REVASCULARISATION OF TYPE 2 DIABETICS WITH CORONARY ARTERY DISEASE: INSIGHTS AND THERAPEUTIC TARGETING OF *O*-GlcNAcYLATION

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Word count: 3,439 plus 225 Abstract

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ABSTRACT

Background and Aim:

Coronary artery bypass graft (CABG) using autologous saphenous vein continues to be a gold standard procedure to restore the supply of oxygen-rich blood to the heart muscles in coronary artery disease (CAD) patients with or without type 2 diabetes mellitus (T2DM). However, CAD patients with T2DM are at higher risk of graft failure. While failure rates have been reduced through improvements in procedure-related factors, much less is known about the molecular and cellular mechanisms by which T2DM initiates vein graft failure. This review gives novel insights into these cellular and molecular mechanisms and identifies potential therapeutic targets for development of new medicines to improve vein graft patency.

Data Synthesis:

One important cellular process that has been implicated in the pathogenesis of T2DM is protein *O*-GlcNAcylation, a dynamic, reversible post-translational modification of serine and threonine residues on target proteins that is controlled by two enzymes: *O*-GlcNAc transferase (OGT) and *O*-GlcNAcase (OGA). Protein *O*-GlcNAcylation impacts a range of cellular processes, including trafficking, metabolism, inflammation and cytoskeletal organisation. Altered *O*-GlcNAcylation homeostasis have, therefore, been linked to a range of human pathologies with a metabolic component, including T2DM.

Conclusion:

We propose that protein *O*-GlcNAcylation alters vascular smooth muscle and endothelial cell function through modification of specific protein targets which contribute to the vascular re-modelling responsible for saphenous vein graft failure in T2DM.

Keywords: Coronary artery disease; Coronary artery bypass graft; Type 2 diabetes mellitus; Protein *O*-GlcNAcylation.

Abbreviations list: CAD, coronary artery disease; CABG, coronary artery bypass graft; IMA, internal mammary artery; T2DM, type 2 diabetes mellitus; HSV, human saphenous vein; SMC, smooth muscle cell; EC, endothelial cell; OGT, *O*-GlcNAc transferase, OGA, *O*-GlcNAcase; SVG, saphenous vein graft.

Introduction

Cardiovascular diseases (CVDs) are the leading non-communicable cause of mortality worldwide [1]. Of key interest and significance among these is coronary artery disease (CAD). CAD arises due to accumulation of cholesterol and chronic inflammation at susceptible sites on the coronary arterial wall, resulting in the formation of atherosclerotic plaques. Plaque formation is due in part to dysfunctional responses of the endothelium to haemodynamic stress and changes in blood flow, resulting in increased expression of proteins such as superoxide dismutase which activates intracellular signalling pathways to sustain a prothrombotic and proinflammatory phenotype [2, 3]. As plaques grow, they can narrow the arteries and reduce blood flow to the heart muscle, resulting in angina or, in response to plaque rupture and thrombosis, myocardial infarction and risk of death.

One important approach to manage CAD is coronary artery bypass graft (CABG) surgery, which typically utilises autologous saphenous vein as a conduit vessel to improve coronary blood flow in these patients [4](Figure 1). A large body of evidence suggests that grafting using the internal mammary artery (IMA) gives better outcomes than saphenous vein [5]. However, the greater saphenous vein, which is the longest vein in the body, is more utilised than IMA because it is not always possible to attain full revascularisation with arterial grafts [5]. Meanwhile, CAD patients with type 2 diabetes mellitus (T2DM) are more vulnerable to vein graft failure following CABG, a phenomenon which arises from specific alterations in human saphenous vein smooth muscle cell (HSV-SMC) and endothelial cell (EC) phenotype that trigger vascular re-modelling [6]. There is a strong possibility that molecular mechanisms specific to T2DM are responsible for this and one of such possible mechanisms is protein *O*-GlcNAcylation. This process requires the enzymatic synthesis of *O*-GlcNAc, the donor substrate for target proteins in *O*-GlcNAcylation, which is entirely dependent on availability of glucose. Furthermore, maintenance of *O*-GlcNAc

homeostasis is essential for optimal cellular function, and its disruption may contribute to the pathogenesis of human diseases with a metabolic component such as T2DM [7]. While cellular *O*-GlcNAcylation levels are maintained by the mutual regulation of *O*-GlcNAc transferase (OGT) and *O*-GlcNAcase (OGA), sustained hyperglycaemia which is typical in T2DM can alter the balance in favour of OGT-mediated *O*-GlcNAcylation [7]. This strengthens the possibility that protein *O*-GlcNAcylation, a glucose-dependent post-translational modification that links multiple metabolic pathways with protein function, can trigger HSV-SMC and EC dysfunction through modification of key protein targets [8].

1. Coronary artery disease

CAD develops when the coronary arterial vasculature cannot supply enough oxygen- and nutrient-rich blood to the heart. CAD is responsible for over 65,000 deaths per year in the UK alone [10]. It has a significant impact on people's lives, including their quality of life, future employment and personal relationships, as well as increasing the risk of premature death [10]. A major factor that limits effective management of CAD is that symptoms are not detectable until affected coronary arterial branches are profoundly dysfunctional. Symptoms may include angina, shortness of breath, fatigue and weakness. There are several complications of CAD and while some, such as abnormal heart rhythm or arrhythmia, heart failure and blood clots in the artery due to ruptured plaque(s) can be managed without hospitalisation; myocardial infarction which is the major cause of mortality among CAD patients would require immediate hospitalisation and management [11].

Patients with diabetes, particularly T2DM which accounts for over 90% of diabetes cases, are at increased risk for developing cardiovascular disorders, including CAD and stroke. Globally, 50 to 80% of T2DM patients have CAD [12]. T2DM is a risk factor for CAD and, when these two disease conditions co-exist in an individual, there is typically a worse prognosis compared to their individual presence [12]. Importantly, several mechanisms involved in the pathophysiology of CAD and T2DM are conserved; these include obesity as a risk factor, chronic inflammation, oxidative stress and insulin resistance [13], all of which have been reported to display altered O-GlcNAc homeostasis [8].

3. Coronary artery bypass graft (CABG)

CABG remains a goal standard in the management of patients with CAD [14, 15], and around 20,000 are carried out in England every year [15]. It utilises blood vessels from other parts of the body, such as the IMA from the chest, the radial artery from the arm and the greater saphenous vein from the leg [15]. These blood vessels are attached to the coronary artery below the area of atherosclerotic narrowing, thereby "bypassing" the affected vessel [16] (Figure 1). However, CABG failure is a well-established phenomenon which puts patients at risk of recurrent angina, with the need for repeated coronary revascularisation to reduce the risk of myocardial infarction. The use of IMA has been demonstrated to improve outcomes, with vessel patency 10 years post-surgery reported to be 85-91% [16]. In contrast, the rates of saphenous vein graft (SVG) failure at 1-year post surgery have been quoted at between 10% and 25% (5, 17). From 1 to 5 years a further 5% to 10% SVGs will occlude, and from years 6 to 10 an additional 20–25% will fail, [5, 17, 18] meaning that after 10 years, SVG patency rates are approximately 50%, with only half of these devoid of vessel atheroma [5, 19]. However, even with the greater risk of SVG failure

compared to arterial grafts, it continues to be the preferred option for CABG as it is not always possible to attain full revascularisation by arterial grafts [5].

Multiple factors are thought to be responsible for SVG failure. Most of the focus is on graft-related, patient-related and surgery-related factors, meanwhile, minimal attention has been given to underlying cellular and molecular mechanisms responsible for SVG failure. For the graft-related factor, the type of artery or vein grafted and the coronary flow are key factors to be considered. Similarly, graft diameter, the presence of focal stenosis and the size of the distal perfusion bed affects the desired perfusion post-CABG, while reduced flow has been associated with greater neointimal proliferation in SVGs [20]. SVGs to the left anterior descending artery have the best patency, followed by those to diagonals, circumflex branches and the posterior descending artery, with grafts to the main right coronary artery least likely to have long-term patency [21]. Also, patient-related factors such as age, gender, and other underlying morbid conditions such as T2DM, left ventricular hypertrophy, and renal insufficiency could result in CABG failure [22]. Improved understanding of these factors has helped improved CABG patency.

3.1. Surgery-related factors predisposing to SVG failure

Many factors predispose patients to complications after CABG, some of which include size differences between the graft and the artery, graft kinking, poor distal runoff, and small target vessel diameter [5]. Over the years, studies have shown that variation in surgical techniques influences SVG patency and outcomes [5]. Some of these variations are highlighted below.

3.1.1. Variation in on-pump and off-pump surgery

In on-pump CABG, the heart is rendered motionless using cardioplegia solution and blood supply to the rest of the body is ensured with the use of the cardiopulmonary bypass machine (also known as the heart-lung machine or the pump). In this case, the operative conditions are more favourable to attain good vascular anastomoses. Conversely, off-pump CABG which is considered as the newer method aims to achieve the same outcome without using a heart-lung machine or cardioplegia solution. The procedure is performed with the heart beating and special devices are used to mechanically stabilise the relevant part of the heart so that suturing can be performed on a relatively immobile platform [23, 24]. Large randomized studies and meta-analyses have shown that off-pump procedures result in poorer 1-year composite outcomes and graft patency compared to on-pump CABG [25, 26]. These differences may be due to the relative hypercoagulability seen with off-pump compared to on-pump procedures [5]. The use of cardiopulmonary bypass for on-pump surgery induces platelet dysfunction and coagulopathy that are desirable for promoting SVG patency [5, 27].

The choice of procedure normally depends on the comfort level of the surgeon performing the procedure, but, of the 2 techniques, on-pump CABG is the most commonly used method [23]. Although peri- and post-CABG complications, such as stroke, kidney or liver failure, decrease in cognitive function and bleeding, are more associated with the on-pump technique, these complications are lower with the off-pump technique especially in high risk patients [23].

3.1.2. Sequential and composite grafting

The use of sequential and composite SVG grafting is normally reserved for cases with lack or shortage of conduits. In contrast to the single graft, which is composed of a single distal anastomosis for every proximal anastomosis, sequential and composite grafting involves the use of more than one distal anastomosis for every proximal anastomosis so as to attain full revascularisation (Figure 2). The sequential anastomosis may allow for a larger combined perfusion bed, resulting in reduced vascular resistance and increased flow velocity compared to a single graft [28]. The complete revascularisation achieved with sequential and composite grafting is, therefore, more beneficial in patients with multi-vessel CAD as the limited SVG conduit is utilised more efficiently [29].

Early data suggested that clinical outcomes from multiple distal target SVGs were either comparable or better than single distal target SVGs when the graft anastomoses are performed correctly [31]. However, larger more recent studies suggest that those with multiple distal target SVGs are more likely to have graft failure and are at higher risk of death, myocardial infarction, or repeat revascularisation five years post-CABG [32]. This is common with diabetic patients, where both the end-to-side and side-to-side anastomosis may insert into a poor-quality target vessel [33]. In practice composite grafts are normally reserved for arterial conduits when two IMAs or IMA and radial arteries are used.

3.1.3. No-touch technique

Prior to the development of “no-touch” SVG harvesting by Souza *et al.* 1996 [5], the formation of thrombi within grafts due to intra-operative manual disruption of the endothelium and

hydrostatic dilation was a key challenge post-SVG [34]. No-touch SVG harvesting allows for the pedicled SVG to be removed with the perivascular tissue still intact. This technique has improved graft patency compared to the conventional harvesting technique [35]. More recent data obtained from randomized studies have further suggested that no-touch harvesting results in less intra-operative vascular SMC activation compared with conventional harvesting, thereby reducing the risk of neointimal hyperplasia responsible for long-term CABG failure [36].

3.1.4. Compression therapy

Luminal diameters of HSVs are typically larger than that of the coronary artery, potentially creating abnormal blood currents within grafted veins that can damage the vessel and increase the risk of thrombus formation. Also, as veins do not have the thick muscular walls found in arteries, the increased flow rates found in the arterial circulation can potentially damage the SVG and induce an adaptive thickening of the vessel wall that induces the development of neointimal hyperplasia [5, 37]. A technique involving use of external compression from a support device implanted during surgery has been developed to mitigate this variation. External compression of SVGs prevents dilation and promotes down-sizing, which has proven to enhance arterial-like healing and reduce the development of neointimal hyperplasia [5, 37].

4. Insights on the role of protein *O*-GlcNAcylation in vein graft failure in T2DM

Over the years, advances in surgical techniques have improved outcomes, yet, there is currently no therapy targeting the molecular mechanisms responsible for vein graft failure (VGF). One of these molecular mechanisms which is implicated in the pathogenesis of T2DM diseases is protein *O*-GlcNAcylation. This is a dynamic, reversible post-translational glycosylation of serine

and threonine residues in target proteins which is controlled by just two enzymes: OGT and OGA. Protein *O*-GlcNAcylation impacts a range of cellular processes, including trafficking, metabolism, inflammation and cytoskeletal organisation. Altered *O*-GlcNAcylation profiles have, therefore, been linked to a range of human pathologies with a metabolic component, including T2DM [8].

From the findings of Olsen *et al* [38], glucose metabolism through the hexosamine biosynthetic pathway as determined by the rates of glycolysis and UDP-N-acetylglucosamine (UDP-GlcNAc) synthesis in *ex vivo* mouse heart is ~0.006% of the glycolytic efflux. The hexosamine biosynthetic pathway is a unique nutrient-sensing metabolic pathway that produces the activated amino sugar UDP-GlcNAc, a critical substrate for protein glycosylation. In this pathway (Figure 3), rate-limiting enzyme L-glutamine-D-fructose 6-phosphate amidotransferase transfers an amino group from glutamine to fructose-6-phosphate to form glucosamine-6-phosphate (GlcN-6-P). GlcN-6-P is then rapidly acetylated by glucosamine 6-phosphate *N*-acetyltransferase and isomerized to *N*-acetyl-1-phosphate glucosamine. Then the nucleoside is added to the sugar by UDP-*N*-acetylhexosamine pyrophosphorylase 1 to yield UDP-GlcNAc [8, 39].

UDP-GlcNAc serves as the sugar donor for classical glycosylation events occurring in the endoplasmic reticulum and Golgi as well as *O*-GlcNAc modification of proteins by OGT in the nucleus, cytoplasm and mitochondria which are the major intracellular sites of OGT expression [40]. OGT, an enzyme which is encoded by the *OGT* gene in humans, is responsible for catalyzing the addition of a GlcNAc moiety through an *O*-glycosidic linkage to the free hydroxl group on either serine or threonine residues in target proteins [8, 40]. Three isoforms (ncOGT, mOGT and sOGT) of human OGT are produced from the *OGT* gene. ncOGT, which has been localized to both the nucleus and cytoplasm, is the longest isoform. It contains a unique *N*-

terminal sequence, followed by 12 tetratricopeptide repeats (TPR) motifs, a linker region, and the catalytic domain. mOGT contains a different *N*-terminal sequence, which also encodes a mitochondrial targeting sequence. The *N*-terminal sequence is then followed by 9 TPR motifs, a linker region, and the catalytic domain. sOGT which is the shortest isoform is ubiquitously expressed within the cell. It consists of only 2 TPR motifs, a linker region, and the catalytic domain. The catalytic region in all three isoforms is identical and contains two domains, the CD I domain and the CD II domain [40]. Conversely, the enzyme OGA reverses this *O*-GlcNAc modification of proteins by catalyzing the hydrolysis of *O*-GlcNAc from protein targets [40]. The *O*-GlcNAc modification occurs on a wide variety of proteins such as nuclear pore proteins, RNA polymerase II, transcription factors, cytoskeletal proteins, proteasome components, synapsins, oncogenic proteins and tumor suppressor proteins [40]. Over 4000 *O*-GlcNAcylated proteins have now been identified and these play key roles in cellular and biological processes including transcription, epigenetic regulation, homeostasis and stress responses [41]. This post-translational modification, therefore, has an important role in all cell types and altered homeostasis will impact on the function of ECs and SMCs [8].

Experimental studies have shown that acute increases in protein *O*-GlcNAcylation in response to stress can suppress inflammation and enhance cell survival as part of a protective adaptive mechanism [42-44]. For example, Xing et al. [42] demonstrated that increasing O-GlcNAc levels through administration of either glucosamine or non-selective OGA inhibitor PUGNAc in rats reduced induction of the adhesion molecules P-selectin and VCAM-1 and neutrophil-selective chemokine CINC-2β in following carotid artery injury. However, a large body of literature now supports that altered *O*-GlcNAcylation can also impact on a number of cellular processes that are pertinent in vascular dysfunction. Increased *O*-GlcNAc in T2DM is an established phenomenon

and this has been shown to cause overproduction of reactive oxygen species (ROS) via activation of NADPH oxidase [45]. Similarly, increased activation of HBP with high-glucose which is typical in T2DM patients, induces the production of ROS [46, 47]. Furthermore, *O*-GlcNAcylation depletion by shRNA-mediated knockdown of OGT has been shown to prevent high glucose-induced ROS production in mesangial cells [46, 48]. These findings strongly suggest significant interplay between redox signaling and *O*-GlcNAcylation modification in diabetes [46]. While our understanding of how the *O*-GlcNAcylation-induced ROS role in vascular dysfunction continues to grow, recent findings have proposed that ROS modulates the activities of miR-200 family of microRNAs [49]. MicroRNAs (miRs) play an essential role in mediating the post-transcriptional regulation of the endothelial oxidative response [46, 50] and particularly, members of the miR-200 family are highly sensitive to ROS [46]. Specifically, H_2O_2 has been shown to regulate the miR-200c at the transcriptional level, as pri-miR-200c-141 and miR-200c and miR-141 common promoters were upregulated by H_2O_2 [49]. More so, overexpression of miR-200 has been reported to be involved in diabetes-induced inflammation, and diabetes-induced endothelial dysfunction [46].

Furthermore, ROS stimulates protein kinase C (PKC) activity, which leads to increased production of vascular endothelial growth factor (VEGF) and activation of the pro-inflammatory transcription factor nuclear factor-κB (NF-κB) [51]. These result in the activation of ECs which secrete a range of inflammatory cytokines such as tumor necrosis factor alpha (TNFα) and interleukin 1 (IL-1) [52]. There is also an increase in expression of adhesion proteins on the cell surface of ECs, facilitating the recruitment and infiltration of immune cells such as monocytes [52]. The monocytes differentiate into macrophages, which is accompanied by increased expression of pattern recognition receptors on their surface, that participate in the promotion of

inflammation and uptake of modified LDL, ultimately leading to the formation of lipid laden foam cells. Continued accumulation of modified LDL together with disturbed cellular lipid homeostasis causes apoptosis of foam cells resulting in lipid deposition and amplification of the inflammatory response [52]. SMCs therefore migrate from the media to the intima where they proliferate, uptake modified lipoproteins and secrete extracellular matrix proteins that stabilize the plaques [53]. Continued inflammation orchestrated by cytokines destabilizes such plaques via decreased production of extracellular matrix proteins, increased production of extracellular matrix-degrading matrix metalloproteinases and reduced expression of inhibitors of these enzymes [52]. Foam cells, which are laden with accumulated lipids, eventually rupture in the tunica intima. The accumulated lipids, pro-inflammatory cytokines and growth factors combine to trigger atheroma formation in grafted veins that can result in SVG failure [52, 53].

Also, Lo *et al* [46] further showed that high glucose induced OGT expression in human aortic endothelial cells and that increased OGT expression and protein *O*-GlcNAcylation is implicated in endothelial inflammation, as high glucose induced ICAM-1, VCAM-1, and E-selectin mRNA expression; ICAM-1 expression; and THP-1 monocytic cell adhesion were reduced after OGT depletion by targeted short inhibitory RNA [46]. *O*-GlcNAcylation induced endothelial inflammation is constitutively augmented in a chronic hyperglycaemic state typical of poorly controlled T2DM [46]. In HSV ECs, this would sustain a pro-inflammatory environment critical for formation of a neointima, resulting in a progressive loss of patency that could cause VGF. Conversely, when HSV segments are grafted into the coronary circulation, they need to adjust to the increased shear of arterial blood flow by increasing SMC proliferation, thereby making the wall thicker. However, this ultimately becomes pathological as the cells migrate towards the lumen to form a neointima.

306 Furthermore, studies have also suggested that excessive *O*-GlcNAc modification can occur at
307 multiple loci within the insulin receptor/IRS/PI3K/Akt/eNOS pathway to reduce nitric oxide
308 production in the endothelium [54-57]. This impacts adversely on vascular function due to a
309 downregulation of the vasodilatory and protective roles of nitric oxide pertinent in vascular
310 dysfunction. A recent study [58] also showed that *O*-GlcNAcylation mediated glucose-induced
311 impairment of eNOS activation in endothelial cells from patients with T2DM, resulting in altered
312 endothelial cell phenotype. In this study [58], freshly isolated endothelial cells obtained by J-
313 wire biopsy from a forearm vein of patients with T2DM were compared with those from non-
314 diabetic controls. The study further showed that endothelial *O*-GlcNAcylated protein levels were
315 higher in T2DM patients when compared with non-diabetic controls. It was also observed that
316 while the normal physiological glucose concentrations (5 mmol/L) lowered *O*-GlcNAc levels
317 and restored insulin-mediated activation of eNOS in the endothelial cells from patients with
318 T2DM, elevated glucose concentrations (30 mmol/L) maintained both *O*-GlcNAcylated protein
319 levels and impaired insulin action. Treatment of endothelial cells with the OGA inhibitor
320 Thiamet G increased *O*-GlcNAc levels and blunted the improvement of insulin-mediated
321 endothelial nitric oxide synthase phosphorylation in response to glucose normalization [58].
322 These findings strongly suggest that *O*-GlcNAc is an important mediator of vascular endothelial
323 dysfunction in T2DM.

324 These evidences strongly suggest that augmented protein *O*-GlcNAcylation play a key role in the
325 pathogenesis of vascular dysfunction and VGF in T2DM. In recent years, targeting protein *O*-
326 GlcNAcylation has yielded viable therapeutic options in disease conditions such as cancer [8, 59-
327 62], and neurodegenerative disorders [8, 63-66], which we believe can be further explored in
328 VGF. More so, as the principal enzymes that control the protein *O*-GlcNAcylation process and

its reversal have been determined and in recent years, the development of an *O*-GlcNAc-specific antibody and other affinity purification approaches coupled with advances in mass spectrometry to identify *O*-GlcNAcylated targets have all aided our understanding of this dynamic cellular process. Also, further advances in the identification of *O*-GlcNAc sites and generation of highly specific inhibitors of the enzymes afford us the opportunity to further explore this dysregulation in specific cell types and disease states for development of new therapeutic agents.

Funding

Work in TMP's laboratory is supported by the Hull and East Riding Cardiac Trust Fund. IOB is supported by scholarships from the Tertiary Education Trust Fund (Tetfund), Nigeria (TETF/ES/UNIV/EDO STATE/TSAS/2019/VOL.1) and University of Benin, Nigeria.

Declaration of Competing Interest

The authors declare no conflict of interest.

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Figure Titles and Legends

549 Figure 1: Saphenous vein bypass graft. (a) Section of the leg showing the great and small
550 saphenous veins (b) Grafted vein bypassing point of coronary artery blockade. Originally, the
551 picture (a) was from (http://www.surgery.usc.edu/vascular/
552 varicoseveinsandvenousdisease.html), the picture (b) was after (https://atlasofscience.org/a-
553 novel-treatment-for-saphenous-venous-graft-thrombosis/). Images adapted from (9).

554

555 Figure 2**:** Patterns of composite grafting with sequential bypass. (Top row, from left to right)
556 Left internal thoracic artery *(LITA)* with a Y-composite graft; right internal thoracic artery
557 *(RITA)* with a Y-composite graft; and RITA with an I-composite graft. (Bottom row, from left to
558 right) RITA with a U-composite graft; gastroepiploic artery *(GEA)* with an I-composite graft;
559 and GEA with a U-composite graft. Adapted from (30).

560

561 Figure 3: The hexosamine biosynthetic pathway. Glucose enters the cell through the glucose
562 transporter and is metabolized to yield UDP-GlucNAc that serves as common precursor for all
563 amino sugars used for the synthesis of glycoproteins, lipids, and proteoglycans. Adapted from
564 (39). GFAT, L-glutamine-D-fructose 6-phosphate amidotransferase.

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