to obesity-related diseases (Allender and Rayner, 2007).

In the case of ischaemic stroke, being overweight or obese increases the risk of occurrence to 1.22 and 1.64, respectively (Strazzullo et al., 2010). A BMI exceeding
27 kg/m\(^2\) also increases the risk of fatality from stroke to 1.30 (Shinton et al., 1991). Other aspects of metabolic syndrome are also associated with higher stroke risk. For example, having diabetes doubles the chance of stroke and having high cholesterol carries an increased risk of stroke (Stroke Association, 2013).

However, not all studies are in agreement concerning the relationship between BMI and ischaemic stroke risk. A three year study of six thousand people did not find high BMI to be predictive of stroke. Instead the distribution of adiposity was found to be more predictive, with larger waist to hip ratios correlating with stroke incidence (O'Donnell et al., 2010). Other studies have echoed this relationship though only in males (Hu et al., 2007; Tanne et al., 2005).

While obesity, whether defined by BMI or hip to waist ratio, relates to an increased risk of stroke, the relationship to post-stroke survival is more controversial. Poorer rehabilitation has been reported in individuals with very high BMIs (Kalichman et al., 2007), for example obese patients tend to be kept in hospital longer after stroke and are more often discharged to a nursing facility than patients of a healthy weight (Razinia et al., 2007). Conversely other studies have reported that stroke patients with higher BMIs have favourable odds in terms of long-term survival (Kim et al., 2012; Olsen et al., 2008; Vemmos et al., 2011) and incidence of a second stroke (Ovbiagele et al., 2011). It has been suggested that this apparent beneficial effect of higher BMIs on stroke outcome is more prominent in the elderly. Indeed, a previous study of stroke mortality reports a disparity between age groups for BMI prediction of stroke outcome. In persons aged 40-54, a BMI over 25.4 increased risk of stroke mortality, however in persons aged 55-64 who were moderately overweight (BMI 25.4-27 kg/m\(^2\)) saw the inverse relationship (Shinton et al., 1991).

Pre-clinical studies largely indicate a role for obesity in stroke exacerbation, with more severe ischaemic strokes seen in obese animals compared to controls, evidenced by larger lesions and poorer functional recovery (Deutsch et al., 2009; Drake et al., 2011; Langdon et al., 2011; Maysami et al., 2015; McColl et al., 2010).

An additional factor to consider in the relationship between weight and ischaemic stroke outcome is the viability of treatment. Several studies report that patients with obesity and other aspects of metabolic syndrome are resistant to tPA (Arenillas et al., 2009; Calleja et al., 2011; Deguchi et al., 2012; Lou and Selim, 2009). As well as poor efficacy for thrombolysis, a greater occurrence of haemorrhagic transformation
in response to tPA administration has been noted in patients exceeding 100 Kg in weight (Diedler et al., 2011). The high numbers of overweight/obese patients suffering ischaemic stroke and the lack of effective and safe treatment for this patient group highlight the need for alternative therapeutic options for ischaemic stroke.

1.3.6 Obesity, systemic inflammation and mechanisms of increased stroke risk and damage

In obesity, hypertrophy and hyperplasia of the adipose tissue initiates an inflammatory response characterised by the production of pro-inflammatory cytokines, including interleukin-1 (IL-1) and tumour necrosis factor-α (TNFα; Jernås et al. 2006), which are secreted into the circulation causing a chronic state of low-grade systemic inflammation. Indeed, circulating levels of IL-6, TNFα, C-reactive protein (CRP; Malavazos et al. 2007), monocyte chemotactic protein-1 (MCP-1; Christiansen et al. 2005; Sartipy & Loskutoff 2003; Takahashi et al. 2003) and leukocytes are elevated in obesity (Chae et al., 2013). Systemic inflammation is a common feature of several stroke co-morbidities and has been shown to increase the risk and severity of stroke in both clinical and pre-clinical studies (Murray et al., 2013; Rothwell and Luhehsi, 2000).

The increased risk of stroke in obesity is thought to be due to the low-grade chronic inflammation inducing a pro-thrombotic state. Blood levels of von Wilebrand factor, Factor VIII and other components of the coagulatory pathway are raised by IL-6 (Kerr et al., 2001), as seen in obesity (Malavazos et al., 2007), thus predisposing clot formation.

The mechanisms by which obesity exacerbates cerebral damage after stroke are not yet well understood. Mechanisms have been suggested, including heightened neuroinflammatory response (Drake et al., 2011) and disruption of vascular elasticity and permeability (Deutsch et al., 2009; McColl et al., 2010), which may lead to deficits in cerebral blood flow after stroke. Indeed studies of peripheral inflammation (Montaner et al., 2003; Murray et al., 2014) and other components of metabolic syndrome (Ayata et al., 2013; Kawai et al., 1998) note abnormalities in CBF after stroke, indicating dysfunction of cerebral perfusion may also be a mechanism contributing to the greater ischaemic damage seen in obesity.
1.3.7 The contribution of obesity to cerebral blood flow changes in stroke

Little is currently known about CBF after stroke in obesity, although changes in the expression of and sensitivity to certain vasoactive mediators, including resistin and endothelin-1, have been reported in obese rodents (Savopoulos et al., 2011; Subramanian and MacLeod, 2003) which may cause dysregulation in general vascular tone.

Studies of peripheral inflammation and other components of metabolic syndrome report abnormalities in CBF after stroke. For example, acute administration of IL-1 to rats undergoing experimental stroke causes CBF deficits in a greater area than in vehicle-dosed rats, as detected by MRI (Parry-Jones et al., 2008). Expansion on these initial findings demonstrated that IL-1-induced hypoperfusion post-stroke occurs via an endothelin-1 dependent vasoconstrictive mechanism (Murray et al., 2014). These reports of hypoperfusion after stroke are mirrored in studies of metabolic syndrome conditions including hyperglycaemia (Kawai et al., 1998; Tarr et al., 2013), hyperlipidaemia (Ayata et al., 2013) and hypertension (Reid et al., 2012).

There is strong overlap between the obese phenotype and the previously reported factors effecting post-stroke CBF, including inflammation (Malavazos et al., 2007), hyperlipidaemia (Chang et al., 2012; Joerin et al., 2013) and hypertension (Savopoulos et al., 2011; Subramanian and MacLeod, 2003), suggesting a similar mechanism may occur following stroke in obesity.

1.4 Introduction summary

To summarise, obesity predisposes the occurrence of ischaemic stroke and is associated with greater levels of brain damage and poorer functional recovery, as shown in both clinical and animal studies. There is a lack of effective treatments for ischaemic stroke, particularly for obese individuals who often do not respond well to thrombolysis by tPA. This highlights the need for greater understanding of ways in which obesity exacerbates stroke damage in order to inform the development of future therapies. Recent studies have suggested systemic inflammation and several metabolic syndrome related-diseases results in hypoperfusion and therefore greater damage during ischaemic stroke. The chronic state of systemic inflammation which develops in obesity suggests it may also affect the progression of ischaemic
damage by similar mechanisms. Therefore, a study regarding the effect of obesity on hypoperfusion in stroke is an important step in expanding our current understanding of how obesity exacerbates ischaemic brain damage.
2. Aims

The long term aim of work stemming from this project will be to investigate whether obesity exacerbates ischaemic stroke damage due to deficits in cerebral perfusion. This can be studied using an MRI technique known as perfusion weighted imaging (PWI), which quantifies cerebral blood flow non-invasively (Calamante et al., 1999; Murray et al., 2014; Parry-Jones et al., 2008).

In preparation for this future study, a rat model of ischaemic stroke in obesity must be established. Therefore, the current MPhil project aims to:

1) Establish and characterise a rat model of diet-induced obesity (DIO)
2) Assess the effect of the diet model on ischaemic stroke

Male Wistar rats will be fed a high fat diet (HFD) for 12 weeks. Previous literature indicates that not all rats fed a HFD will develop an obese phenotype (Mrad et al., 1992; Nascimento and Monte-Alto-Costa, 2011; Oliveira Junior et al., 2010). Therefore, we predict the HFD group will form DIO and a DIO-resistant (DIO-R) subgroups distinguishable by body weight. We hypothesise that this diet period will be sufficient to produce DIO rats which are distinct from control diet fed animals based on body weight and relative body fat. Blood pressure and blood glucose levels will also be monitored in case of the development of hypertension or hyperglycaemia.

Ischaemic stroke will be induced using an adaption of a previously published method of middle cerebral artery occlusion (MCAo) using an intraluminal filament (Longa et al., 1989). We hypothesise that DIO rats will experience more severe strokes than control animals as reported in previous studies of DIO rats (Deutsch et al., 2009; Langdon et al., 2011). The outcome of stroke will be assessed by lesion volume and behavioural scores (24 or 48 h post re-perfusion).

Future work will aim to use MRI to monitor CBF and evolution of lesion volume acutely following reperfusion. Therefore, DIO and control animals will also have lesion volumes at 3 h post reperfusion analysed using MRI to ensure lesions are detectable using DWI at this time point. We hypothesise that lesions will be present at this time in both diet groups and that lesion volumes will be greater in DIO animals compared to controls.
3. Methods

3.1 Animals

Male Wistar rats were purchased from Charles-River (UK) and housed in groups of three in standard housing conditions (12 h light/dark cycle, 21 ± 2 °C). All experiments were conducted in accordance with the UK Animals Scientific Procedure Act (ASPA) 1986 under Home Office licence 40/3617. See table 1 below for animal numbers and usage.

<table>
<thead>
<tr>
<th>Cohort</th>
<th>Animal numbers</th>
<th>Recovery period after stroke surgery</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>17 (HFD 12, Control 5)</td>
<td>24 h reperfusion</td>
</tr>
<tr>
<td>2</td>
<td>26 (HFD 18, Control 8)</td>
<td>48 h reperfusion (HFD n=12, control diet n=2)</td>
</tr>
<tr>
<td>3</td>
<td>17 (HFD 12, Control 5)</td>
<td>48 h reperfusion</td>
</tr>
<tr>
<td>4</td>
<td>34 (HFD 22, Control 12)</td>
<td>4 h reperfusion</td>
</tr>
</tbody>
</table>

Table 1: Animal numbers and usage

3.2 Diet

After a 1 week acclimatisation period, rats (weighing 219-300 g; ~6 weeks old) were started on either a high fat diet (60% energy from fat, TestDiet, UK) or a control diet (12% energy by fat, Test diet) for a period of 12 weeks (see table 2 for diet compositions). Throughout the diet period animals had free access to both food and water. Body weight and food intake were monitored weekly.
Previous studies of obesity in rats have shown that not all rats fed a HFD develop an obese phenotype (Mrad et al., 1992; Nascimento and Monte-Alto-Costa, 2011; Oliveira Junior et al., 2010). Therefore in each cohort, twice as many animals were placed in the HFD group as in the control group to allow the HFD animals to be divided into DIO and DIO-R groups at the end of the diet period.

An inclusion weight range was used to reduce the effect of start weight variability on final body weight measurements. Animals were only included in the study if their weight at the start of the diet period was 215-300 g. The inclusion weight range selection was guided by the mean ± 2 SD of the starting body weights in each cohort.

**Table 2: Composition of high fat and control diets.**

<table>
<thead>
<tr>
<th></th>
<th>High fat diet</th>
<th>Control diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kilocalories (kcal) per gram</td>
<td>5.21</td>
<td>3.87</td>
</tr>
<tr>
<td>Fat (% of energy)</td>
<td>59.9</td>
<td>12.0</td>
</tr>
<tr>
<td>Carbohydrate (% of energy)</td>
<td>21.4</td>
<td>69.3</td>
</tr>
<tr>
<td>Protein (% of energy)</td>
<td>18.6</td>
<td>18.6</td>
</tr>
</tbody>
</table>

3.3 Non-invasive blood pressure measurement via tail cuff system

Blood pressure was measured using BP-2000 series II tail cuff blood pressure analysis system (Visitech Systems, USA). To ensure adequate blood flow in the tail, animals were kept warm by an initial 3 min incubation in a 36 °C heating chamber and placed on a 35 °C integrated heating platform during the measurement process. Measurements were averaged across a minimum of 3 readings excluding any readings disrupted by movement artefacts. All animals in cohorts 2 and 3 were habituated to the restrainer and tail cuff on two separate occasions prior to blood pressure measurement. Unfortunately, animals in cohort 1 were not habituated prior to blood pressure measurements due to time restraints.

3.4 Blood glucose level measurement

Tail vein blood was sampled immediately before stroke surgery and analysed using an Accu-Chek Aviva Blood Glucose Meter System and Accu-Chek Aviva Test Strips (Boots, UK). Reported values are an average of two readings.
In cohorts 1-3, tests were performed 1-3 days prior to surgery (baseline) and again after surgery immediately prior to sacrifice (24 h or 48 h reperfusion). Motor-neurological and behavioural assessments were performed using the criteria detailed in tables 3 and 4. For the Motor-neurological assessment, animals were given a score between 0 and 5, where a higher score relates to greater deficit levels. For the behavioural assessment, a cumulative points system was used in which a score was given for each of the six criteria categories and summed together to give a maximum score of 21, where a lower score indicates greater impairment. Both scoring systems are based on previously described post-stroke functional tests (Hunter et al., 2000).

**Table 3: Motor-neurological assessment criteria**

<table>
<thead>
<tr>
<th>Criteria</th>
<th>Score awarded</th>
</tr>
</thead>
<tbody>
<tr>
<td>No deficit</td>
<td>0</td>
</tr>
<tr>
<td>Failure to extend contralateral forepaw</td>
<td>1</td>
</tr>
<tr>
<td>Weakened grip of contralateral forepaw when tail pulled</td>
<td>2</td>
</tr>
<tr>
<td>Circling to contralateral side when tail pulled</td>
<td>3</td>
</tr>
<tr>
<td>Spontaneous circling to contralateral side</td>
<td>4</td>
</tr>
<tr>
<td>Coma</td>
<td>5</td>
</tr>
</tbody>
</table>
**Table 4: Behavioural assessment criteria**

<table>
<thead>
<tr>
<th>Criteria</th>
<th>Cumulative points awarded</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Spontaneous activity</strong></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>Occasional movement</td>
<td>1</td>
</tr>
<tr>
<td>Frequent but sluggish movement</td>
<td>2</td>
</tr>
<tr>
<td>Repeated vigorous movements (e.g. rearing, movement between cage floor and shelf)</td>
<td>3</td>
</tr>
<tr>
<td><strong>Transfer arousal</strong></td>
<td></td>
</tr>
<tr>
<td>Coma</td>
<td>0</td>
</tr>
<tr>
<td>Awake, no movement</td>
<td>1</td>
</tr>
<tr>
<td>Some initial movement</td>
<td>2</td>
</tr>
<tr>
<td>Exploratory behaviour with limited movement</td>
<td>3</td>
</tr>
<tr>
<td>Slightly reduced exploratory behaviour</td>
<td>4</td>
</tr>
<tr>
<td>Excited continued exploration of new cage</td>
<td>5</td>
</tr>
<tr>
<td><strong>Gait</strong></td>
<td></td>
</tr>
<tr>
<td>Absolute incapacity</td>
<td>0</td>
</tr>
<tr>
<td>Obvious limp</td>
<td>1</td>
</tr>
<tr>
<td>Slight limp</td>
<td>2</td>
</tr>
<tr>
<td>No impairment</td>
<td>3</td>
</tr>
<tr>
<td><strong>Escape response to touch</strong></td>
<td></td>
</tr>
<tr>
<td>No response</td>
<td>0</td>
</tr>
<tr>
<td>Responds to touch but minimal movement</td>
<td>1</td>
</tr>
<tr>
<td>Delayed or reduced response</td>
<td>2</td>
</tr>
<tr>
<td>Strong, immediate response</td>
<td>3</td>
</tr>
<tr>
<td><strong>Struggle response when held</strong></td>
<td></td>
</tr>
<tr>
<td>No struggle</td>
<td>0</td>
</tr>
<tr>
<td>Slight, brief struggle</td>
<td>1</td>
</tr>
<tr>
<td>Moderate struggle</td>
<td>2</td>
</tr>
<tr>
<td>Vigorous struggle</td>
<td>3</td>
</tr>
<tr>
<td><strong>Ability to rear to vertical body position</strong></td>
<td></td>
</tr>
<tr>
<td>No attempt to rear</td>
<td>0</td>
</tr>
<tr>
<td>Paws at cage wall but cannot rear</td>
<td>1</td>
</tr>
<tr>
<td>Rears to maximum of ~50% of full rear</td>
<td>2</td>
</tr>
<tr>
<td>Rears to maximum of ~75% of full rear</td>
<td>3</td>
</tr>
<tr>
<td>Rears to full vertical position</td>
<td>4</td>
</tr>
</tbody>
</table>
3.6 Surgical procedures

The experimental plan was designed to minimise variation of external factors between the diet groups, such as the time of day surgery was conducted.

In cohort 1, all animals underwent surgery within the same week. This was possible as the surgical procedures were performed by another member of the lab, Katie Murray, while behavioural assessments and perfusions were performed concurrently. Animals were purposefully assigned to surgery time slots so that animals from each diet group underwent surgery at times spread evenly over each day and over the week.

In cohort 3, stroke surgeries were performed over a space of three weeks. Animal orders and diet induction were staggered so that each week one cage of HFD (n=3) and one cage of control rats (n=3) finished the 12 week diet ready for surgery. Again surgery time slots were assigned evenly between the diet groups.

In cohort 4, the study design allowed for three animals to undergo surgery and MRI scanning per week. Animal orders and diet induction were staggered so that three animals were ready for surgery each week. Each week of experiments would consist of only one diet group (i.e. either HFD or control diet) and the weeks were alternated between the diet groups. For each animal the experimental protocol lasted the full day and always commenced at the same time.

In cohort 2, the study was initially designed to include MRI scanning as in cohort 4. However, due to a mechanical failure of the MRI scanner part way through the study, the remaining animals underwent stroke surgery followed by 48 h reperfusion. Therefore in this study, each week three animals from either the HFD or the control group underwent surgery. Surgery weeks were alternated between the diet groups and the surgery start times were matched between the groups.

All surgical procedures were conducted under aseptic conditions using tools and surgical components that were either purchased sterile for single use or sterilised prior to each operation by autoclaving or UV irradiation. For animals in cohort 1, all surgical procedures were performed by another member of the lab, Katie Murray. All other surgeries were performed personally.
3.6.1 Preparation of animal for surgical procedures

General anaesthesia was induced in a gas anaesthetic chamber by delivery of 3% isoflurane in a gas mixture of 30% oxygen and 70% nitrous oxide at a rate of 1 L/min. A surgical plane of anaesthesia was maintained throughout the procedure by delivery of 1.5-3% isoflurane in the above gas mixture via a mouth mask.

The skin surrounding the operative sites were shaved then cleaned using Videne surgical scrub solution (Adams, UK). The animal was placed on a heat mat (Harvard Apparatus, UK) and a rectal thermometer was inserted to maintain physiological body temperature.

3.6.2 Attachment of the laser Doppler probe

During stroke surgery a laser-flow blood perfusion monitor (Oxford Optronix, UK) was used to detect a reduction in blood flow in the MCA territory to indicate correct placement of the occluding filament at the origin of the MCA. The system transmits and detects laser light via two fibre optic channels of a laser Doppler probe. The disparity between transmitted and detected light levels caused by the scattering of light within tissues of the body is used to calculate the Doppler frequency which is proportionally related to blood flow velocity.

A rostral-caudal midline incision was made into the scalp and lower tissue dissected bluntly to expose the skull. A partial burr-hole was made in the right parietal bone immediately caudal to the coronal suture using a dental drill. The laser Doppler probe was placed into the hole and secured to the skull with a cotton wool and VetBond scaffold.

3.6.3 Intraluminal filament model of middle cerebral artery occlusion

Cerebral ischaemia was achieved by occluding the origin of the MCA for 60 min with a silicone tipped filament (Doccol, USA) using an adaptation of a previously published method (Longa et al., 1989). Filament dimensions were as follows: total length=30 mm, un-coated diameter=190 μm, silicon coated length=5-6 mm, silicon coated diameter=350±20 μm, 390±20 μm, 410±20 μm, 430±20 μm or 450±20 μm.
Figure 2: Intraluminal filament model of middle cerebral artery (MCA) occlusion in rat. A Doccol filament (black suture with silicone tip [shown in green]) was inserted into the internal carotid artery (ICA) via the external carotid artery (ECA) and extended up into the Circle of Willis to occlude the base of the MCA. Also shown are the common carotid artery (CCA), the basilar artery (BA) and the anterior cerebral artery (ACA). Figure adapted from Chen et al. (2006).

With the animal in a supine position, a rostral-caudal incision was made slightly to the right of the midline reaching from the mandible to the sternum. Underlying tissue was bluntly dissected and the salivary gland retracted to reveal the intersection of the sternohyoid and sternocleidomastoid muscles into which a retractor was inserted and expanded. The underlying omohyoid muscle was cut to expose the vagus nerve and the common carotid artery (CCA), which were carefully separated from one another. The CCA bifurcates at the rostral end to form the ICA, dorsolaterally, and the external carotid artery (ECA), ventromedially. The occipital artery (OPA) and superficial temporal artery (STA) projecting from the ECA were electrocoagulated using a bipolar coagulator (Aesculap, UK) and dissected. The pterygopalatine artery (PPA) branching from the ICA was also electrocoagulated. A microvessel aneurism clip (Fine Science Tools, Germany) was applied to the CCA and the ECA electrocoagulated and dissected approximately 10 mm from the CCA bifurcation to form a stump. A second clip was placed on the ICA forming a closed section of vessel containing the ECA stump between the two clips. Surgical thread (Fine Science Tools) was loosely tied at the origin of the ECA and a small incision
made half way up the stump for the insertion of the Doccol filament. The ECA was manoeuvred 180° to form a continuous line with the ICA permitting the filament to be advanced to the clip of the ICA. The ICA clip was then removed and the filament inserted approximately 20 mm until resistance was felt and/or and a significant Doppler drop was noted on the laser-flow blood perfusion monitor. The filament was then secured with the thread at the base of the ECA stump and the clip removed from the CCA. The animals remained under general anaesthesia for the duration of the 60 min occlusion, at the end of which the CCA clip was briefly reapplied while the filament was fully removed and the ECA stump ligated. Finally, incision sites were closed with 3-0 Mersilk sutures (Ethicon, USA).

3.6.4 Post surgery recovery

Animals in cohorts 1-3 were recovered following surgery. At the end of the surgery, 2.5 mL saline was injected subcutaneously to aid rehydration following surgery. Analgesia was given in the form of topical application of EMLA (2.5% lidocaine, 2.5% prilocaine; AstraZeneca, UK) and a subcutaneous dose of buprenorphine (0.01 mg/kg for 24 h reperfusion rats and 0.03 mg/kg for 48 h reperfusion rats; Alstoe Animal Health, UK). An additional dose of buprenorphine was given to 48 h reperfusion rats the morning following surgery (16-21 h post-surgery). Rats were housed individually during the recovery period with free access to water, standard rodent chow mashed with water and either control or high fat diet pellets dependent on their diet group.

3.6.5 Remote reperfusion of animals undergoing MRI

Animals undergoing MRI scanning after MCAo surgery were remotely reperfused within the scanner. To allow for this, a length of thread was attached to the Doccol filament and left protruding from the operative site which was sutured closed immediately after occlusion. The animal was then transferred to the scanner cradle in a prone position. At the end of the 60 minute occlusion, remote reperfusion was achieved by pulling the thread taught and retracting approximately 1.5 cm thus withdrawing the silicone tip of the filament from the MCA origin to the ICA. Anaesthesia was maintained throughout scanning and until termination by transcardial perfusion (4 h post reperfusion).
At the end of the reperfusion period animals in cohorts 1-3 were anaesthetised and culled by transcardial perfusion with 0.9% saline (NaCl; Fisher Scientific, UK) delivered at a rate of 20 mL/min by a peristaltic pump (Watson Marlow, UK). 4% paraformaldehyde (PFA; 4% PFA in 0.1 M phosphate buffer [PB]) was then delivered at the same rate to fix the bodily tissues. Brains were incubated in PFA overnight then transferred into 30% sucrose (Fisher Scientific) in phosphate buffered saline (PBS; Sigma-Aldrich, UK) for 48 h, after which they were flash frozen in -40 °C isopentane and stored at -80 °C.

Anaesthesia was maintained in cohort 4 animals between scanning and termination and animals were perfused with RNAse-free 0.9% saline (0.1 % DEPC; Sigma).

3.8 Measurement of adiposity and abdominal circumference to body length ratio

At the time of sacrifice, abdominal circumference (just above the pelvis) and body length (nose to base of tail) were measured with a tape measure. The ratio was calculated as follows: abdominal circumference ÷ body length.

Following perfusion with saline, the epididymal and/or perirenal fat deposits were extracted and weighed. Adiposity was calculated as a percentage of final body weight before sacrifice as follows:

(fat pad weights ÷ final body weight) x 100.

3.9.1 Haematoxylin and Eosin (H&E) staining of brain sections

Haematoxylin and Eosin stain the cell nuclei blue and the cytoplasm pink, respectively. Infarcted tissue can be distinguished from healthy tissue on H&E stained sections due to its relative pallor and the observation of shrunken nuclei of dead cells under the microscope. Frozen brains were cut into 30 μm thick coronal sections using a freezing sledge microtome (Bright Instruments, UK) and stored in cryoprotectant solution (20% glycerol, 30% ethylene glycol in 0.1 M PB) at -20 °C. Brain sections were mounted onto Superfrost Plus slides (ThermoScientific, UK) and stained by sequential incubation in the following solutions: xylene (2 min;
FisherScientific, UK), 100% ethanol (2 min; FisherScientific), 70% ethanol (2 min), 50% ethanol (2 min), running water (2 min), Gill III haematoxylin (5-8 min; ThermoShandon, UK), running water (until water ran clear), acid alcohol (2 dips; 0.003% hydrochloric acid in 70% ethanol), Scott’s tap water (30 s; 0.02% magnesium sulphate 0.002% sodium hydrogen carbonate in distilled water), eosin Y (30 s; ThermoShandon), running water (until water runs clear), 50% ethanol (2 min), 70% ethanol (2 min), 100% ethanol (2 min), xylene (5 min). Cover slips were mounted over the tissue section using DPX mounting medium (Sigma-Aldrich).

3.9.2 Lesion volume quantification from H&E stained brain sections

Images of the H&E stained sections were captured using a MicroPublisher 3.3 RTV camera (QImaging, Canada) and Northern Eclipse Software V8.0 (Empix, Canada). Image J software (NIH, 2011) was then used to measure the area of infarction in 11 pre-defined sections of the brain spanning from -5.4 mm to +3.24 mm relative to bregma. Infarct area was plotted against distance from bregma using GraphPad Prism v6.0 (GraphPad Software Inc., USA) and lesion volume calculated as volume under the curve.

3.10.1 Magnetic resonance imaging

MRI was performed using a Magnex 7 T horizontal magnet (Agilent Technologies, UK) and a transmit-receive 2.5 cm surface coil (Obese rat brain coil; Rapid Biomedical, Germany) interfaced with a Bruker Biospec Avance III console (Bruker, UK) supporting Paravision 5 software (Bruker, Germany).

An angiography scan was run before and after remote reperfusion to confirm occlusion and re-canalisation of the MCA, respectively. For this purpose, a fast low angle shot time of flight 2D (FLASH-TOF-2D) sequence was used (time to repeat [TR]: 15 ms; echo time [TE]: 3.8 ms; flip angle [FA]: 80°; slice thickness [ST]: 0.4 mm; field of view [FOV]: 4 cm x 4 cm; matrix size: 256 x 256). The lesion was visualised by generating apparent diffusion co-efficient (ADC) maps from diffusion weighted imaging echo planar imaging (DWI-EPI; TR: 4,000 ms; TE: 22.9 ms; averages: 2; ST: 3 mm; FOV: 4 cm x 4 cm; matrix size: 96 x 96; 3 directions: x, y and z; b values = 0 and 1,000 s/mm²; 5 continuous slices).
3.10.2 Lesion volume quantification from ADC maps

Quantitative ADC maps were generated from DWI using Paravision 5 software and analysed in ImageJ. A threshold of 77\% of the average ADC of the non-stroke hemisphere was calculated; ADC values below this threshold indicate the presence of an ischaemic lesion (Meng et al., 2004). DWI spanned the brain in five 3 mm slices. Thus lesion volume was calculated by combining the lesion volumes of each slice (ADC deficit area x 3 mm).

3.11 Statistical analysis

Data were analysed and presented using Microsoft Excel 2007 (Microsoft Corporation, USA) and GraphPad Prism V6. Where appropriate, data are presented as mean values ± one standard deviation. Statistical significance was tested using either paired or unpaired student’s t-test (two data sets), one way analysis of variance (ANOVA)/two-way repeat measures ANOVA followed by Tukey’s multiple comparisons test (three data sets). Correlation was tested using Pearson’s Correlation test using a two-tailed p-value. Statistical significance is denoted by one of the following symbols: *, $ or # (see key in each figure) such that one symbol=p<0.05, two symbols=p<0.01 and three symbols=p<0.001.
4. Results

4.1 Characterising obesity in 12-week high fat diet fed rats

In cohorts 1-3, initial body weights were comparable between the diet groups. However, in cohort 4, the mean initial body weight of the DIO-R group was significantly lower than in the control group (fig. 3; control: 261±17 g, DIO-R: 243±11 g [p<0.01]). When data was grouped together from the four cohorts (fig. 3E) no significant difference in initial body weight was seen between the diet groups.

Any animal with an initial body weight outside of the inclusion range of 215-300 g was excluded from the study. The range of body weights varied between the cohorts (fig. 3; cohort 1: 220-255 g; cohort 2: 219-300 g; cohort 3: 223-269 g; cohort 4: 223-288 g) and the mean initial body weight of cohorts 2 and 4 were greater than seen in cohort 1 (cohort 1: 234±10 g; cohort 2: 254±20 g [p<0.01]; cohort 4: 250±16 g [p<0.01]).
Male Wistar rats fed a high fat diet (HFD) for 12 weeks were divided into diet induced obese (DIO) or DIO-resistant (DIO-R) groups dependent on their weight gain compared to control diet fed rats. Initial body weights prior to the start of the 12-week diet period are shown (A-E; mean ± 1 standard deviation; A-D n=3-18; E n=20-44). Statistical significance was tested using one way analysis of variance (ANOVA) followed by Tukey’s multiple comparisons test and denoted as $$=p<0.01.$$

Figure 3: Initial body weight (cohorts 1-4). Male Wistar rats fed a high fat diet (HFD) for 12 weeks were divided into diet induced obese (DIO) or DIO-resistant (DIO-R) groups dependent on their weight gain compared to control diet fed rats. Initial body weights prior to the start of the 12-week diet period are shown (A-E; mean ± 1 standard deviation; A-D n=3-18; E n=20-44). Statistical significance was tested using one way analysis of variance (ANOVA) followed by Tukey’s multiple comparisons test and denoted as $$=p<0.01.$$

$ DIO-R vs. control
Figure 4: Absolute body weight gain over 12-week control or high fat diet (HFD) period (cohorts 1-4). Body weight gain is plotted for male Wistar rats fed a control or HFD (A-E; mean ± 1 standard deviation; A-D n=3-18; E n=20-44). HFD animals above the limit of the control mean + 2SD for absolute weight gain (A-D dotted black line) were classed as diet induced obese (DIO) and the remainder as DIO-resistant (DIO-R). Statistical significance was tested using one way analysis of variance (ANOVA) followed by Tukey’s multiple comparisons test and denoted as $=p<0.05, ** or ##=p<0.01$ and *** or ###=p<0.001.
At the end of the 12 week diet period, body weight and weight gain were not greater in all HFD animals compared to controls indicating that a portion of the HFD animals were resistant to obesity. Due to the variation in initial body weights, weight gain rather than final body weight was used to define obesity. HFD rats whose weight gain was greater than the mean weight gain of the control group plus two standard deviations were defined as DIO, and the remainder of the HFD group were defined as DIO-resistant (DIO-R). Divided this way, in all cohorts DIO rats had significantly greater weight gain than control and DIO-R animals (fig. 4; cohorts 1, 2 and 4: p<0.001; cohort 3: p<0.01). The same trend was seen when data across the four cohorts was grouped. However, when grouped, weight gain in the DIO-R group was significantly greater than seen in controls (fig. 4E; p<0.05).

In all cohorts, the body weight of the DIO group diverged from that of the control and DIO-R groups by 2-5 weeks and 2-7 weeks on diet, respectively (fig. 6). By 12 weeks of diet, in each cohort, DIO animals had greater body mass than control and DIO-R animals (fig. 5 and 6; cohorts 1, 2 and 4: p<0.001; cohort 3: DIO vs. control p<0.01, DIO vs. DIO-R p<0.05). When data was grouped for the four cohorts the same relationship was seen (fig. 5E; p<0.001). In cohort 2, DIO-R animals were also consistently heavier than control animals from 8 weeks on diet (fig. 6B) until the end of the study (fig. 5B; p<0.01).

Note in all cohorts, while the rate of weight gain appears to be declining by the end of the diet period, body weight is still increasing in both HFD and control diet groups at this time (fig. 6). This tendency is particularly noticeable in the latter three cohorts.
Figure 5: Final body weight after 12-week control or high fat diet (HFD) period (cohorts 1-4). Male Wistar rats fed a HFD were divided into diet induced obese (DIO) or DIO-resistant (DIO-R) groups dependent on their weight gain compared to control diet fed rats. Final body weights are plotted for control, DIO and DIO-R groups (A-E; mean ± 1 standard deviation; A-D n=3-18; E n=20-44). Statistical significance was tested using one way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test and denoted as *, # or $=p<0.05, **, ## or $$=p<0.01 and ### or $$=$p<0.001.
Figure 6: Body weight over 12-week control or high fat diet (HFD) period (cohorts 1-4).
Male Wistar rats fed a HFD were divided into diet induced obese (DIO) or DIO-resistant (DIO-R) groups dependent on their weight gain compared to control diet fed rats. Body weight over the diet period are plotted for control, DIO and DIO-R groups (A-D; mean ± 1 standard deviation; n=3-18). Statistical significance was tested at each displayed time point using one way analysis of variance (ANOVA) followed by Tukey’s multiple comparisons test and denoted as *, # or $$=p<0.05, **, ## or $$$=p<0.01 and ### or $$$=p<0.001.
Figure 7: Adiposity from epididymal fat in control and high fat diet (HFD) fed rats (cohorts 1-4). Male Wistar rats fed a HFD for 12 weeks were divided into diet induced obese (DIO) or DIO-resistant (DIO-R) groups dependent on their weight gain compared to control diet fed rats. At the time of sacrifice the epididymal fat deposits were weighed and adiposity calculated as a percentage of final body weight before sacrifice (A-E: mean ± 1 standard deviation; A-D n=3-16; E n=19-40). Statistical significance was tested using one way analysis of variance (ANOVA) followed by Tukey’s multiple comparisons test and denoted as **, ## or $$$=p<0.01 and *** or $$$$=p<0.001.
Figure 8: Adiposity from epididymal and perirenal fat in control and high fat diet (HFD) fed rats (cohorts 2-4). Male Wistar rats fed a HFD for 12 weeks were divided into diet induced obese (DIO) or DIO-resistant (DIO-R) groups dependent on their weight gain compared to control diet fed rats. At the time of sacrifice the epididymal and perirenal fat deposits were weighed and adiposity calculated as a percentage of final body weight before sacrifice (A-D; mean ± 1 standard deviation; A-C n=3-16; D n=13-34). Statistical significance was tested using one way analysis of variance (ANOVA) followed by Tukey’s multiple comparisons test and denoted as $=p<0.05$, ## or $$=p<0.01$ and $$$=p<0.001$.

The proportion of body weight from fat was analysed by comparing final body weight to epididymal and/or perirenal fat pad weights (fig. 7 and 8). DIO animals in all cohorts, and when data across the cohorts was combined (fig. 7E and 8D), had a greater percentage of fat than controls (p<0.01-p<0.001).

Despite DIO animals having a greater body mass than DIO-R animals, relatively greater fat deposits in DIO compared to DIO-R animals were only measured in cohort 4 (fig. 7D and 8C; p<0.01) and when data was grouped together between the four cohorts (fig. 7E and 8D; p<0.01). DIO-R groups also had a greater percentage of fat than control groups in all cohorts (p<0.05-p<0.001; in cohort 4 only when both epididymal and perirenal fat deposits were considered).
Body proportions of animals in cohorts 1, 3 and 4 were measured and abdominal circumference to body ratios calculated (fig. 9). DIO groups in the three cohorts had proportionally larger abdominal circumferences compared to controls (p<0.05- p<0.001). In cohorts 1 and 3, DIO-R animals had ratios similar to DIO animals and greater than that of controls (p<0.05). Conversely, DIO-R animals in cohort 4 were more similar in proportion to the control group and had relatively smaller abdominal circumferences than the DIO group (p<0.001). When data from the three cohorts was combined, DIO animals had relatively greater abdominal circumference in comparison to both control and DIO-R animals, while DIO-R animals were also proportionally larger in circumference than controls (fig. 9D; p<0.001).

**Figure 9**: Abdominal circumference to body length ratio of control and high fat diet (HFD) fed rats (cohorts 1, 3 and 4). Male Wistar rats fed a HFD for 12 weeks were divided into diet induced obese (DIO) or DIO-resistant (DIO-R) groups dependent on their weight gain compared to control diet fed rats. At the time of sacrifice, abdominal circumference (just above the pelvis) and body length (nose to base of tail) were measured (A-D; mean ± 1 standard deviation; A-C n=3-16; D n=13-28). Statistical significance was tested using one way analysis of variance (ANOVA) followed by Tukey’s multiple comparisons test and denoted as * or $=p<0.05$, ** =p<0.01 and ***, ### or $$$=p<0.001.
Figure 10: Systolic blood pressure of control and high fat diet (HFD) fed rats (cohorts 1-3). Male Wistar rats fed a HFD for 12 weeks were divided into diet induced obese (DIO) or DIO-resistant (DIO-R) groups dependent on their weight gain compared to control diet fed rats. Systolic blood pressure was measured using a non-invasive tail cuff system after 12 weeks of diet. All data presented as mean ± 1 standard deviation (A-C n=3-11; D n=14-26). Statistical significance was tested using one way analysis of variance (ANOVA) followed by Tukey’s multiple comparisons test and denoted as *=p<0.05.

Blood pressure was measured using a non-invasive tail cuff method in cohorts 1-3 after 12 weeks of diet (fig. 10 and 11). Systolic blood pressure (fig. 10) was significantly higher in DIO animals than in the control group in cohort 1 (DIO: 148±10 mmHg, control: 125±11 mmHg [p<0.05]) and when data was grouped between the three cohorts (DIO: 146±10 mmHg, control: 135±14 mmHg [p<0.05]). In both cases the systolic blood pressure of the DIO-R group was slightly higher than in controls but not significantly so (cohort 1: 133±14 mmHg; cohorts 1-3 combined: 144±12 mmHg). In cohort 2, DIO and DIO-R animals showed a trend for slightly higher systolic blood pressure compared to controls but there was not a significant difference (control: 135±16 mmHg, DIO: 142±14 mmHg, DIO-R: 143±9...
Systolic blood pressure was similar between diet groups in cohort 3 (control: 145±5 mmHg, DIO: 148±2 mmHg, DIO-R: 150±8 mmHg). Notably, mean systolic blood pressure of control groups was 10 and 20 mmHg higher in cohorts 2 and 3, respectively, compared to cohort 1. No effect of diet group was seen on diastolic blood pressure (fig. 11).

**Figure 11: Diastolic blood pressure of control and high fat diet (HFD) fed rats (cohorts 1-3).** Male Wistar rats fed a HFD for 12 weeks were divided into diet induced obese (DIO) or DIO-resistant (DIO-R) groups dependent on their weight gain compared to control diet fed rats. Diastolic blood pressure was measured using a non-invasive tail cuff system after 12 weeks of diet. All data presented as mean ± 1 standard deviation (A-C n=3-11; D n=14-26). Statistical significance was tested using one way analysis of variance (ANOVA) followed by Tukey’s multiple comparisons test (no significant results).
Blood glucose levels were also comparable between diet groups in all four cohorts (fig. 12).

**Figure 12: Blood glucose levels of control and high fat diet (HFD) fed rats (cohorts 1-4).** Male Wistar rats fed a HFD for 12 weeks were divided into diet induced obese (DIO) or DIO-resistant (DIO-R) groups dependent on their weight gain compared to control diet fed rats. Blood glucose levels were measured from fresh tail vain blood samples. Reported values are an average of two readings. All data presented as mean ± 1 standard deviation (A-D n=3-17; E n=19-43). Statistical significance was tested using one way analysis of variance (ANOVA) followed by Tukey’s multiple comparisons test (no significant results).
**4.2 The effect of obesity on ischaemic stroke**

Animals from cohort 1 underwent 60 min MCAo surgery with a 350 μm filament followed by a 24 h reperfusion period. The stroke surgery only produced lesions in just over half of the rats. However, in those animals with lesions, DIO animals had significantly greater infarct volumes (144±43 mm³) than DIO-R (62±24 mm³ [p<0.05]) and control (43±7 mm³ [p<0.05]) groups (fig. 13A). Poorer stroke outcome in DIO rats was also evident from deficits in behavioural and motor-neurological abilities 24 h post stroke compared to control and DIO-R groups (fig. 13B; p<0.05-0.01).

*Figure 13: The effect of diet induced obesity on ischaemic stroke (cohort 1). Male Wistar rats fed a high fat diet (HFD) for 12 weeks were divided into diet induced obese (DIO) or DIO-resistant (DIO-R) groups dependent on their weight gain compared to control diet fed rats. Stroke was induced by 60 min middle cerebral artery occlusion (MCAo) with a 350 μm filament followed by 24 h reperfusion before sacrifice. 30 μm thick brain sections were stained with haematoxylin and eosin to visualise the infarct. Lesion volumes (Ai) and representative images are shown (Aii; lesion outlined in black). Behavioural (Bi; high score healthy) and motor neurological (Bii; low score healthy) tests were run before stroke surgery and 24 h post reperfusion. All data presented as mean ± 1 standard deviation (n=2-4). Statistical significance was tested using one way analysis of variance (ANOVA; lesion volume) or 2-way repeated measures ANOVA (behavioural/motor neurological scores) followed by Tukey’s multiple comparisons test and denoted as * or #=p<0.05.*
For cohort 2, the filament size was increased to 390 μm with the aim of improving stroke surgery success rate. The occlusion time remained the same but the reperfusion time was increased to 48 h. Animal numbers were low in this experiment (control: n=2, DIO: n=4, DIO-R: n=7) as the first half of animals from cohort 2 were used in an MRI based study which was unexpectedly cut short due to a mechanical malfunction of the scanner. Despite increasing the filament size, still just less than half of animals developed lesions following stroke surgery.

Figure 14: The effect of diet induced obesity on ischaemic stroke (cohort 2). Male Wistar rats fed a high fat diet (HFD) for 12 weeks were divided into diet induced obese (DIO) or DIO-resistant (DIO-R) groups dependent on their weight gain compared to control diet fed rats. Stroke was induced by 60 min middle cerebral artery occlusion (MCAo) with a 390 μm filament followed by 48 h reperfusion before sacrifice. 30 μm thick brain sections were stained with haematoxylin and eosin to visualise the infarct. Lesion volumes (Ai) and representative images are shown (Aii; lesion outlined in black). Behavioural (Bi; high score healthy) and motor neurological (Bii; low score healthy) tests were run before stroke surgery and 48 h post reperfusion. All data presented as mean ± 1 standard deviation (n=1-3). Low animal numbers did not permit statistical analysis.
Animal numbers were too low to statistically analyse the effect of diet on lesion size. However, lesion volumes of each diet group (fig. 14A; control: 37 mm³, DIO: 119±23 mm³, DIO-R: 53±59 mm³) were comparable to those in cohort 1. DIO-R lesion size was much more variable than in cohort 1, with the occurrence of a large cortical lesion in one animal. Deficits in behaviour score was reflective of damage levels (fig. 14Bi) while motor neurological scores indicated greater deficits in the control animal (fig. 14Bii) than had previously been observed in this project in rats with infarcts confined to the striatum.

Figure 15: The effect of diet induced obesity on ischaemic stroke (cohort 3). Male Wistar rats fed a high fat diet (HFD) for 12 weeks were divided into diet induced obese (DIO) or DIO-resistant (DIO-R) groups dependent on their weight gain compared to control diet fed rats. Stroke was induced by 60 min middle cerebral artery occlusion (MCAo) with a 410 μm filament followed by 48 h reperfusion before sacrifice. 30 μm thick brain sections were stained with haematoxylin and eosin to visualise the lesion, for which volumes are shown (A). Behavioural (Bi; high score healthy) and motor neurological (Bii; low score healthy) tests were run before stroke surgery and 48 h post reperfusion. All data presented as mean ± 1 standard deviation (n=2-4). Statistical significance was tested using one way analysis of variance (ANOVA; lesion volume) or 2-way repeated measures ANOVA (behavioural/motor neurological scores) followed by Tukey’s multiple comparisons test (no significant results).
In cohort 3, the filament was again increased in diameter to 410 μm in order to improve the rate of lesion formation. Of animals that underwent successful surgery, just over half went on to develop a detectable lesion by 48 h post reperfusion (control: 3/5, DIO: 2/3, DIO-R: 4/6). The differences in lesion volumes and behavioural/motor neurological scores between the diet groups were non-significant. Control lesion volumes (59±6 mm³) were very similar to the previous two cohorts. Lesion volumes within the DIO and DIO-R groups were very variable (DIO: 96±83 mm³, DIO-R: 63±67 mm³) with large cortical infarcts seen in one DIO and one DIO-R animal (fig. 15A).

**Figure 16:** The effect of diet induced obesity on ischaemic stroke (cohorts 2 and 3). Male Wistar rats fed a high fat diet (HFD) for 12 weeks were divided into diet induced obese (DIO) or DIO-resistant (DIO-R) groups dependent on their weight gain compared to control diet fed rats. Stroke was induced by 60 min middle cerebral artery occlusion (MCAo) with a 390 or 410 μm filament followed by 48 h reperfusion before sacrifice. 30 μm thick brain sections were stained with haematoxylin and eosin to visualise the lesion. Lesion volumes are presented for each diet group (Ai) and for HFD animals as a correlation with 12-week body weight (Aii). Behavioural (Bi; high score healthy) and motor neurological (Bii; low score healthy) tests were run before stroke surgery and 48 h post reperfusion. Data (Ai, B) presented as mean ± 1 standard deviation (n=4-7). Statistical significance was tested using one way analysis of variance (ANOVA; lesion volume) or 2-way repeated measures ANOVA (behavioural/motor neurological scores) followed by Tukey’s multiple comparisons test and denoted as (no significant results). Correlation was tested using Pearson’s Correlation and a two-tailed p-value.
To better understand the stroke outcome of animals 48 h post reperfusion, data from cohorts 2 and 3 were grouped. Grouping did not reveal any significant effect of diet on lesion volume (fig. 16Ai) or post stroke functional outcome (fig. 16B) and there was no significant correlation between body weight and lesion volume in HFD animals (fig. 16Aii).

Although a significant effect of diet on lesion volume was only seen in cohort 1, it is should be noted that across cohorts 1-3 lesion volumes extending beyond the striatum to a large portion of the cortex were only seen in HFD animals. Lesion volumes in control animals ranged between 38 and 60 mm³ while 5 of the 7 DIO animals and 2 of the 11 DIO-R animals had lesions greater than 100 mm³ (fig. 13Ai and 16Ai).

In cohort 4, animals were transferred into the MRI scanner during the occlusion period. Lesion values were calculated from ADC maps generated from DWI scans run during occlusion and 3 h post reperfusion. Once again the filament diameter was increased (410-450 μm) but the rate of lesion formation did not improve. No significant effect of diet was detected at the 3 h time point. However, in contrast to the previous cohorts, relatively large lesion volumes over 100 mm³ were seen in 3 of the 5 control animals at this time point (fig. 17Aii). The range of lesion sizes was also vast in the control group (36-273 mm³) compared to DIO (29-116 mm³) and DIO-R (14-45 mm³) groups (fig. 17Aii). Similar trends were seen in lesion size during occlusion, with very large lesions (>200 mm³) seen in 3 of the 5 control animals (fig. 17Ai). Lesion volume during occlusion was weakly correlated with 3 h lesion volume (fig. 17B; \( r^2 = 0.4978, p<0.05 \)).
Figure 17: The effect of diet induced obesity on ischaemic stroke (cohort 4). Male Wistar rats fed a high fat diet (HFD) for 12 weeks were divided into diet induced obese (DIO) or DIO-resistant (DIO-R) groups dependent on their weight gain compared to control diet fed rats. Stroke was induced by 60 min middle cerebral artery occlusion (MCAo) with a 410 - 450 μm filament followed by 4 h reperfusion before sacrifice. Lesion volumes were derived from apparent diffusion coefficient (ADC) maps generated from diffusion weighted magnetic resonance imaging conducted 45 min through the occlusion period (Ai) and 3 h post reperfusion (Aii). Representative ADC maps are presented (Aiii; red overlay indicates lesion). Data (Ai-ii) is presented as mean ± 1 standard deviation (n=3-5) and statistical significance tested using one way analysis of variance (ANOVA) followed by Tukey’s multiple comparisons test (no significant results). Lesion volumes at the two time points were correlated (B) and analysed using Pearson’s Correlation and a two-tailed p-value.
4.3 Analysing the efficiency of MCAo stroke surgery

In cohorts 1-3, the success rate of MCAo surgery for production of a non-fatal lesion (fig. 18A) was only 60% when using a 350 μm filament (n=9/15), 46% when using a 390 μm filament (n=6/13) and 64% when using a 410 μm filament (n=9/14). Success rates were consistent between the three diet groups, always being within the success range of 50% ± 1 animal (data not shown) and there was no effect of initial body weight on the success of stroke surgery (data not shown).

The mean percentage drop in laser Doppler reading after filament insertion to the base of the MCA was higher in lesioned animals than non-lesioned animals undergoing surgery with a 350 μm filament (fig. 18B). A similar but non-significant relationship is also seen for the other filament sizes (390-410 μm). Note that several non-lesion animals had a Doppler drop over (350 μm: n=3/6; 390 μm: n=3/7, 410 μm: n=2/5) or marginally below (390 μm: n=3/7 [50-59% drop]) the suggested threshold of 60% (Murray et al., 2014), indicating that a Doppler drop ≥60% does not guarantee a lesion forming stroke in these animals (fig. 18B).
Figure 18: The effect of Doccol filament diameter on stroke outcome (cohorts 1-4).
Male Wistar rats fed a high fat diet (HFD) for 12 weeks were divided into diet induced obese (DIO) or DIO-resistant (DIO-R) groups dependent on their weight gain compared to control diet fed rats. Stroke was induced by 60 min middle cerebral artery occlusion (MCAo) with a 350 – 450 μm filament followed by 4, 24 or 48 h reperfusion before sacrifice. The presence of a lesion was assessed visually from brain sections stained with haematoxylin and eosin (350 – 410 μm filaments; 24 and 48 h reperfusion) or from apparent diffusion coefficient (ADC) maps generated from diffusion weighted magnetic resonance imaging (430 – 450 μm filaments; 3 h reperfusion). Frequency of lesion formation (A) and the effect of the reduction in laser Doppler reading above the 60% threshold (red dotted line) at the time of occlusion on lesion outcome (B) are presented. Where appropriate (B) data is presented as mean ± 1 standard deviation (n=2-10) and statistical significance tested using unpaired student’s t-test and denoted as *=p<0.05.
Figure 19: Reduction in laser Doppler reading during and after middle cerebral artery occlusion (cohorts 2 and 3). Stroke was induced in male Wistar rats by 60 min middle cerebral artery occlusion (MCAo) with a 390 – 410 μm filament followed by 48 h reperfusion before sacrifice. The presence of a lesion was assessed visually from brain sections stained with haematoxylin and eosin. The reduction in laser Doppler reading at the time of occlusion, immediately before reperfusion and five minutes after reperfusion are presented as a percentage of pre-occlusion readings. The horizontal red dotted line indicates the 60% reduction threshold.

In cohorts 2 and 3, laser Doppler readings were monitored throughout occlusion and acutely post reperfusion. Of animals who developed a detectable lesion by 48 h post-stroke (n=14), 12 saw a reduction in laser Doppler readings greater than the 60% threshold at the time of occlusion, 11 of which maintained a reduction over this threshold for the duration of the occlusion period (fig. 19Ai). Of the animals which did not develop lesions (n=11), five had an initial reduction in laser Doppler readings over the threshold, four of which maintained the reduction over the full occlusion time (fig. 19Aii).

Acutely following reperfusion (5 min), all except one animal (lesion group) experienced an increase in laser Doppler reading. However, the laser Doppler...
readings did not return to or exceed pre-occlusion levels in all animals (fig. 19A; lesion group: 4/14; non-lesion group: 4/11).

The relationship between the reduction in laser Doppler reading at the time of occlusion and final lesion volume was also examined. However no significant correlation was found (cohort 1: \( r^2=0.01074, p=0.807 \); cohorts 2 and 3: \( r^2=0.1598, p=0.1568 \); cohort 4: \( r^2=0.2916, p=0.1671 \)).

In rats undergoing MRI scanning 430-450 μm filaments were used. Surgery success rates appeared poorer when using these filament sizes (fig. 18A; 430 μm: 40% \([n=3/5]\); 450 μm: 38% \([n=6/16]\)). However, in this experiment, animals were transferred from the surgical bed into the scanner during occlusion. This provides a greater opportunity for premature full or partial reperfusion of the MCA due to movement of the filament. Therefore, the low success rates should not be attributed purely to filament diameter.

To accommodate for the possibility of lack of MCA occlusion, MR angiography was used to visualise blood flow in the MCA in animals in cohort 4. Of animals undergoing successful scanning (filament fully inserted during surgery, angiography and DWI scans performed, survived until 4 h post reperfusion), 56% had MCA occlusions confirmed by angiography (fig. 20Ai; \(n=13/23\)). Lesions were seen during occlusion in all animals with angiography-confirmed occlusions. However, only 69% of these animals still had detectable lesions by 3 h post reperfusion \((n=9/13)\). It should be noted that animals who did not present with 3 h lesions had relatively small lesions during occlusion \((23-99 \text{ mm}^3)\) but that other animals with occlusion lesions in that size range \((n=3)\) had lesions at 3 h post reperfusion (fig. 17B).

To investigate whether an increase in occlusion time would increase the success rate of surgery, an additional small cohort of non-diet rats (554-600 g) underwent stroke surgery with longer occlusion times. A 15 minute increase in occlusion time did not improve surgery success rate \((n=3/6)\). However, the produced lesions all extended into a large part of the cortex (data not shown).
Figure 20: Middle Cerebral Artery (MCA) occlusion occurrence and lesion formation (cohort 4). Male Wistar rats fed a high fat diet (HFD) for 12 weeks were divided into diet induced obese (DIO) or DIO-resistant (DIO-R) groups dependent on their weight gain compared to control diet fed rats. Stroke was induced by 60 min middle cerebral artery occlusion (MCAo) with a 410 - 450 μm filament followed by 4 h reperfusion before sacrifice. Magnetic resonance imaging (MRI) angiography was used to visualise blood flow in the right MCA. Representative images indicate an occluded (top) and a non-occluded (bottom) MCA (Aii). Lesions were identified using apparent diffusion coefficient (ADC) maps generated from diffusion weighted MRI. Occurrence of MCA occlusion and lesion formation are detailed in figure Ai.
5. Discussion

5.1 Characterising the DIO model

As predicted, not all HFD rats gained more weight than controls and therefore HFD groups were divided into DIO and DIO-R groups based on their 12 week weight gain. In all cohorts, DIO animals were distinct from control animals based on final body weight, percentage body fat and abdominal circumference to length ratio, as hypothesised. The DIO-R group defined in each cohort, however, did not have such a well-defined phenotype. Indeed, after 12 weeks of diet, DIO-R animals from cohorts 1, 3 and 4 were similar in body weight to controls, while in cohort 2 DIO-R animals form separate “middle weight” group distinct from either control or DIO groups. Moreover, DIO-R animals have a body-fat content more similar to that of DIO animals than controls in cohorts 1-3. However, in cohort 4 and when data across the four cohorts is considered, body-fat content again indicates DIO-R animals as a “middle weight” group separate from the control and DIO groups. Having a DIO-R group may add a new depth to a future study as it allows the effect of obesity to be separated from the effect of the HFD. However, the primary aim of the current study was to assess the effect of DIO on stroke, thus if DIO-R groups do not present with a consistent phenotype distinct from DIO animals then, unfortunately, this group may not be of use.

It has been suggested that judging obesity in rodents based on body weight and/or weight gain is less reliable than consideration of relative body fat (Nascimento and Sugizaki, 2008). However, in the literature, greater body weight and weight gain are the standard characteristics used to define DIO from DIO-R rats (Mrad et al., 1992; Nascimento and Monte-Alto-Costa, 2011; Oliveira Junior et al., 2010). It should be noted that, in this study, body fat content is based only on epididymal and/or perirenal fat pad weights and thus may not be a true representative of total body fat. It may be that DIO animals have greater fat deposit elsewhere, for example subcutaneously or within organs of the body (e.g. liver; Carmiel-Haggai et al. 2005), which are contributing to their greater weight. Alternative methods of body fat quantification could be used in future to better understand the difference in total body adiposity in the diet groups. For example, quantitative magnetic resonance (QMR) is a non-invasive method which differentiates between lean and fat body mass based on differences in their nuclear magnetic resonance properties (Miller et al., 2011).
In addition to measurement of body weight, proportions and adiposity, it would be informative to assess the inflammatory profile of animals in this dietary model. In obesity, elevated cytokine expression has been reported in the adipose tissue (Jernås et al. 2006) and the circulation (Christiansen et al., 2005; Malavazos et al., 2007; Sartipy and Loskutoff, 2003; Takahashi et al., 2003). Therefore, identification of heightened systemic inflammation would strengthen the definition of DIO in this model. Pre-surgery blood plasma samples were collected and are available for this purpose. Organ samples were also taken but were harvested post stroke and therefore are unsuitable for this purpose as stroke can cause changes in peripheral inflammation (Offner et al., 2006; Wang et al., 2011). A naïve cohort of diet model animals, on which no surgery is performed, may be useful in future for this purpose.

Hypertension is the primary risk factor for stroke (Lawes et al., 2008) and can be caused by obesity (Narkiewicz, 2006). In this study, systolic blood pressure was raised in DIO rats compared to controls in one of the three cohorts tested (cohort 1) and when data was considered across the three cohorts. In cohort 1, rats were not habituated to the restrainer before measurements were taken suggesting that restraint stress may have altered initial findings, as previously reported (Behringer et al., 2009). It should be noted that the systolic blood pressure of HFD animals was largely consistent between the cohorts, while for controls it was lower in the first cohort. This suggests that the indication of elevated blood pressure in DIO animals in cohort 1 may actually be a reflection of unusually low blood pressure in the control group of this cohort. However, the relatively higher systolic blood pressure seen in DIO animals when data from the three cohorts was combined suggests hypertension may be present in this model of obesity.

Though DIO animals could be selected based on weight gain, they accounted for less than half of the HFD animals and the difference in final body weight between the lightest DIO rats and the heaviest control rats was minimal. It was noted that by 12 weeks on diet (~18 weeks of age), control animals are still gaining weight steadily suggesting at this stage rats are not fully grown. Indeed, the growth curve for male Wistar rats levels out after 24 weeks of age (Zhao and Gregersen, 2015). Therefore, if the diet period was extended to 16 or 20 weeks and started at the age of 10 weeks old, final body weights would be much more indicative of adiposity rather than greater lean body mass and thereby produce a stronger DIO phenotype. The suggested diet duration would be in keeping with previously published DIO rat models which range from 8 to 20 weeks in length (Rosini et al., 2012)
5.2 The effect of obesity on ischaemic stroke

Due to the low success of stroke surgery producing lesions, it is hard to draw definitive conclusions regarding the effect of obesity on stroke. However, the preliminary data suggests that DIO rats may experience poorer outcome from ischaemic stroke. In cohort 1 this was indicated by greater infarct volumes and poorer behavioural scores compared to controls and looking across the first three cohorts it was noted that large lesions (extending beyond the striatum into a large part of the cortex) were only seen in HFD animals and not in controls. These results would be consistent with published data from genetically obese mice and rats undergoing transient ischaemic stroke (McColl et al., 2010; Pradillo et al., 2012; Terao et al., 2008) and DIO rats undergoing MCAo (Deutsch et al., 2009; Langdon et al., 2011; Maysami et al., 2015).

However, a very different pattern was seen in cohort 4. No effect of diet was detected and, in contrast to the previous cohorts, very large lesions were present in some members of the control group. This experiment aimed to detect the acute development of lesions using DWI and therefore lesion volume was measured at a much earlier time point and using a different method than in the previous cohorts.

DWI allows the identification of infarcted brain tissue by detecting oedema formation which develops very rapidly after stroke. Water is drawn to the ischaemic core by osmosis due to the high concentration of sodium and calcium ions found within the cells which have undergone metabolic arrest (Dirnagl et al., 1999). Thresholding the ADC map at 77-83.5%, compared to the contralateral hemisphere, has been shown to correlate well to infarct area as assessed by histological staining (Lo et al., 1997; Murray et al., 2014). However, other studies report the unreliability of ADC-maps for accurately representing ischaemic damage.

One study reported lesions calculated from ADC-maps 5 h post ischaemia were much smaller than those detected by histological methods at that time point. Also, in the same study, ADC map derived lesions at 3 h post reperfusion were significantly smaller than at 0.5 and 24 h after ischaemia (Ringer et al., 2001). Fluctuation in the size of lesions derived from ADC-maps in the days following cerebral ischaemia has also been reported in humans (Fiehler et al., 2002). A review of multiple studies using ADC-maps to calculate lesion volumes reports that there is strong overlap in ADC values for of penumbral and lesioned tissue (Kidwell et al., 2003). Together
these studies imply that while DWI can be predictive of infarct formation, histological confirmation of final lesion volume is more accurate.

In this cohort, histological analysis of lesion volume has not been performed to confirm the ADC map derived lesion volumes. When the brains of the rats with large cortical lesions were removed after sacrifice, lesions were visible to the naked eye as areas of pale tissue (data not show). However, the presence of striatal lesions remains unconfirmed.

Consideration of laser Doppler data revealed no significant correlation with lesion size in any of the cohorts, suggesting that variation in blood flow velocity in the MCA territory induced by the surgical occlusion is not responsible for variation in final lesion volumes.

Overall, data produced from the stroke studies is insufficient to confidently conclude an effect of the DIO model on ischaemic stroke outcome. This is partially due to unexpectedly low animal numbers caused by low rates of lesion formation following stroke surgery. Initial data from the first cohort suggested an exacerbation of ischaemic damage in DIO animals but later cohorts did not demonstrate an effect of diet. It is also possible that 12 weeks of HFD was not long enough to produce a DIO phenotype strong enough to have an effect on stroke outcome.

5.3 Optimising MCAo surgery in 12-week diet rats

An ongoing problem throughout the project was the poor rates of lesion production from stroke surgery. Though the surgery technique is established in our research group, the rats used in this study are larger and older (>500 g, ~18 weeks) than those used in previous studies (~350 g, ~9 weeks). Initially, 350 μm filaments were used, as have been used in previous studies. Following the low success rate in the first cohort, a hypothesis was developed that larger rats have wider cerebral vessel lumens and therefore require a wider diameter of filament to fully occlude the MCA. However, despite increasing the filament diameter for each successive cohort, little improvement was made in the surgery success rate.

Previous studies from our research group have confirmed successful occlusion of the MCA by a 60% drop in laser Doppler reading (Murray et al., 2014). In the current study, reaching the 60% threshold did not always predict lesion development,
suggesting perhaps the 60 min occlusion time was insufficient. However, when occlusion time was increased to 75 min, an increase in lesion volume was seen but no improvement in surgery success rate.

Premature partial reperfusion in these rats may account for their lack of lesion, as previously reported (Schmid-Elsaesser et al., 1998). However, when laser Doppler readings were monitored throughout the occlusion period (cohorts 2 and 3), maintenance of a reduction above the 60% threshold did not ensure lesion formation either.

Although increasing the filament size enhanced the average Doppler drop, a number of animals failed to reach the 60% reduction threshold suggesting a lack of occlusion in some animals. In cohort 4, occlusion of the MCA was confirmed by MRI angiography ~45 min through the occlusion time. At this time point, blood flow was still detectable in the right MCA of just under half of the animals indicating lack of occlusion was responsible for the lack of lesion formation. This data may underestimate the rate of occlusion as transfer of the animals into the scanner cradle may have caused premature reperfusion in some animals.

In cohort 4, not all animals with occlusions went on to develop detectable lesions by 3 h post reperfusion, suggesting that a 60 min MCAo was not sufficient to induce an ischaemic infarct. However, as discussed above, ADC-map derived lesions at this time point may not be a reliable indication of the final infarct (Fiehler et al., 2002; Ringer et al., 2001).

Lack of MCA occlusion is the most likely reason for lack of lesion formation. At this body size, variation in cerebral vessel lumens may be more variable meaning there is not an optimum size of filament to occlude the MCA. The intraluminal filament MCAo model was chosen for this study based on the good control over occlusion duration and the option for future MRI studies of CBF following remote reperfusion as performed in previous studies (Murray et al., 2014). However, an alternative method of ischaemic stroke should now be considered. The MCA can be mechanically occluded, for example by use of clips, but this surgery requires a craniotomy. Techniques exist which induce clot formation within the vessel, including thrombin injection and photothrombosis. Reproducible infarcts are produced from the pro-thrombotic models, although time of reperfusion is not easy to control perhaps making these models less suited to studying post-reperfusion
related events (Mergenthaler and Meisel, 2012). Focal vasoconstriction induced by direct injection of endothelin-1 into the brain can also be used to model ischaemic stroke (Windle et al., 2006), but would not be suitable for the future studies of CBF acutely after stroke.
6. Conclusion and future work

Overall, from the current study, a conclusion regarding the effect of the DIO model on ischaemic stroke cannot be drawn. The split of HFD into DIO and DIO-R groups and the poor rate of lesion formation following stroke surgery resulted in very low animal numbers, which in turn left the studies underpowered to produce conclusive data.

The DIO model could be improved by extending the diet period beyond the age at which lean weight gain declines in order to see a more defined DIO phenotype. Further characterisation of the DIO model would be beneficial, including analysis of systemic inflammation and total body adiposity. The intraluminal filament model of MCAo has thus far proved ineffective in rats of this size, therefore an alternative ischaemic stroke model may have to be considered.

7. Summary

In summary, obesity increases ischaemic damage after stroke through mechanisms that are not well understood. Co-morbidities that share common features with obesity have been shown to affect CBF after stroke thus inducing greater cerebral damage. The animal models developed in the current study require further work to enable studies into the effect of obesity on CBF following stroke. However, understanding the relationship between obesity and enhanced ischaemic damage remains an important avenue of research which will aid future development of treatments for this subtype of stroke patients.
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