Final Report Form

Final reports **must be submitted within six months** of the end date of the grant. Please complete sections A and B of this form and email as a Word document to research@heartresearch.org.uk

Please note that the final payment of the grant will be withheld until the Charity receives a satisfactory final report

Grant reference: RG2651
Grantholder(s): Professor Annette Graham and Dr Yvonne Dempsie
Departmental address and email: Department of Biological and Biomedical Sciences, School of Health and Life Sciences, Glasgow Caledonian University, 70 Cowcaddens Road, Glasgow G4 0BA
Grant start and end dates: 01/10/2016 to 30/9/2019
Date of report:
Title of project: 'MicroRNA sequences involved in conversion of human macrophages to 'foam' cells: identification of novel therapeutic targets'

SECTION A: SCIENTIFIC REPORT

This section can be up to **4 sides of A4**, Arial 11 point font

1) Background to the project and aims of the research

Atherosclerosis is the principal cause of coronary heart disease, and is characterised by chronic inflammation and accumulation of lipoprotein-derived cholesterol and cholesteryl esters by arterial macrophages. A screen of microRNA sequences (miRs) differentially regulated during conversion of human macrophages to cholesterol-laden foam cells revealed three sequences (from 84 tested) increased by >2-fold, one of which (miR-302a) is established as a novel modulator of cholesterol homeostasis and atherogenesis in mice. The other two sequences, hsa-miR-150-5p and hsa-let-7d-5p, which we confirmed expressed in human aorta, therefore required further study. This studentship was designed to investigate the therapeutic potential of these miRs by (i) interrogating their expression in sterol loaded macrophages exhibiting a defined inflammatory phenotype, (ii) investigating molecular characteristics and key targets of these sequences and (iii) determining impact of mimics and inhibitors of these miRs on genesis of macrophage 'foam' cells.

2) Details of the results obtained and what you discovered and achieved

(i) Induction of miRNA sequences in THP-1 macrophages exhibiting sterol accumulation and an 'M2-like' phenotype

Human THP-1 macrophages were differentiated for 7 days (PMA; 250nM) and incubated with native LDL (75μg/ml; 24h) or the same concentration of LDL modified using copper ions, hypochlorous acid and acetic anhydride; peroxynitrite modified LDL was also tested, but eliminated due to variable induction of cytotoxicity. Both native and modified forms of LDL induced an anti-inflammatory 'M2-like' phenotype, evidenced by modest reductions in *IL6* and *TNFA*, and marked increases in *IL10* gene expression. Oxidized (Cu^{2+}) and acetylated LDL (Ac-LDL) markedly increased expression of scavenger receptors, CD36 and CD68, and variously induced significant reductions in expression of Sterol Regulatory Element Binding Protein (SREBP)-2, HMG CoA reductase and the LDL receptor. These outcomes, combined with increased expression of LXRα and ABCA1, indicated a high flux of sterol in macrophages treated with Ac-LDL, confirmed by increased total macrophage cholesterol mass and repressed endogenous biosynthesis of cholesterol and cholesteryl ester. The extent of LDL modification indicated a threshold (Relative Electrophoretic Mobility REM > 2.5) for accumulation of sterol mass, further evidenced by Nile Red staining, assessed by flow cytometry and visualised as lipid droplets. Notably, despite significantly increasing macrophage cholesterol mass hypochlorite-modified LDL did not elicit classical cholesterol homeostasis responses, as judged by unchanged expression of *SREBF2*, *HMGCR*, *LDLR*, *NR1H3* or *ABCA1*. Under these precisely defined conditions, macrophage expression of hsa-let-7d-5p, but not hsa-miR-150-5p, was significantly increased at 24h and 48h by treatment with Ac-LDL, compared with control; a modest increase at 12h was noted in macrophages treated with HOCl-LDL (but not native or copper-oxidized LDL) but this was not sustained at later time points.

(ii) Delivery and efficacy of miR-150 and let-7d mimic and inhibitor in THP-1 macrophages and macrophage 'foam' cells

Transfection efficiency in differentiated THP-1 macrophages, in the absence of a cholesterol load, was initially established by Q-PCR following delivery of miR-1 mimic (5nM), as this sequence is minimally expressed in this cell line: substantive increases (4300-fold; $p<0.001$) were noted compared with the relevant siRNA control after 24h. Cellular levels of miR-150 (2231-fold; $p<0.001$) and let-7d-5p (69.3-fold; $p<0.001$) increased compared with control following delivery of each mimic under the same conditions; however, no significant knockdown was observed in the presence of let-7d-5p inhibitor (100nM) after 24h, with very low levels of miR-150 precluding effective knockdown.

Efficacy of the miR-150 mimic was tested against two targets (*ADIPOR1, MMP14*) but neither showed consistent knockdown; by contrast, the let-7d-5p mimic elicited a reduction (25%; $p<0.05$), and the inhibitor an increase (15%; $p<0.05$) in expression of *CCL7* (encoding C-C motif chemokine ligand 7), but not *HMGA2*, in macrophages, compared with the relevant SiRNA control, suggesting an anti-inflammatory role for let-7d. Notably, when macrophages were transfected with let-7d-5p mimic and inhibitor, and then incubated with Ac-LDL (above), marked differences in expression of these target genes, compared with their relevant SiRNA controls, were evident. Let-7d mimic reduced expression of *HMGA2* by a modest amount (17.7%; $p<0.05$), but the presence of the inhibitor elicited a marked 2.9-fold ($p<0.05$) sterol-dependent increase in expression of *HMGA2* (**Figure 1**). No significant impact of either mimic or inhibitor on expression of *CCL7* or cytokine genes (*IL1B, TNFA, TGFB*) were noted in Ac-LDL treated cells.

Importantly, let-7d mimic decreased total (10%; $p<0.05$) free cholesterol (8%; $p<0.05$) and cholesteryl ester mass (21%, $p<0.05$), in Ac-LDL treated macrophages while let-7d inhibitor significantly increased total (29%; $p<0.05$) and free cholesterol (29%; $p<0.05$) mass, compared with their relevant SiRNA controls; a significant difference was also noted between cells treated with mimic and inhibitor (**Figure 2**). Expression of a number of genes involved in cholesterol homeostasis (*SREBF2, HMGCR, LDLR*) and uptake (*CD36, SCARA, LOX1*) of modified LDL were examined, with only LOX-1 upregulated (32%; $p<0.05$) in the presence of let-7d mimic; further investigation of the impact of let-7d inhibition on genes involved in the cholesterol efflux pathway (*ABCA1, ABCG1, APOE, NR1H3*) did not reveal any biological significance. Together, these data suggested the impact of let-7d inhibition to be foam-cell specific, and novel roles for both let-7d and HMGA2 in modulating macrophage lipid accumulation.

(iii) Let-7d-5p inhibition alters cholesterol metabolism in THP-1 macrophage 'foam' cells

The pathway by which let-7d inhibition promotes sterol deposition in macrophages treated with Ac-LDL was investigated using functional assays assessing distinct cholesterol homeostasis responses in the presence of this modified lipoprotein. There were no changes in flux of exogenous [^{3}H]oleate (10μM; 24h) into the cholesteryl ester pool or efflux of [^{3}H]cholesterol in the presence or absence of apoA-I (10μg/ml; 24h) in sterol-loaded cells treated with let-7d inhibitor, compared with SiRNA control. However, incorporation of [^{14}C]acetate into endogenously synthesized cholesterol and cholesteryl ester pools increased by 38% ($p<0.05$) and 39% ($p<0.05$), respectively in the presence of Ac-LDL (**Figure 3**). Addition of apoA-I was able to reverse the increase in total cholesterol mass due to the presence of let-7d inhibitor (**Figure 4**). This suggests let-7d inhibition expands a pool of endogenously synthesized cholesterol amenable to efflux via ABCA1; intriguingly, this pool is not available for esterification to exogenous [^{3}H]oleate and does not alter efflux to apoA-I from cells labelled with exogenous [^{3}H]cholesterol.

(iv) Let-7d-5p targets high mobility group AT-hook 2 (HMGA2) in THP-1 'foam' cells

The impact of let-7d inhibition on expression of *HMGA2* in sterol-loaded cells translated into a significant (32%, $p<0.05$) increase in the level of HMGA2 protein, relative to β-actin, in THP-1 macrophages treated with Ac-LDL (**Figure 5**), suggesting a novel pathway of action for let-7d.

(v) HMGA2 knockdown reduces cholesterol accumulation in THP-1 'foam' cells

Three SiRNA sequences (#1-#3) were tested (5-20nM; 24-48h) for their ability to lower HMGA2 protein levels, and cholesterol levels in THP-1 'foam' cells (**Figure 5**); a maximal reduction of 29% ($p<0.05$) in HMGA2 expression (10nM; 24h) was associated with a 21.1% ($p<0.05$) reduction in free cholesterol mass over the same time period (**Figure 5**).

This is the first report linking HMGA2 with macrophage sterol metabolism: HMGA2 is a small non-histone protein which can bind DNA and modify chromatin status, contributing to fine-tuning of gene expression. Previous work has linked HMGA2 with adipogenesis, via increased expression of transcription factors *CEBPB* and *PPARG* (Xi *et al*., 2016) but no changes in expression of these genes were detected in this study. Certainly, let-7d reductions in expression of HMGA2 have been proven to protect against oncogenesis (Balzeau et al., 2017), and our data suggests that modulation of cholesterol metabolism in rapidly dividing cells may contribute to this protective effect: miR-33b, which also targets HMGA2, has clearly been linked with onco-suppression (Lin et al., 2015). Finally, the inability to induce let-7d-5p in sustained manner, observed in macrophages treated with HOCl-LDL, may be one reason cholesterol homeostasis is overwhelmed in these cells, leading to greater sterol accumulation than seen in the presence of Ac-LDL.

(vi) Microarray *in silico* analysis predicts key pathways implicated in 'foam' cell formation

In silico analysis of pathways targeted by miRNA sequences (DIANA-miRPATH v.30) that exhibited significantly different expression (0.4-fold as threshold) during 'foam' cell formation implicated a number of pathways in this process, including extracellular matrix-receptor interactions and, most markedly, fatty acid metabolism and biosynthesis, indicating new avenues for exploration.

3) Details of any changes in the research compared with the original proposal

Overall, the project remained consistent in its key objectives. However, three changes were made during the evolution of the study. The first was to expand the nature of the oxidizing agents used to generate oxidized LDL used to drive foam cell formation. The second change was to adopt reviewers' comments on our original proposal, to expand our search for miRNA targets in 'foam' cells by using an unbiased screen. Microarray analysis was performed of all mature human miRNAs (>4000) in the miRBase database (release 22, March 2018) and an *in silico* analysis of pathways targeted by miRNA sequences (DIANA-miRPATH v.30) altered in 'foam' cells forms the last chapter of Mr Lightbody's thesis. The third change was to focus on hsa-let-7d-5p in the final year of study, in recognition of (i) the relative inefficacy of the miR-150 mimic, (ii) the very low levels of expression of miR-150 in THP-1 macrophages which preclude effective knockdown

of this sequence and (iii) a role for miR-150-5p in foam cells (Li and Zhang, 2016) published during the first year of this grant.

4) Circumstances which aided or impeded the progress of the research

The progress of the research was greatly aided by the dedication, and precise attention to detail, evidenced by student Richard Lightbody. He has taken great care with the laboratory work, requisite for probing the complex and subtle changes induced by miRNA sequences in cells, and shown excellent understanding of the project and supporting literature. Progress has been consistent, but we note the necessity for careful characterisation of efficiencies of miRNA Q-PCR quantification kits from commercial suppliers.

5) How the significance of the research relates to recent or current work in the field

Since inception of this project (2015/2016), there has been an explosion of interest in miRNA involved in 'foam' cell formation: more than 40 miRNA sequences have been reported in human and murine macrophages. Many genes targeted by these sequences play established roles in lipid metabolism or inflammation, but a significant number have no prior links to either process, highlighting the importance of miRNA research in driving discovery of novel cellular disease processes. Within this rapidly evolving field, our work on let-7d-5p stands alongside a recent report that let-7g, another member of the let-7d family, inhibits both canonical (RelA/p50) and non-canonical (RelB/p52) nuclear factor-kappa B (NF-κB) signalling pathways, limiting inflammatory (IL-1β, IL-6, MCP-1) and apoptotic responses, and decreasing macrophage foam cell formation *in vitro* and *in vivo* (Wang *et al*., 2017). Further, reductions in expression of the let-7 miRNA family have been observed in human carotid plaques from diabetic individuals and diabetic apoE-/- mice (Brennan *et al*., 2017), supporting our conclusion that induction of let-7d-5p is a protective response in sterol-loaded macrophages.

6) Whether the project involved collaboration with other groups (academic or commercial)

The project did not require collaboration with academic or commercial groups, but we made contact with Dr Lorraine Work at the University of Glasgow, who kindly gave a seminar here on her miRNA work.

7) Whether any other funding bodies were involved in funding the research supported by this grant and details No other funding bodies were involved in funding the research supported by this grant.

8) Implications of the research including potential benefits to patients, outputs and outcomes, policy implications and changes in healthcare practice

Our current findings, and those of Brennan *et al* (2017) and Wang *et al* (2017) posit members of the let-7 miRNA family as protective against the lipid accumulation and inflammation associated with atherosclerotic lesions. Therapies based on these findings could be developed: a number of miRNA therapeutics are currently in clinical trials: 'Miraversen' (miR-122) which targets hepatitis C is in a Phase II clinical trial, and MRX34, a double stranded miRNA mimic of miR-34 has entered a Phase I clinical trial for treatment of advanced liver cancer. Since differing miRNA sequences impact distinct stages of the atherogenic process, delivery of a pool of mimics and/or inhibitors may be an attractive therapeutic strategy for this progressive disease. Many factors need consideration in developing miRNA therapeutics, including effective vectors and delivery options, and 'off-target' side-effects and/or toxicities due to disruption of multiple target genes and/or cell signalling networks. However, given the intense interest and rapid advances in this field, exploiting the therapeutic potential of miRNA may become fully realised, offering effective medicines to treat this costly (>£30 billion to the UK economy) and complex disease.

9) Likely timescale for clinical impact

As with all basic science research, studies in the laboratory are inevitably some years (9-10y) away from therapeutic exploitation for clinical use. However, the loss of let-7d found in human carotid plaques from diabetic individuals (Brennan *et al*., 2017), and the protective functions of let-7d and let-7g described in this study, and by Wang *et al* (2017), posit a novel therapeutic strategy focused on the let-7d family to resolve lipid accumulation and inflammation within the arterial wall. Dissemination of results, and recognition of their significance by academic and industrial (pharma) scientists is now key in fostering further development of these findings.

10) Whether the research has stimulated further research and details of other grants applied for or awarded

The research has stimulated further work on the interactions between lipoproteins and microRNA: two PhD students are examining distinct microRNA sequences altered by exposure to high density lipoprotein (HDL), and their roles in protecting beta cells from glucolipotoxicity, and in promoting wound healing, respectively. A grant application to Tenovus UK (Scotland) was submitted in support of one of these studies but was not successful; an application to Diabetes UK is in preparation.

11) Details of any potentially patentable/commercially exploitable intellectual property which has resulted from the research and a suitable contact at your Technology Transfer Office None at

present. Susan Armstrong is our point of contact for Intellectual Property Advice and the University's Technology Transfer Portfolio T: 0141 331 3069; E:susan.armstrong@gcu.ac.uk.

12) How the findings may be disseminated to the general public and publicised in order to raise the profile of HRUK. Please provide a quote which can be used for publicity/fundraising purposes.

'Small non-coding RNA, let-7d-5p, helps to protect against lipid accumulation in cells that can promote atherosclerosis and coronary heart disease: exploiting the let-7d microRNA family may provide useful new therapeutic strategies to fight this complex and progressive condition.'

13) Titles and full references of all research publications which have resulted from this research (in press and published). If manuscripts are in preparation, please give expected date of submission. Copies of all publications should be forwarded to HRUK

As part of Richard's thesis preparation, he has contributed as first author to a review article:

Lightbody RJ, Taylor JMW, Dempsie Y, Graham A 'MicroRNA sequences modulating inflammation and lipid accumulation in macrophage 'foam' cells: implications for atherosclerosis' currently under review by World J Cardiology (attached).

A full paper is currently in preparation for submission April/May 2020.

Lightbody RJ, Taylor JMW, Dempsie Y, Graham A 'Induction of microRNA hsa-let-7d-5p contributes protection against lipid accumulation by macrophage 'foam' cells'

14) Details of conference presentations (oral and poster) and any prizes, awards etc received as a result of the research

British Pharmacological Society poster prize (2016) GCU 'Transforming Lives Through Life Science'

European Atherosclerosis Society Advanced Course (poster presentation) in 'Atherosclerosis at the interface of hyperlipidaemia and inflammation' December 2018 (Vienna)

Scottish Cardiovascular Forum (oral presentation) February 2019 (Inverness)

European Atherosclerosis Society meeting (poster presentation) May 2019 (Maastricht).

Supporting Figures

Figure 1

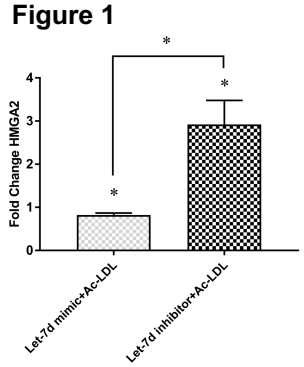


Figure 2

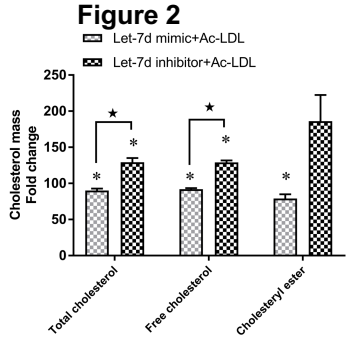


Figure 3

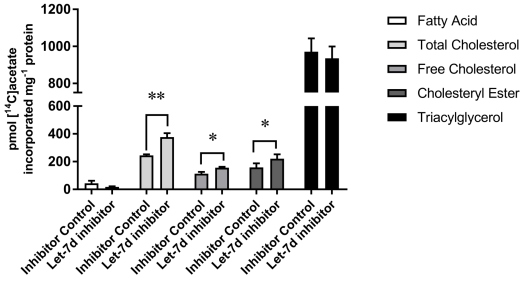


Figure 4

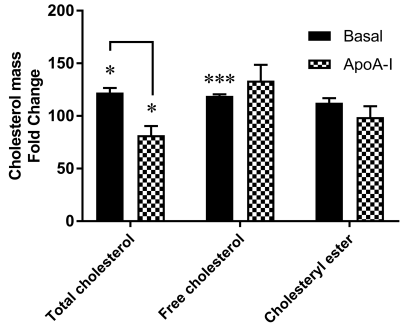


Figure 5

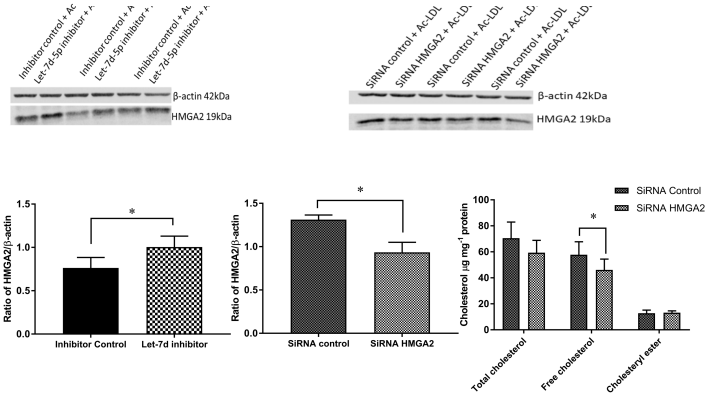
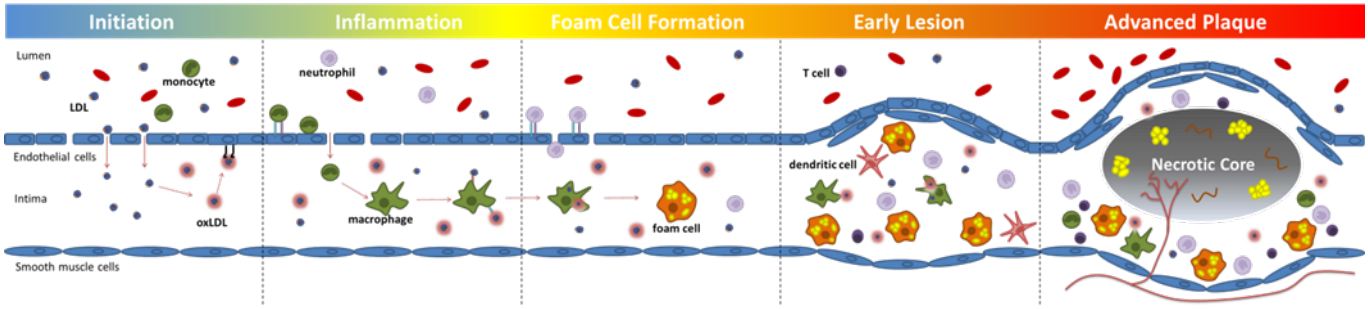


Figure legend All values are mean± SEM for the number of indepe [illegible] of Ac-LDL (75µg ml^{-1}; 24h). **[1]** Fold changes in expression of let-7d-5p in macrophage 'foam' cells, determined by Q-PCR (n=3); **[2]** Fold changes in cholesterol mass, compared with the relevant SiRNA control, for let-7d-5p mimic (5nM, 24h) and inhibitor (100nM; 24h) in 'foam' cells (n=4); **[3]** Incorporation of [^{14}]acetate into lipid pools in 'foam' cells treated with let-7d inhibitor (n=4, 5nM, 24h); **[4]** Fold changes in cholesterol mass, compared with the SiRNA control, due to let-7d-5p inhibitor (5nM, 24h) ± ApoA-I (10µg ml^{-1}, 24h) (n=3); **[5]** Changes in HMGA2 protein expression after let-7d-5p inhibition (n=3, 24h) and treatment with SiRNA (n=3; 10nM, 24h) against HMGA2; changes in cholesterol mass due to HMGA2 knockdown using SiRNA (n=4). *p<0.05; **p<0.01.

SECTION B: LAY SUMMARY



Atherosclerosis is the main cause of heart attacks and stroke. It is a process whereby lipid ('fat': cholesterol) accumulates within the walls of major blood vessels (arteries). Initially this occurs within white blood cells (macrophages) which build up in the artery wall at sites of physical or chemical damage (inflammation; above). Lipid accumulates in macrophage 'foam' cells, derived from the 'bad' cholesterol-transporting particles found in the bloodstream. These particles are damaged by oxidation within the artery wall, leading to excess fat deposition and inflammation, and further recruitment of white blood cells to an increasingly complex lesion (plaque) which eventually contains a necrotic (dead) core. Recent evidence suggests that a class of small RNA molecules (microRNA) can regulate clusters of genes involved in different events involved in this process and clinical trials (Phase II) in humans have shown microRNA are viable therapeutics.

The aim of this project was to investigate microRNA that regulate genes involved in the development of atherosclerosis, and to see if their activity can to slow accumulation of lipid and/or associated inflammation in macrophages.

1. First, we showed that one of the sequences (called 'let-7d') was consistently increased in macrophage 'foam' cells which displayed an effective response to the accumulation of 'fat', but was not in those which did not, suggesting a **protective effect** for this sequence.
2. The delivery of a mimic and inhibitor of let-7d was performed, and their effectiveness assessed by looking at the levels of gene targets for this sequence. What should happen here, is that the level of the target gene should decrease after treatment with the mimic, and increase after treatment with the inhibitor.
   Interestingly, we saw differing outcomes when we did this in cholesterol-laden 'foam' cells, compared to normal macrophages. In the latter, an anti-inflammatory effect of the let-7d mimic was seen, as judged by decreased expression of a gene called CCL7. This effect was lost in 'foam' cells, but we saw a marked increase in levels of another target, called HMGA2, after treatment with let-7d inhibitor. This suggested a **novel, 'foam'-cell specific effect for let-7d and HMGA2.**
3. We showed let-7d mimic and inhibitor had opposite effects on the accumulation of 'fat' (cholesterol) in macrophage 'foam' cells: the **mimic decreased cholesterol mass**, and the **inhibitor increased cholesterol mass**, by increasing the amount of cholesterol made by the macrophage itself. This again suggested that **induction of let-7d in 'foam' cells is a protective response.**
4. The level of HMGA2 protein increased after treatment with let-7d inhibitor, suggesting a role for this protein in promoting cholesterol accumulation. In order to prove this, we showed that reducing the levels of HMGA2 protein (using SiRNA) protected against accumulation of cholesterol.

We conclude that induction of let-7d-5p, and consequent reductions in its target protein (HMGA2), can protect against the accumulation of 'fat' in white blood cells. This may provide the basis of novel therapies for treatment of atherosclerosis in humans. Despite substantial decreases in death from cardiovascular disease over the last two decades, coronary heart disease and stroke are still major causes of morbidity and mortality in the UK, although the increasingly prevalent incidence of obesity and diabetes may counter these positive changes. Using small RNA molecules may be one way to prevent, or even reverse, the damaging process of formation of atherosclerotic plaques.

Signature of Grantholder A Graham Date:18/03/2020

Name (block capitals): A GRAHAM