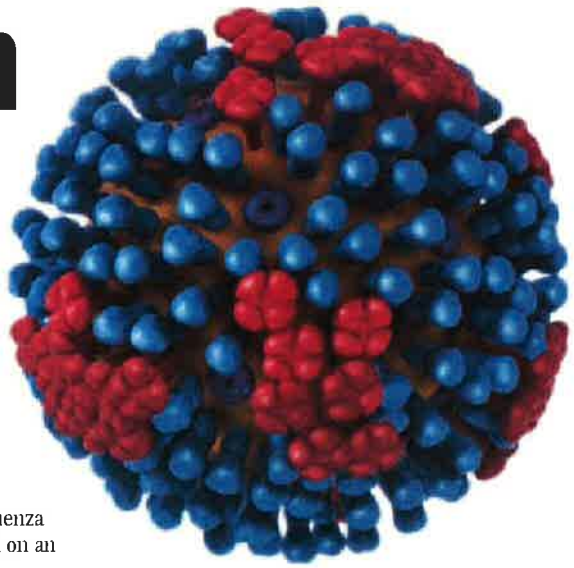
Respiratory viral pathogens: evolution and molecular characterisation

Respiratory illness has come under more intense scrutiny over the past decade with the emergence of new viral pathogens. Here, Sanjiv Rughooputh and Mitradev Pattoo provide an update on ongoing genetic research.



CDC/ Douglas Jordan

Influenza A viruses are divided into subtypes based on the presence of two surface proteins: haemagglutinin and neuraminidase. There are 16 different haemagglutinin subtypes and nine different neuraminidase subtypes.

Acute respiratory infections (ARI) are the leading cause of morbidity and mortality worldwide. These diseases are responsible for at least 6% of the world's disability and death. Many viruses known to cause ARI manifesting as influenza-like illness (ILI) include adenovirus, respiratory syncytial virus (RSV), human enterovirus, rhinoviruses, human metapneumovirus, bocavirus, human coronavirus, human parainfluenza virus and influenza virus.

Clinical presentation of ILI is non-specific, but, with strong epidemiological evidence and thorough virological investigation, the aetiological agent can be identified. The development of molecular methods such as the reverse transcriptase-polymerase chain reaction (RT-PCR) has facilitated rapid and sensitive detection of the whole spectrum of ILI-causing viruses.

According to conservative estimates by the World Health Organization (WHO), infections caused by influenza viruses alone on a global scale result in between three and five million cases of severe illness and between 250,000 and 500,000 deaths annually. The majority of these can be prevented by the uptake of influenza vaccines that are manufactured on an annual basis.

SEASONALITY AND SPREAD

Despite our increasing knowledge of the molecular biology of viruses associated with respiratory infections, there remains a remarkable lack of knowledge about why respiratory pathogens exhibit seasonality in their incidence. If, however, seasonality for some viruses (eg influenza) is clearly defined in temperate countries, with epidemic peaks occurring in winter, in tropical areas the seasonality appears to be less marked. Nonetheless, certain meteorological conditions (eg wet and cold) favour a higher circulation of certain viral pathogens such as influenza and RSV.

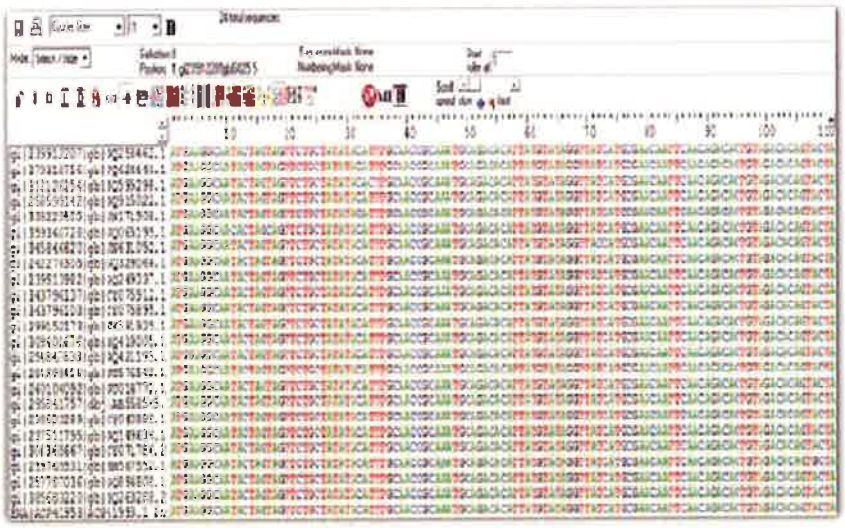
On the other hand, some viruses or particular strains are endemic to specific areas and can be distinguished genetically from strains circulating in other regions. In contrast, for some other viruses, genetically similar strains can spread and circulate around the world. For example, the international spread of respiratory viruses has been epitomised by the global pandemic of influenza A subtype H1N1 (swine influenza) in 2009. This virus emerged in Mexico and spread to the USA in April 2009 and eventually was detected in no less than 207 countries within a few months of identification of the index case.

'Development of molecular methods such as RT-PCR has facilitated rapid and sensitive detection of the whole spectrum of influenza-like illness-causing viruses'

CORONAVIRUSES

The spread of respiratory viruses worldwide is not limited to this influenza pandemic. In February 2003 there was the first reported case of severe acute respiratory syndrome (SARS), a viral respiratory illness caused by a coronavirus, subsequently termed SARS-associated coronavirus (SARS-CoV), in Asia. Over the next few months, the illness spread to more than two dozen countries in North America, South America, Europe and Asia before the global outbreak of 2003 was finally contained. A total of 8098 people worldwide became ill with SARS during the 2003 outbreak, of which 774 died.

A new coronavirus has since emerged,



Multiple alignment of different influenza sequences using BioEdit. The primers are based on the conserved regions from a consensus sequence.

identified in September 2012 in a patient who died from a severe respiratory infection in June 2012. Subsequently, the Coronavirus Study Group (CSG) of the International Committee on Taxonomy of Viruses (ICTV) decided in May 2013 to call this coronavirus Middle East respiratory syndrome coronavirus (MERS-CoV). Globally, from September 2012 to the end of August 2013, WHO has been informed of a total of 108 laboratory-confirmed cases of MERS-CoV infection, currently including 50 deaths but the death toll is on the rise.

INFLUENZA VIRUSES

Almost at the same time, a new influenza virus emerged, influenza A subgroup H7N9. This subgroup of influenza virus normally circulates among birds. The first human cases of influenza A (H7N9) were reported to WHO in April 2013 by the Chinese Health Authority and, as of 12 August, 135 laboratory-confirmed cases have been reported, with 44 deaths. April seems to be a favourable month for new influenza viruses to emerge.

Human infections with other subgroups of H7 influenza viruses (eg H7N2, H7N3 and H7N7) have been reported in The Netherlands, Italy, Canada, USA, Mexico and the UK. Presently, there is only a limited amount of information about the pathogenesis and transmission of this new influenza virus.

TIME AND SPACE

The behaviour of strains of viral respiratory pathogens of certain genetic make up varies in different populations where the virus may be more adapted. Hence, detection, characterisation of respiratory pathogens temporally and spatially will provide very useful information on the adaptability of these viruses in different subsets of the population over time.

The authors have just embarked on a temporal-spatial study looking at the evolution of respiratory pathogens, particularly at influenza viruses in the first instance, eventually extending to other respiratory pathogens. Molecular analysis of haemagglutinin (*HA*) and neuraminidase (*NA*) genes of influenza viruses is crucial in the monitoring of modification in the viral genome related to pathogenesis and susceptibility to antiviral drugs. The segmented nature of the influenza viral genome enables exchange (termed reassortment) between the eight segments when distinct viruses co-infect the same host cell and generate progeny with a mixed genome. For HA and NA, this reassortment process is called antigenic shift. If this reassortment takes place between viruses originating from different species, it can generate viruses with pandemic potential, which includes HA and/or NA proteins from the avian or swine influenza viruses against which humans have no pre-existing immunity.

To conduct this study, in-house RT-PCR assays have been developed based on whole genome sequences of the *HA* gene of pandemic influenza A H1N1 retrieved from the Influenza Virus Resource database (www.ncbi.nlm.nih.gov/genomes/FLU) from different parts of the world. In this exercise, the sequence of H1N1 A/California07/09, the vaccine strain, was also included.

In order to design primers, bioinformatics tools such as Clustal W and BioEdit Sequence Alignment Editor (version 7.0.9.0) were used, and a consensus sequence developed. Using the consensus sequence, different primer sets were designed to span the entire length of the *HA* gene of the influenza virus.

WORK IN PROGRESS

In an RT-PCR method, the primers will be used to amplify the *HA* gene of different isolates of influenza A collected at different times and in different locations. The amplified products will then be sequenced and the sequence data compared to the reference sequence to determine whether or not there is any change in the virus genome and eventually in the amino acid sequences. If changes in the amino acid sequences are identified, these will alter the properties of the haemagglutinin, a protein that helps the virus bind to cells.

Several gene mutations have been found in those coding for the haemagglutinin of influenza A. One such mutation in the H1N1 (pdm) 2009 subtype is at position 222 in the receptor binding site, which is believed to play an important role in HA binding specificity. A mutation in this region (D222G) has been described in influenza A H1N1 (pdm) 2009 that enhances selection for avian receptors and reduces selection for human receptors, and has been linked to severe clinical outcome.

This study aims to identify other mutations that will help to elucidate the adaptation of influenza viruses in humans of different genetic make up and ethnicity. ■

FURTHER READING

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This article describes ongoing work being supported by an IBMS Overseas Research Grant to Dr Rughooputh.

In 2014, the Institute awarded six research grants totalling £18, 308. Brief summaries of the proposed research have been requested from this year's recipients, with the first three received being reproduced here.

Research grants awarded in 2014

HOSSEIN ASHRAFI

Hossein Ashrafi's IBMS research grant is helping to fund a project entitled *An investigation on the association of human papillomavirus (HPV) and breast cancer*, being undertaken by a team at Kingston University and Kingston Hospital.

Studies on the role of HPV in breast carcinogenesis have generated much controversy and it is still not clear whether or not HPV is present in breast tumours. Preliminary results have shown evidence for the presence of HPV viral DNA in freshly obtained human breast cancer tissue (UK) and now provides a solid basis to advance research in a crucial health priority affecting women. These early findings strengthen the association of HPV and breast cancer and will address important questions on the causative agents in breast cancer. In particular, further research is needed to advance the understanding of an HPV role, if any, in the pathogenesis of breast cancer.

The major benefits for a successful outcome include greater understanding, new knowledge, improved expertise, and in advancing technology. These benefits will impact on UK society through the implementation of an effective programme involving currently licensed HPV vaccines. and enable clinicians and biomedical scientists to pursue the long-term goals of the project.

MICHAEL CARROLL

Dr Michael Carroll is a lecturer in reproductive science in the School of Healthcare Science at Manchester Metropolitan University and is interested in how lifestyle and environmental exposures can affect male fertility. He is currently developing an *in vitro* assay to investigate the effects of advanced glycation end-products (AGEs) on sperm integrity and function. Formed by reactions between sugars with proteins, nucleic acids or lipids, AGEs cause cellular damage directly by cross-linking and indirectly by binding to specific AGE receptors on cell surfaces, thus increasing the production of reactive oxygen species (ROS).

Advanced glycation end-products are known to accumulate in body tissues during ageing, in diabetes and autoimmune disease, and are found in increasing concentrations in the Western diet. The of role AGEs in human reproduction has increased in importance, as it has been shown that they accumulate in the reproductive tract of men, impairing spermatogenesis and sperm function. The prevalence of infertility in diabetic men is reported to be approximately 35%. Impaired sperm quality and function has been attributed as a major cause of infertility. In diabetes, there is increased production of ROS. Additionally, with the excess blood sugar associated with diabetes, proteins, DNA and lipids can become glycated, producing AGEs, which are directly responsible for DNA and cell damage, and have been found at high levels in the reproductive tract in the diabetic male.

Dr Carroll's IBMS research grant will be used to set up an *in vitro* system, using human sperm, to investigate the effects of AGEs on sperm integrity and function. This research will also investigate possible therapies to reduce AGE-related sperm damage.

MARLENA PIRIE

Marlena Pirie's IBMS research grant is helping to fund a project entitled *Determining the role of macrophage phenotype switching in conferring apoptosis in Kikuchi-Fujimoto disease*.

Kikuchi Fujimoto disease (KFD) is a rare non-neoplastic, self-limiting inflammatory disease predominantly affecting the lymph nodes. Although rare cases have been reported in association with viral agents such as Epstein-Barr virus, human immunodeficiency virus, human herpes simplex virus, dengue virus and parvovirus B19, the majority of cases lack an identifiable precipitating factor, and thus the true aetiology of this condition remains to be determined.

Pathologically, KFD is characterised by zones containing abundant apoptotic debris and large numbers of associated macrophages, together with varying numbers of activated T cells and plasmacytoid dendritic cells. The macrophages encountered in KFD are unique in their expression of myeloperoxidase, unlike macrophages seen in other situations of high apoptosis, such as the germinal centre reaction or aggressive lymphomas. However, little else is know regarding their phenotype and function.

This project aims to explore more fully the pathogenetic mechanisms at work in KFD, with particular emphasis on the role played by macrophages and their relationship with the apoptotic process. RNA will be extracted from formalin-fixed, paraffin wax-embedded lymph node biopsies of known cases of KFD, and gene expression profiling performed using Fluidigm technology. A targeted set of genes associated with different pathways of macrophage activation (ie classically and alternatively activated) will be examined, together with genes know to be associated with activation of different T-cell subsets.

Results will be compared with those of similar experiments conducted on tissues containing prominent reactive germinal centres, and on aggressive B-cell lymphomas showing high rates of apoptosis. It is anticipated that differences in expression patterns of these genes will highlight mechanisms at work in these biologically distinct processes, and further the understanding of interactions between apoptotic bodies and macrophages in physiological situations (reactive germinal centres), KFD and malignant lymphoma.

In 2014, IBMS research grants were awarded also to the following members:

- David Bean
- Abigail McMahon
- Sanjiv Rughooputh

Synopses of their grant-funded work will appear in a future issue of *The Biomedical Scientist*.

OBITUARY

It is with regret that we report the death of the following Member:

Haeusler, Kristins Rosalind (Italy)

In 2014 the Institute awarded six research grants totalling £18,308. Brief summaries of the proposed work have been requested from this year's recipients, with the first three synopses published in the September issue (page 502). A further two, from Abigail Hicks (nee McMahon) and Sanjiv Rughooputh, are reproduced here.

More research grant synopses

ABIGAIL HICKS

Abigail Hicks' IBMS research grant is helping to fund a project entitled *Bacteraemia: rapid identification of pathogens and determination of local antimicrobial resistance patterns* being undertaken at Barnsley Hospital BHS Foundation Trust.

With an uncertain future regarding the continued use of antibiotics, it is imperative for clinicians to be able to prescribe appropriate antibiotics at the earliest possible opportunity. Much work is being done internationally to enable clinicians to have this information. This includes the recent awarding of the Longitude Prize, a £10 million grant to address this issue.

The project being undertaken at Barnsley Hospital has two aims: first, to find a faster method of identifying bacteria present in positive blood cultures, which is suitable for use in a small district general hospital; and second, to determine local resistance patterns to inform empirical antibiotic advice.

Bacteraemia, the presence of bacteria in the bloodstream, is a leading cause of mortality in hospitalised patients. The rate of recovery strongly correlates with how quickly the correct antibiotic treatment is administered. Broad-spectrum antibiotics are administered as empirical therapy for bacteraemia before microbiology results are available. Using conventional methods for culture, identification and susceptibility testing of bacteria, it takes at least 24–48 hours for results, at which time the prognosis for the patient is significantly worse if the infecting bacterium is not susceptible to the initial antibiotic therapy. Furthermore, if the patient does not have bacteraemia, they are receiving unnecessary antibiotic treatment which has its own complications, including allergic reaction, toxicity, antibiotic-associated *Clostridium difficile* infection, and antibiotic resistance, as well as increased cost to the NHS and increased length of stay.

Hain Lifescience (Nehren, Germany) produces the GenoType BC Gram-positive and GenoType BC Gram-negative panels which utilise DNA Strip technology. Bacterial DNA in the blood will be amplified, isolated and applied to a DNA Strip matrix which contains specific DNA probes at specified positions. These probes hybridise with the DNA in the sample, producing an enzymatic colour change which can be detected by eye. The strips contain probes for the 32 most common bacterial species that cause bacteraemia, and also probes for the resistance genes *mecA* and *van*, which confer resistance to methicillin and vancomycin, respectively, in Gram-positive bacteria.

Rapid ESBL Screen and Rapid Carb Screen (Bioconnections, Knypersley, UK) use a colour change to phenotypically detect resistance mechanisms in Gram-negative bacilli. Within two hours the clinicians will know if the bacterium is an extended-spectrum β-lactamase (ESBL) or a carbapenemase producer, which will dramatically affect treatment options and may induce infection control measures.

For the epidemiological study of Gram-negative β-lactamase resistance genes present in the local area, further differentiation of ESBLs, AmpCs and carbapenemases will be performed. The Total ESBL + AmpC Confirm disc set (Bioconnections) can differentiate ESBLs and AmpCs in Enterobacteriaceae. The KPC, MBL and OXA-48 Confirm disc set (Bioconnections) can differentiate the carbapenemases *Klebsiella* producing carbapenemase (KPC), metallo β-lactamase (MBL) and oxacillinase-48 (OXA-48) in Enterobacteriaceae. The KPC/MBL in *P. aeruginosa* and *Acinetobacter* Confirm disc set (Bioconnections) can differentiate KPCs and MBLs in *Pseudomonas aeruginosa* and *Acinetobacter* species.

It is hoped that these data will provide improved management of the individual patient and epidemiological information to improve the empirical therapy of suspected bacteraemia.

SANJIV RUGHOOPUTH

Dr Sanjiv Rughooputh works in the Department of Molecular Biology and Virology, Central Health Laboratory, Victoria Hospital, Candos, Mauritius. His IBMS research grant will support a *Prospective study to determine the molecular evolution and characterisation of respiratory pathogens*.

Respiratory infections are the leading cause of mortality worldwide, accounting for 6% of the world's disability and death. Several previous studies highlighted the fact that behind the respiratory infections there is a whole cohort of infectious agents involved, of which respiratory viruses are the most common. Respiratory viruses are usually transmitted by airborne droplets or nasal secretions and affect every age group. However, some individuals are more prone to severe complications.

Viruses known to cause acute respiratory infections include influenza viruses, adenovirus, respiratory syncytial viruses, human enteroviruses, rhinoviruses, human metapneumovirus, bocavirus, human coronaviruses and human parainfluenza viruses. These can lead to a wide spectrum of illness.

Many of these viruses are seasonal in their activity and tend to circulate at higher levels during the winter months. In certain parts of the world, such as the UK, the seasonality of these viruses is well defined. In other places such as the tropics, where seasonality does not change drastically over the year, it can be confounding and also misleading.

Preliminary work carried out so far, supported by a previous IBMS grant, has shown that mutations not previously described were observed in some isolates of H1N1 (pdm2009). Hence, it is very important to expand this study to look at variations that occur in other respiratory viruses along with influenza. This knowledge will help to evaluate the circulating pathogens, understand their pathogenicity and also determine whether or not the mutations contribute to increased morbidity and mortality.

This will be a prospective study whereby respiratory samples collected in 2014 and partly in 2015 will be tested for all respiratory viruses including influenza.

Research grants awarded in 2015

In 2015, the Institute awarded six research grants totalling £22,100. Brief summaries of the proposed research have been requested from this year's recipients, with the first four received reproduced here.

Hossein Ashrafi

Dr Hossein Ashrafi is Associate Professor in Pathology and Course Director for Cancer Biology at Kingston University, London. His IBMS research grant is helping to fund a project entitled *An investigation on the association of human papillomavirus (HPV) and breast cancer.*

Although cancer mortality is decreasing, the incidence of breast cancer is increasing due in part to an ageing population, rising socioeconomic status, increasing obesity and several lifestyle changes. As important as it is to engage in primary breast cancer prevention with lifestyle measures, the search for prophylactic or preventative measures is likely to play an important role, particularly for women at high risk. Human papillomavirus (HPV) is known to play a significant role in cancer initiation and progression, and the association of HPV and cervical cancer is well documented.

The relationship between HPV and breast cancer is imperative for a number of reasons. The exposure of the mammary ducts to the external environment increases the risk of HPV infection. Most breast cancers originate from mammary duct epithelia.

The possible association of HPV with breast cancer has been studied for a long time, with the two most commonly identified HPV types being HPV 16 and 18.

The research team at Kingston University and Kingston Hospital have now reported the first documented identification of 12 other high-risk HPV types in fresh breast tumours. The discovery of HPV in fresh breast cancer tissue samples now provides a strong impetus to spur further research in an important global health imperative involving women. The knowledge acquired will lead to better understanding of risk factors other than those established to date.

Cancer prevention, viral carcinogenesis and breast cancer are rapidly developing sectors of the field of biomedical science and the prospect of investigating the use of vaccines to prevent cancer is very appealing.

Chrystalla Ferrier

Chrystalla Ferrier's grant-support project is entitled *The development of a fully automated panel for the assessment of pro-oxidant and antioxidant status in body fluids.*

This study aims to set up and validate an automated panel for the simultaneous measurement of uric acid, gamma glutamyl transferase, total antioxidant status, superoxide dismutase, glutathione peroxidase, glutathione reductase and C-reactive protein, and to establish local reference ranges from volunteer healthy individuals. In addition, ratios for different combinations of the analytes will be calculated to establish any emerging associations or trends.

Although methods for these analytes are well established, their provision as a profile on a single platform will enable high-throughput analysis of individual pro-oxidant and anti-oxidant status in health and disease.

Martin Gonzo

Martin Gonzo is currently studying at the University of Bath, and his research grant-supported project is entitled *The potential usefulness of hepcidin as a routine diagnostic test in patients presenting with iron deficiency anaemia.*

Hepcidin was first discovered in human blood ultrafiltrate and urine samples as small antimicrobial peptides, the name originating from their place of synthesis in the liver and its antimicrobial activity. Since its discovery, much work has gone into characterising this molecule.

Current guidelines on the diagnosis of iron deficiency anaemia suggest the use of full blood count (FBC) results and serum ferritin levels. The FBC generates a battery of parameters that must be interpreted in conjunction with serum ferritin, and this is a situation that complicates the decision-making process.

The measurement of hepcidin is a promising diagnostic tool, especially for the diagnosis and management of medical conditions in which iron metabolism is affected, such as iron deficiency anaemia. Hepcidin is sensitive to the dynamics of iron utilisation and could be used as a routine laboratory test to help in the diagnosis and management of patients with iron deficiency anaemia.

This project intends to investigate how hepcidin can be used as a more sensitive routine diagnostic test for patients presenting with iron deficiency anaemia in Namibia, a country where prevalence is around 40%. It intends to achieve this by establishing qualitative and quantitative differences of hepcidin molecules in patients with iron deficiency anaemia.

Establishment of the usefulness of hepcidin assays in clinical settings will reduce the lead time to decision-making and may also shorten patients' hospital stays.

Daniel Isemede

Daniel Isemede is a specialist biomedical scientist at Torbay General Hospital. His IBMS research grant is helping to fund a project entitled *High-sensitivity cardiac troponin-T measurement in obese type 2 diabetics enrolled on a weight-loss study: a randomised comparative study of the impact of low-carbohydrate diet versus prescribed energy deficit (low-fat) diet.*

Type 2 diabetes (T2DM) is a metabolic disorder characterised by chronic hyperglycaemia. It is the most prevalent form of diabetes, and accounts for 90–95% of all adult cases of diagnosed diabetes. There is considerable morbidity and mortality associated with T2DM and the disease is associated with increased risk of cardiovascular disease (CVD). Studies have shown that patients with T2DM have a two-fold

absolute risk of CVD, with mortality rates also about two-fold higher compared to non-diabetic individuals. Diabetic patients have a propensity to develop coronary atherosclerosis long before exhibiting any symptoms of myocardial ischaemia. Current measures for CVD risk predictions are suboptimal, especially in diabetic patients, necessitating a more reliable method of risk stratification.

Cardiac troponin-T (cTnT) is a component of the thin filaments that make up the contractile apparatus of striated muscle. It is approximately 39 kDa in size, and usually exists as a complex with troponin-I (TnI) and troponin-C (TnC). The troponin complex functions as a modulator of the contractile function of sarcomeres in response to intracellular calcium ion (Ca^{2+}) concentration, and regulatory protein phosphorylation. Although the troponin subunits are respectively encoded on both cardiac and skeletal muscles, the cardiac isoform of TnI and TnT are restricted to cardiac tissues, and are not expressed on differentiated skeletal muscles, making cTnI and cTnT highly specific biomarkers of myocardial pathology.

Obesity is an important risk factor for the development of T2DM. It is responsible for the development of obesity-related metabolic syndrome. Previous studies have demonstrated an association between obesity and levels of circulating hs-cTnT and other inflammatory and metabolic markers that are elevated in T2DM. Thus, various studies have exploited the impact of weight loss to reduce the burden of obesity on T2DM and associated metabolic and physiologic complications.

Dietary macronutrient (ie carbohydrate, fat and protein) management in T2DM has become common practice in the routine clinical management of the disease. In particular, the use of reduced carbohydrate intake to achieve glycaemic control has gained widespread use. Low carbohydrate diets (LCD), the most popular of which is the one extensively promoted by Atkins with its recommended very low carbohydrate (<10% of daily calorie intake) and high protein intake, are very popular for achieving weight loss compared to prescribed energy deficit diets. The long-term effect of such carbohydrate restriction has never been elucidated in previous research.

Thus, the aim of this study is to determine retrospectively the concentrations of high-sensitivity cardiac troponin T in frozen serum samples obtained from a previous diet and weight loss study, and to determine i) if a correlation exists between weight loss and concentration of circulating hs-cTnT, ii) if the magnitude of weight loss achieved from the LCD arm of the study resulted in more reduction in hs-cTnT compared to an LFD during the six months of intensive dietary therapy, iii) if diet-based attenuation of hs-cTnT was maintained in the 18-month follow-up period, and iv) if an LCD results in increased levels of hs-cTnT in the long term and the relative impact on CVD risk. It is hoped that the outcome of this study will be useful in informing the decision-making process of T2DM risk stratification and management.