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Valetta 2018: Medical heritage

Charles Savona-Ventura

In the wake of the 1565 Great Siege, the Order of the Hospital of Saint John committed itself to building a new fortified city on the peninsula straddling the two harbours. After the foundation stone of the city was laid on the 28th March 1566, the work on *Valletta Humillissima* progressed without interruption and the Convent formally transferred its abode from *Birgu Vittoriosa* to the new city on the 18th March 1571. The administrative move required also the development of plans to build a new *Sacra Infermeria* in the new city for the eventual transfer of the medical services so essential to the original *raison d'être* of the Order. Building works on the hospital commenced in 1574 and completed four years later. The Valletta *Sacra Infermeria*, supplied by a dedicated hospital pharmacy, was managed by defined regulations laid down and codified by the Chapter-General of the Order in 1588.[1] In 1725, the past regulations were collected and published in one volume entitled *"Notizia della Sacra Infermeria, e della carica delli Commissari delle Poveri Inferme"*.[2] These latter regulations covered all facets of health care services provided by the Order. A copy of the hospital regulations was hung up in the hospital for the guidance of the patients in order that the rules of the institution could be more surely and exactly observed. The hospital doors were open to all, except "to assassins, to such as pillage the country by night, to incendiaries, to sodomites, to conspirators, nor to robbers.

Charles Savona Ventura
M.D.(Melit.), D.Sc.Med.(Warsaw), Accr.C.O.G.(Leuven), F.R.C.O.G., F.R.C.P.I., F.R.C.P.Ed.
Department of Obstetrics & Gynaecology
Faculty of Medicine & Surgery
Medical School, Mater Dei Hospital, Msida, Malta
Centre for Traditional Chinese Medicine
Triq Esperanto, University of Malta, Msida, Malta
charles.savona-ventura@um.edu.mt

Such likewise as are guilty of murdering anybody by ambuscade, wilfully in cold blood, treacherously, or by poison; the servants too of our brothers, such as have attempted the lives of our brothers, judges, or ministers of justice, persons that are in debt, such as have committed crimes within the infirmary itself, or designedly in hopes of finding sanctuary there, false witnesses, sacrilegious persons, robbers on the highway." The same Regulations confirmed "that the hospital of the infirmary may be always free and open to receive sick persons, as well as subjects of the orders, as others that shall repair thither to be cured of their diseases, according to its ancient and laudable custom."[1] The Valletta *Sacra Infermeria* was to enjoy a prominent reputation throughout the subsequent centuries with several visitors commenting favourably on the care and hospitality given to its inmates irrespective of creed and social status.

The *Sacra Infermeria* however only catered for males, having no provisions for females. To cover for the needs of the female gender, the philanthropic-minded lady Catherina Scarpi opened a small house for the reception of sick women known as *Santa Maria della Scala*. In 1625, the establishment was transferred to a new building sited in the vicinity of the *Sacra Infermeria*. Financial subsistence by the Order's Treasury became necessary for the continuance of the establishment, and the management of the establishment for female patients, now called the *Casetta delle donne*, was totally taken over by the Order after Scarpi's death in 1655. The management of the establishment was regulated by the same rules and regulations applicable to the *Sacra Infermeria* to ensure that in the care of the infirm, everything must be done for their comfort and treatment.[3]

The Valletta *Sacra Infermeria* and *Casetta delle donne* also had provisions to receive and care for mentally infirm individuals. At the *Sacra Infermeria*, the manageable patients were kept in a room especially reserved for them where they were bound and chained to their beds. If these became

unmanageable and dangerous to themselves and others, they were transferred to the basement ward of the *Infermeria*. Here they were restrained by pinioning, and by chaining their arms and at times even their legs to the walls of the chamber.[2,4] The total number of monthly inmates in the *Sacra Infermeria* ward for the mentally infirm amounted to 18 individuals during the period covering 1st May 1787 to 30th April 1789.[5] At the *Casetta delle donne*, mentally infirm female patients after 1725 were cared for in two rooms reserved for them. This accommodation was augmented in 1783 by annexing an adjacent building.[3] In 1816, both male and female mentally infirm patients were transferred to the *Casa di Carita`* or *Ospizio* at Floriana. During the latter years of the Order's rule in Malta, the cost of running the *Sagr' Ospedale* and the *Ospedale delle Donne* amounted to about 8.0% of the total expenditure of the Order, with the mean annual expenditure during this seven-year period amounting to about 93500 *scudi.*[6]

The Valletta *Sacra Infermeria* further provided health care services to ill galley slaves in the Great Magazine Ward situated below street level after these were transferred from their previous facility in Birgu during the plague epidemic of 1592. Land-bound slaves however as yet had no provisions for care in case of illness. By 1631, an *Infermeria delle Schiavi* was therefore set up in the Slave Prison at Valletta catering for publicly and privately-owned land-bound slaves. A set of regulations to manage the *Infermeria delle Schiavi* was issued on the 22nd December 1631 "so that the sick may not die for want of care and from miserable living".[7]

Medical services in Valletta were not simply restricted to hospital services but also extended to the community either in the form of an extension of the services provided by the *Sacra Infermeria* or in private practice system. The 1631 Hospital Regulations made provision for the issuing of pittances and medicines from the Hospital stores "to poor women, upon the prescription of physicians that have salaries out of the treasury, or from the people, signed by one of the commissaries deputed to visit the poor sick." Remedies and visits by medical personnel were also to be furnished "gratis to the monasteries of St. Ursula, the penitent sisters of the city Valletta, and the capuchins." All pittances given out of the infirmary to poor sick women were to be given in money rather than victuals.[8] Similar provisions were made in the 1725 Regulations whereby the *Sacra Infermeria* dispensary supplied food, medication and a daily pittance to the blind, the leprous, the scrofulous, and the poor incurables of the city. The dispensary also provided to members of the religious orders and the institutional homes for girls and women penitents. Assistance in the form of accommodation and allowance was also given to all pilgrims.[2] In addition, the Order maintained a district nursing service covering the Grand Harbour region for the care of the sick poor in the community. This service was regulated by the *Regolamento per le Povere Inferme di Malta* published as a supplement to the *Sacra Infermeria* Regulations in 1725. Two Knights of the Order, termed "Commissioners of the Sick Poor", were deputed as supervisors to enable the provision to "many poor incurables, who are incapable of providing for themselves, with daily alms, and distribute to others, in addition to what remains in the caldrons, a large quantity of soup and vermicelli, which is purposely cooked each day; also a large number of old sheets and coverlets are given to poor women, and many bandages and crutches to cripples".[2] The Commissioners had the assistance of four "*elderly*" women, known as *Pitanziere,* in their daily rounds. These were to ensure that the supplies arrived to the sick poor, to see that the physicians appointed to visit them attended to their duties, and that the patients received the proper food and medicines. Five physicians and five surgeons were occupied with this duty, two of each for Valletta and the remainder for the other towns around the Harbour region. The annual cost of this community service amounted to about 39,000 *scudi.*[9]

The free hospital and community medical services made available in Valletta by the Order was augmented by a private health service that covered medical and surgical treatment and apothecary services. In 1782, six private-run apothecary shops serviced the community residing in Valletta.[3] Besides the medical and surgical personnel serving in the Order's hospitals in Valletta, a number of practitioners were providing a private practice service to Valletta residents [*vide* table below].[10]

***Table 1**: Medical & paramedical personnel resident in Valletta - 1766*

Professional category	*Hospital*	*District service*	*Private practice*	*Naval service*	*Unknown*
Physicians	10	1	1	-	3
Surgeons	5	-	6	5	-
Barberotti	10	-	4	4	-
Insangnatori	2	-	5	-	-
Fa occhiali	-	-	1	-	-
Student barberotti	2	-	-	-	-
Apothecaries	4	-	2	-	4
"Nurses"	24	-	-	-	-

The Valletta *Sacra Infermera* also played an important role in medical education. Those individuals aspiring to become surgeons generally started their career as barber-surgeons (*barberotti*) in the *Sacra Infermeria* and after proof of competence they became Junior Surgeons (*Prattici*). These trainees were subsequently encouraged to proceed abroad to further their surgical training. Attempts at introducing formal medical education in the Maltese Islands can be dated to the establishment of the *Scuola di Medicina e Chirurgia* set up at the *Sacra Infermeria* by Grandmaster Nicholas Cottoner on the 19th December 1676. Instruction in theoretical anatomy and surgery, and later in the surgical aspects of physiology, pathology, semiotics, hygiene and therapeutics, was given to the barber-surgeons of the *Sacra Infermeria* and to all other youths who aspired to join the surgical profession provided that they could read and write. By 1682 the course in surgery lasted ten years. In 1716 a dissection room was built in the cemetery of the *Sacra Infermeria* and the necessary instruments were obtained from Paris. A set of rules governing the teaching of surgery and anatomy were published in December 1729 and revised in 1739.[3]

In 1768, Grandmaster Pinto requested authorisation from Pope Clement XIV to appropriate the *Collegium Melitense* in Valletta owned by the expelled Society of Jesuits to set up the *Pubblica Universita` di Studi Generali*. The Papal brief *Sedula Romani Pontific* was given on the 20th October 1769 and the decree constituting the University was signed by Grandmaster Pinto on the 22nd November 1769. The institution comprised two sections: a "*Collegium*" aimed at elementary and secondary education, and a "*Universitas*" to confer doctoral degrees. The *Collegio Medico* was set up on the 25th May 1771 and was managed by the *Accademia dei Medici*. The *Accademia* was responsible for conducting the student examinations.[3] With the occupation of the Islands by the French, formal University teaching was abolished by General Napoleon Bonaparte by degree published 18th June 1798. A few weeks after the French were forced to leave, Sir Alexander Ball re-instituted the University on 6th November 1800 and medical studies were resumed that same year with the first three doctors qualifying in 1804.[11]

In 1798, Napoleon Bonaparte ousted the Order of St. John from Malta. With the capitulation of the Order of Saint John, the sick French troops, initially housed in Mdina, were transferred on the 16th June 1798 to the Valletta *Sacra Infirmeria* which was converted into a military hospital and renamed the *Grand Hôpital*. A full account of the *Sacra Infirmeria* during the French occupation is given by the Physician-in-chief of the hospital Dr. Claude Etienne Robert who published his experiences in 1802. Only a few wards were considered fit to accommodate patients, while the pharmacy, the laboratory and the storerooms were considered inadequate. Dr. Robert carried out a number of modifications to improve sanitation, ventilation and lighting, but he condemned the *Sacra Infermeria* as a hospital saying "*Ainsi, si l' hôpital de Malte etoit si vante du temps de l'ordre, ces louanges ne peuvent tomber que sur la maniere avec laquelle il etoit administre*". The wards were cleared from all incumbent objects including pictures on the walls, the bed canopies and curtains. The *Falanga*, previously reserved to treat venereal patients, was modified with the provision of large windows and connected to the Great Ward to increase the number of beds available for febrile patients.[12] Further arrangements became necessary to manage venereal

disease among the French troops and dedicated venereal disease hospitals were set up at the Santa Scolastica monastery and the Anglo-Bavarian Auberge in Valletta. These were closed down during the early British Administration.

The conversion of the *Sacra Infermeria* to a dedicated military hospital required the transfer of civilian patients to an alternative edifice. Accommodation for male civilians was arranged by January 1799 at the Convent of Mary Magdalene in Valletta, this being renamed *Hôpital Civil*, and was subsequently extended by adapting a de-consecrated church as a casualty ward, while the choir was converted into a dispensary. The upper floor of the monastery was used for fever cases, while the lower rooms were used for surgical cases and as stores. Part of the basement housed mental patients. A mortuary was built in the yard. The French Commissioner on 31st August 1798 re-organised the management structure of the hospital. The medical staff of the hospital consisted of two senior physicians assisted by three junior physicians, and two senior surgeons assisted by three junior surgeons and two barber-surgeons. The *Casetta delle Donne* continued to function as previously.[3,13]

The French occupation in 1798 thus saw a reorganization of the hospital services in Malta with a segregation of civil and military patients. After two years of civil strife, the Islands fell under the management of the British Colonial Office. The civil authorities continued to strife to provide adequate continuous hospital services retaining the establishments as set up by the French government. The original *Sacra Infermeria*, now variously named General Hospital or Garrison Hospital or Station Hospital, continued to function as a military hospital; while the *Infermeria delle Schiavi* in the Slave Prison, that saw its closure with the abolition of slavery imposed by Napoleon Bonaparte, was transformed in 1803 into a British Naval Hospital. This building continued to serve as a naval hospital until 1st July 1819, when the naval patients were again moved to the *Armeria* after a decision by the naval authorities to concentrate their various departments in Vittoriosa. At the beginning of the nineteenth century facilities for the treatment of sick merchant seamen - The Merchant Seaman Hospital - were set up in Malta, but the hospital closed down in 1822. Sick Merchant seamen were subsequently admitted to the Civil General Hospital in Valletta. In November 1850, a ward designated "The British Merchant Seamen Ward" was set aside in the surgical division of the Civil Central Hospital at Floriana. The Valletta Garrison Hospital continued to be used until the opening of new Military Hospital at Mtarfa in 1920.[14] The building was subsequently passed on to the civil government and served, until 1940 as a Police Depot. During the Second World War, the building sustained significant damage. In the post-War period, the remaining halls served several minor functions, including that of an Examinations Hall. In 1978, the building was converted into the Mediterranean Conference Centre.

The *Hôpital Civil*, now Civil Hospital, and the *Casette delle donne* continued to provide a service for the civil population right through the first half of the 19th century. These civil establishments were managed under the provisions of the *"Piano per il regolamento dell'ospedale di Malta decretato il 20 Marzo 1802"*.[15] All forms of disease were treated in the civil hospitals. The increasing demands made on the establishments ensured that ward overcrowding became the norm by 1837. In May 1850, the inmates from the Civil Hospital and the *Casetta delle donne* were transferred to the newly-established Central Hospital at Floriana. The *Casetta delle donne* building became the Hospital for Incurables reserved exclusively for inmates of both sexes suffering from incurable disease. It retained this role until the inmates were transferred to a new hospice at Imgieret in 1892.[16] The *Casetta* building was destroyed during the Second World War. The site was utilized in the 1950's to erect the science laboratories of the University. The former Male Civil Hospital was in 1851 reorganized as an orphan asylum. The building was destroyed during the Second World War and only the church survives. The site was used to build a new government school. It subsequently served as a Service Club and as an elementary school for children.

The transfer of the civil hospital services to Floriana in 1850 saw the end of hospital service provision from the capital. However, community services continued to be furnished throughout the British administration. Following the 1814 administrative reforms, the *medici dei poveri* or "Physicians to the Poor" were incorporated with the Executive Police Department with the doctors being referred to as *medici di polizia* or "Police Physicians" with the Chief Police Physician being

the physician responsible for Valletta and Inspector of Dispensaries. In the early decades of the nineteenth century [14th April 1832], the administration instituted the first public dispensary *Farmacia dei poveri* at the *Auberge d'Italie* at Valletta with the aim of screening admission to the Civil Hospitals. This was staffed by two physicians and two surgeons who offered their services without payment, and a paid apothecary. It subsequently started being referred to as *albergo dei poveri* abridged as *berga.*[17, 18]

Today the only health-related services that remain in Valletta are limited to the Ministry of Health building at Castella Palace which houses also the offices of the Department of Health. In 2012, Valletta was declared European Capital of Culture (ECoC) for 2018 with a partner Dutch city, Leeuwarden. In order to commemorate and honour Valletta, the Malta Medical School journals will feature front covers with artworks that are in some way related to Valletta, commencing with this very issue.

References

1. The Old and New Statutes of the Order of St. John of Jerusalem, translated from the Edition of Borgoforte, AD M,DC,LXXVI by Order of the Chapter of the great Priory of France, p. 25. In: de Vertot R. The history of the Knights of Malta. London: G. Strahan 1728; vol. 2:24-29,137-141.
2. Notizia della Sacra Infermeria e della carica delli Commissarj delle Povere Inferme. Rome: R. Bernaba 1725.
3. Cassar P. Medical history of Malta. London: Wellcome Historical Medical libr. 1965: 69-78, 364, 437-464, 509.
4. Inglott FV. Brief Historical Account of the treatment of the Insane in Malta. National Malta Library Manuscript 377, 1867.
5. National Malta Library. Archives of the Order of Malta P.R. 6408. As reported in Mifsud A. Knights Hospitallers of the ven. Tongue of England in Malta. Malta: Herald Print 1914: 140 fn.
6. Wismayer JM. The Seven-year balance sheet of the Sovereign Military and Hospitaller Order of St. John of Jerusalem of Rhodes and of Malta from 1st May 1778 to end of April 1785 by the Chevalier Bosredon de Ransijat Secretary to the Venerable Common Treasury with an Introduction and Glossary. Malta: Universal Intelligence Data Bank of America 1984.
7. Cassar P. A Medical Service for Slaves in Malta during the rule of the Order of St. John of Jerusalem. Medical History 1968, 12:270-277.
8. Vertot R. The history of the Knights of Malta. London: G. Strahan 1728, vol. 2:24-29.
9. Hume EE. Medical Work of the Knights Hospitallers of St. John of Jerusalem. Baltimore: John Hopkins University Press 1940: 109-111.
10. Cassar P. Residential areas of Medical and Paramedical Personnel in Valletta in the late eighteenth century. Maltese Medical Journal 1989; 1(2):21-25.
11. Cassar Pullicino J. The re-opening of the University in 1800. Journal of the Faculty of Arts, 1958; i:144.
12. Robert CE. Memoire sur la topographie physique et medicale de Malte, suivi de l'histoire des malades qui ont regne dans cette ville parmi les troupes francaise, sur la fin de l'an 6, et pendant les annees 7 et 8. Paris: P. Didotlaine 1802: 32-37.
13. Testa C. The French in Malta. Malta: MidSea Publ. 1997: 184-186.
14. Savona-Ventura C. Military and Naval Hospitals in the last two centuries. Historia Hospitalium. Zeitschrift der Deutschen Gesellschaft fur Krankenhausgesschichte 2000-01; 22:156-181.
15. Piano per il Regolamento dell'Ospedale di Malta decretato il 20 Marzo 1802. Malta: Palace Press 1802.
16. Savona-Ventura C. Civil Hospitals in Malta in the last two hundred years. Historia Hospitalium. Zeitschrift der Deutschen Gesellschaft fur Krankenhausgesschichte 1998-99; 21:45-46.
17. Malta Government Gazette 11 April 1832:112; 15 December 1832:52
18. Il Filocamo 1 May 1841, 5:35.

Editorial note

For the December 2017 issues and for all of 2018, the *Malta Medical Journal* and the *Malta Medical School Gazett*e will feature front covers that depict Valletta. This is in honour of Valletta 2018, wherein Valletta will be the European Capital of Culture, with all attendant programmes. The editorial board sincerely hopes that you will appreciate these covers as much as the actual contents.

Prof. Victor Grech

Cover Picture:
'Valletta'
Oil on canvas with palette knife
By Victor Grech

Victor Grech is a consultant paediatrician with a special interest in paediatric cardiology. He has a PhD in this field and another in science fiction. He is the editor of the journals Images in Paediatric Cardiology and the Malta Medical Journals and co-chairs HUMS, the Humanities, Medicine and Sciences Programme at the University of Malta.

Original Article

Rapidity of diagnosis and management of H. Pylori in the endoscopy unit at Mater Dei Hospital

Daniela Zammit, Thelma Xerri, Pierre Ellul

Abstract

Introduction: H.pylori infection has been associated with various gastric pathologies and its prevalence varies between different countries. Furthermore, there is an increasing antibiotic resistance and the eradication rates have declined. There is clinical and administrative pressure as to provide the Rapid Urease Test (RUT) result as quickly as possible and ideally prior to discharge from the endoscopy unit.

Results: A total of 542 patients fulfilled the inclusion criteria. The patient`s mean age was 54.6 years and 52.4% were female. The main clinical indications for an Oesophago-Gastro-Duodenoscopy (OGD) were dyspepsia (44.7%) and GORD (24.5%). The overall positivity rate was 15% of which 8.7% were early positive and 6.3% were late positive. Analysis of patients' age with RUT positivity revealed that patients above the age of 60 years were more likely to have a positive result (p=0.013). There was no statistical significance between the H.pylori results and smoking (p= 0.6).

In this study, there was a variety of 10 different eradication regimes prescribed, the most popular being the use of a PPI 20mg BD + Amoxicillin 1g BD + Clarithromycin 500mg BD for 10 days (total of 27 cases) versus 14 days (23 cases).

Conclusion: This study demonstrates the importance of checking the RUT taken at endoscopy at 24 hours as this has given a 42% increase in the yield for H.pylori. It also demonstrates that various regimens are used in clinical practice. In view of the relatively low prevalence of H.pylori, especially amongst young patients, maybe it is prime time that treatment of H.pylori is specifically managed by culture and sensitivity to avoid worsening clarithromycin-resistance.

Daniela Zammit M.D.*
daniela.c.zammit@gov.mt

Thelma Xerri M.D.

Pierre Ellul M.D., PhD, FRCP, MSc

Msida, Malta

**Corresponding Author*

Keywords

Helicobacter pylori; triple therapy; OGD; Proton Pump Inhibitor; eradication regime.

Abbreviations

- CLO: Campylobacter-like organism test
- CT scan: computerized tomography
- GI: Gastrointestinal
- GORD: Gastro-oesophageal Reflux Disease
- H.pylori: Helicobacter pylori
- MALT: Mucosa-associated lymphoid tissue lymphoma
- OGD: oesophago-gastro-duodenoscopy
- PET scan: Positron Emission Tomography

- PPI**:** Proton Pump Inhibitor
- RUT: Rapid Urease Test

Introduction and Aims

Helicobacter pylori (H.pylori) is a gram-negative bacterium found in the stomach of more than 50% of the population worldwide.[1] It is linked to chronic gastritis, gastric and duodenal ulcers and stomach cancer.[2] Up to 85% of patients infected with H.pylori are asymptomatic;[3] therefore a 'test-and-treat' approach is adopted and is most effective in a population with a high prevalence of H.pylori infection (defined as a prevalence of more than 20%), especially in patients under the age of 50 years without any alarm symptoms.[4] There are various methods of testing; these can be divided into minimally-invasive (blood antibody test, stool antigen test or using the carbon urea breath test), and invasive (analysis of gastric biopsies through Rapid Urease Test (RUT test), histology and culture). None of the methods are 100% specific and sensitive. One of the most commonly used is the RUT test upon performing an oesophagogastroduodenoscopy (OGD).[5]

This gastric biopsy test is based on the activity of the H. pylori urease enzyme. This splits the urea test reagent to form ammonia. Ammonia, being alkaline, increases the pH. This is detected by a colour indicator. Tests that produce rapid reliable findings have been shown to reduce the overall cost of management of these patients by decreasing the need for telephone calls to be made to patients after 24 hours.

The standard first-line treatment option for H.pylori eradication is a 7-10 twice daily (b.d.) triple therapy of proton-pump inhibitor (PPI) together with amoxicillin and clarithromycin or metronidazole.[6] Unfortunately, such a regime is becoming increasingly ineffective worldwide due to clarithromycin resistance, with data showing that eradication rates have declined to less than 80% in both the United States and Europe.[7]

Several alternative regimes for eradication have been proposed, including the extension of the treatment duration to 10 or 14 days; using a different PPI; quadruple therapy with the use of bismuth with a PPI and two antibiotics; concomitant and sequential regimes; use of probiotics-supplemented triple therapy or using other antibiotics such as levofloxacin [8-11]. Despite such antibiotic regimens, a 100% eradication rate is rarely achieved. [13] The success of eradication therapy depends on patient compliance and bacterial factors such as antibiotic resistance.

Various studies have demonstrated different results on the correlation of smoking and prevalence of H.pylori infection. It is proposed that smoking is negatively associated with H.pylori infection due to an increase in gastric acidity following smoking,[9] whilst other studies showed a positive relationship in view of damage to gastric mucosal protection [10] and reduced efficacy of eradication therapy.[11-12] Others studies have demonstrated that there is no statistically significant difference in the rate of positive infections in relation to current or previous smoking status.[14] While most of the recent studies have concentrated on the choice and prescription of antibiotics there is minimal recent data on the timing of the interpretation of the RUT after an oesophago-gastro-duodenoscopy (OGD).

The primary aims of this study were:

1. To assess the diagnostic accuracy of the RUT by assessing it at 4 hours and 24 hours.
2. To determine whether there is a correlation between smoking and H.pylori infection.

3. To identify the different treatment regimes prescribed at our local hospital.

A secondary aim of the study was to determine the prevalence of positive H.pylori infections among patients undergoing an OGD.

Methodology

This was a prospective study performed between January 2016 and July 2016. Approval was obtained from the Malta University Research Ethics Committee. Ninety-five percent (95%) of the endoscopists who perform OGD's at Mater Dei Hospital agreed to participate. The following data was obtained and entered into the database: date of procedure; patient`s age; gender; clinical indication; RUT; treatment regime prescribed and smoking status. The exclusion criteria were: (1) patients had already been tested and/or treated for H.pylori (2) use of proton pump inhibitors in the 2 weeks prior to the test and (3) the use of antibiotics in the preceding 4 weeks.

The RUT used in each case by all endoscopists involved was the Kimberly-ClarK CLOtest Rapid Urease Test. The test was defined as either negative, early positive or late positive.

The patients' OGD reports were checked on the same day of the procedure and the following day by two investigators. If the RUT test showed a colour change up to 4 hours after the procedure, it was marked as "early positive". If the RUT test demonstrated a positive test more than 4 hours later and within 24 hours, it was marked as "late positive".

Results

A total of 580 consecutive patients who performed an OGD at our centre were recruited. Thirty – eight (38) patients were excluded as 31 patients had been previously tested and/or treated for H. Pylori and another 7 patients did not have a CLO test taken during the OGD. From the 542 eligible patients, 52.4% were female (284 patients).

The minimum age was 15 and the maximum was 88. The patient`s mean age was 54.64 years (median age: 58 years; range 15 - 88 years). Figure 1 demonstrates the age distribution of the patients' cohort.

The main clinical indication was dyspepsia (44.7%). Other main indications were gastro-oesophageal reflux disease (GORD) (24.5%) and in the investigation of anaemia (10.1%). The other clinical indications are demonstrated in Table 1.

From the cohort, 85% of the RUTs were negative for H.pylori. The rest were positive and were classified into early positive (8.7%) and late positive (6.3%). Analysis of patients' age with RUT positivity revealed that patients above the age of 60 years were more likely to have a positive result (p=0.013). Figure 2 demonstrates the percentage of positive RUT tests for every age group.

From the patients with a negative RUT test, 25.4% were smokers. Similarly from those with a positive result, 28.4% were smokers. There was no statistical significance between the H.pylori results and smoking (p=0.6).

The most commonly prescribed regimens were omeprazole 20mg BD + Amoxicillin 1g BD + Clarithromycin 500mg BD for 10 days (33%) and 14 days (28%). In the rest (39%), 10 different regimens were prescribed, as can be demonstrated in Figure 3.

Figure 1: *Age distribution of patients who underwent OGD*
The minimum age was 15 and the maximum was 88. The patient`s mean age was 54.64 years (median age: 58 years; range 15 - 88 years).

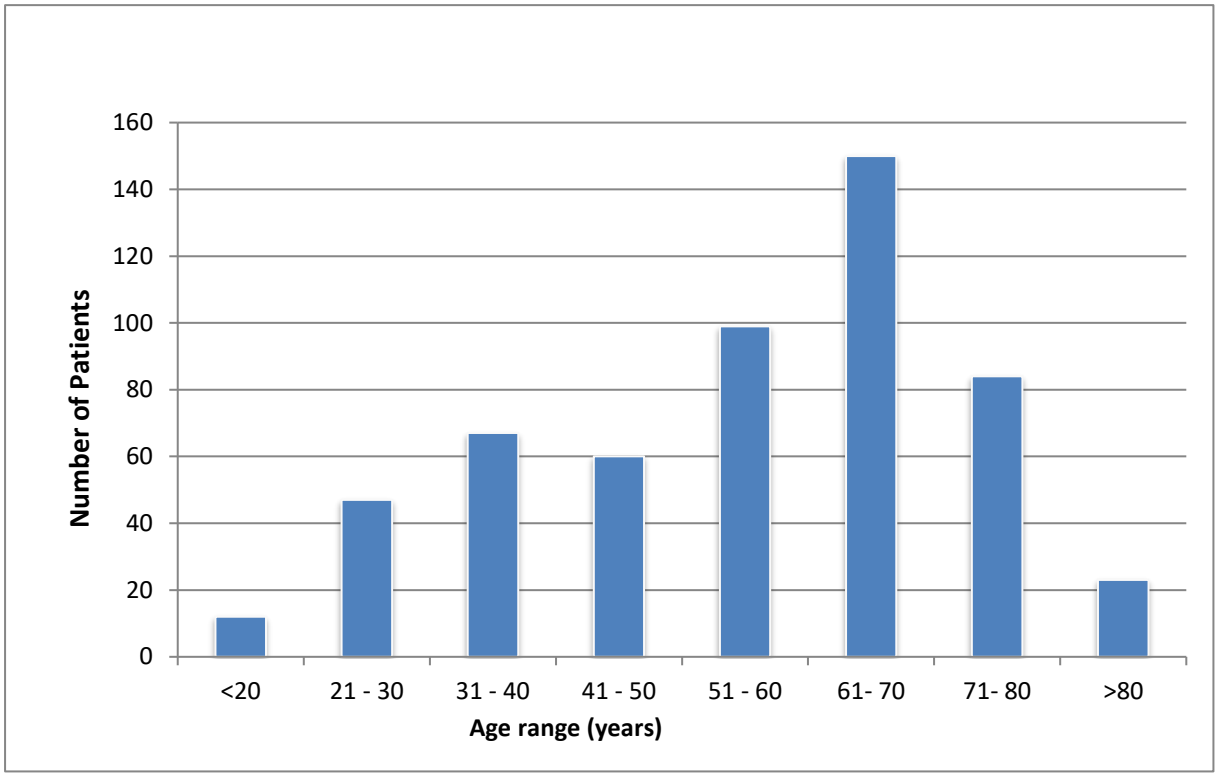
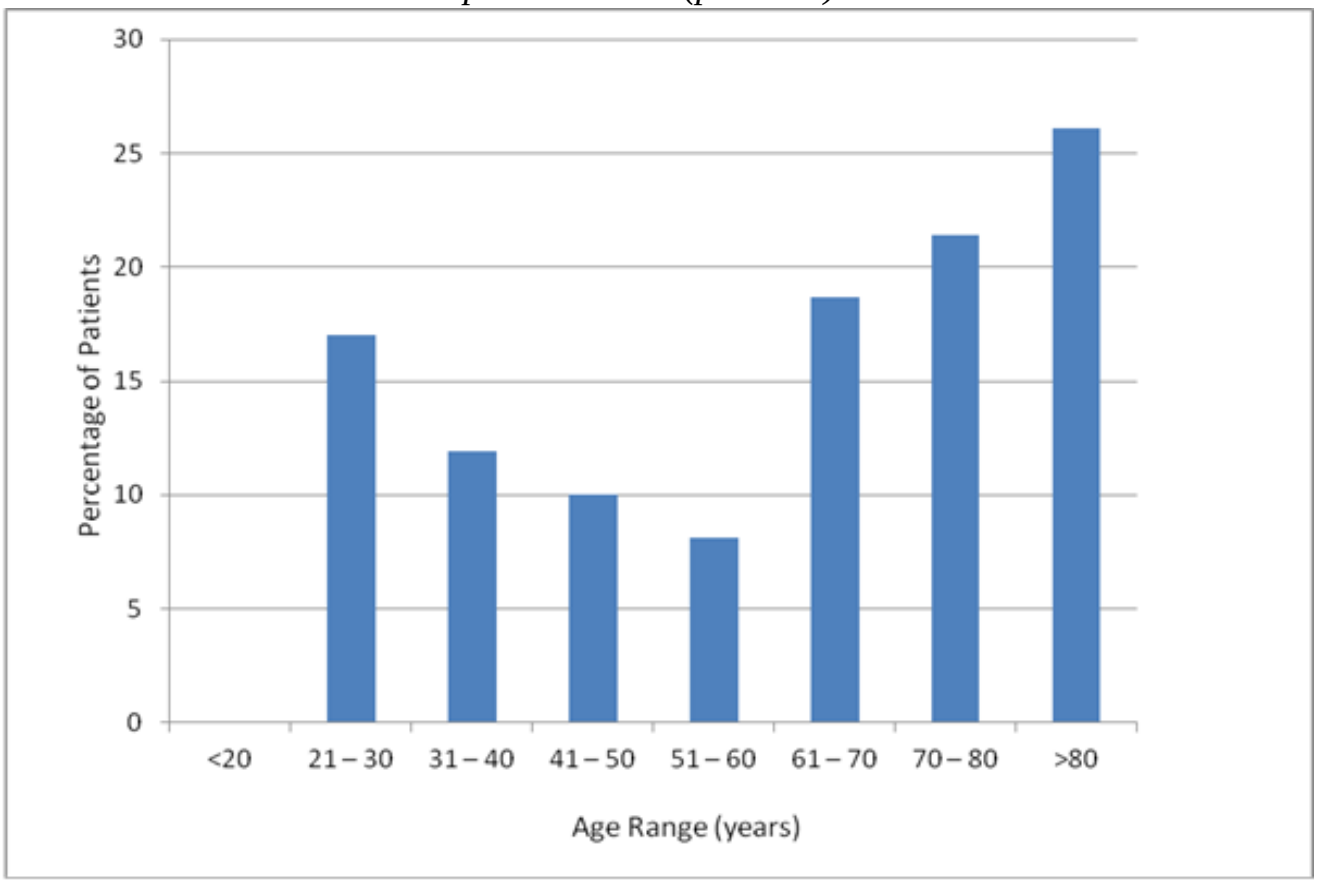


Table 1*: Clinical indication for OGD*
The main clinical indication was dyspepsia (44.7%). Other main indications were gastro-oesophageal reflux disease (GORD) (24.5%) and in the investigation of anaemia (10.1%)

Clinical Indication	Percentage (%)
Dyspepsia	44.7
GORD	24.5
Anaemia	10.1
Weight loss	2.2
Variceal Screening due to cirrhosis	2
Dysphagia	1.1
Not specified	3
Screening for upper GI cancer	6.5
Work-up for coeliac disease	2.2
Suspected upper GI bleeding	2
Gastric mass on CT scan and/or increased uptake on prior PET scan	1.7

***Figure 2**: Percentage of Positive RUT in each age group*
From the cohort, 85% of the RUTs were negative for H.pylori. The rest were positive and were classified into early positive (8.7%) and late positive (6.3%). Analysis of patients' age with RUT positivity revealed that patients above the age of 60 years were more likely to have a positive result (p=0.013).

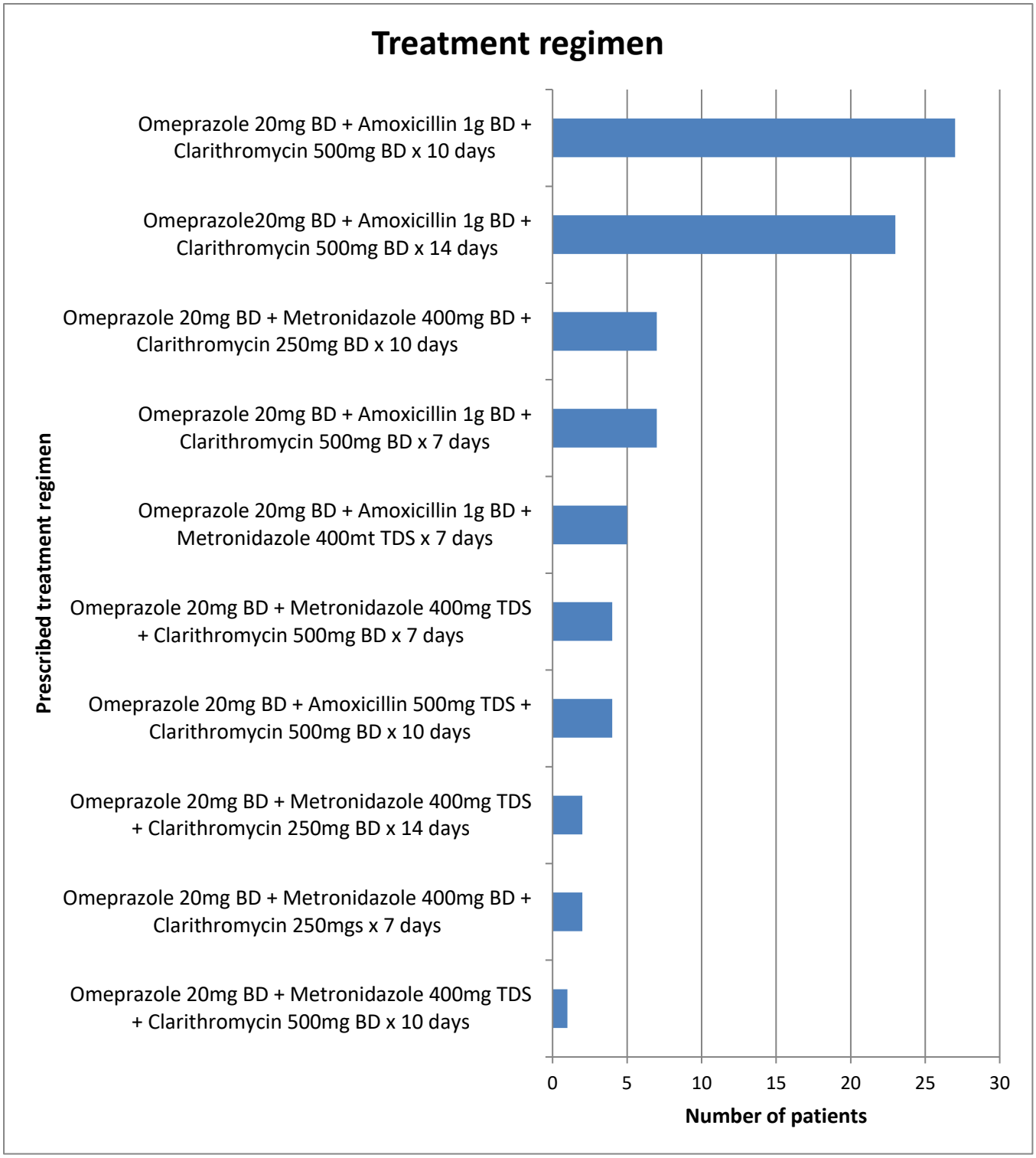


Discussion

H. pylori infection is a cause of peptic ulcer disease, gastric mucosa-associated lymphoid tissue (MALT) lymphoma and gastric cancer. RUTs are used widely throughout the world in endoscopy units to determine if patients are infected with H. pylori. The accuracy of these tests is important because a missed diagnosis of H. pylori infection can thus result in various pathologies.[15]

In the RUT, the gastric biopsy is put into a gel medium containing urea and a pH indicator. If present, the urease enzyme of H. pylori splits urea into ammonia and carbon dioxide. Ammonia alters the pH of the medium which then causes the colour change of the pH indicator. This process can take a variable amount of time and potentially there are various variables such as previous exposure to PPI`s, antibiotics and amount of urease that is produced by the bacterium.[16] The expectation from a clinical perspective and also administrative is to provide a quick and reliable result before the patient leaves the endoscopy unit in view of patient satisfaction and as to decrease the need for phone calls on the next day and other related paperwork.

***Figure 3**: Treatment regimen prescribed to patients with a positive RUT*
The most commonly prescribed regimens were omeprazole 20mg BD + Amoxicillin 1g BD + Clarithromycin 500mg BD for 10 days (33%) and 14 days (28%). In the rest (39%), 10 different regimens were prescribed



The overall prevalence of 15% does not support the empirical treatment for H. Pylori amongst symptomatic patients within our population. There was a statistically significant difference in prevalence between patients below and above the age of 60 years (p=0.013). Furthermore in patients under the age of 20 years, none of the patients had H. Pylori. One possible bias within the study group could have been that although patients were told to stop proton pump inhibitors 2 weeks prior to the OGD, they might not have done so and they might have consumed antibiotics in the preceding 4 weeks. This would have led to an under representation of H.pylori. Similarly, we only tested for H. pylori with one modality (the RUT) and did not compare the result with histology and/or culture as a reference standard. This could also have led to a lower incidence. However, previous studies have demonstrated that the RUT is 97% specific and 98% sensitive when compared to histology which was 100% specific and 91% sensitive.[16] Furthermore, previous data from studies has demonstrated that overall positivity is 75% within 20 minutes, 85% are detected at 1 hour, 90% by 3 hours and 95% by 24 hours.[17] Data from our study contrasts significantly with this data as 42% of the RUT turned positive after 4 hours.

In view of this previous data and the costs involved, we did not check for H.pylori with other modalities. However, we assessed what really happens in the day-to-day clinical practice. It is important to note that there is limited recent analysis about the timing of the colour change for this RUT and most data is coming from the late 1980s. Thus, in view of increasing or changing antibiotic resistance patterns it might be prime time to review this, as analysis of the RUT at an early stage and not reviewing at 24 hours might lead to missing out on the diagnosis of H.pylori and thus not treating it.

Previous studies have demonstrated both positive and negative associations of smoking with H.pylori.[14] In this study there was no correlation between the presence of H.pylori and smoking. No H.pylori related malignancies were noted within this cohort.

Inconsistencies regarding the eradication regimes prescribed in the endoscopy unit are evident and depend on consultant preference and the junior doctors who prescribe the treatment after checking RUT. As culture with antibiotic sensitivities is not routinely performed when H.pylori infection is diagnosed, it is generally recommended that different antibiotics be given at higher doses for 14 days. [18] Our study concluded that only 28% of the positive RUT patients were prescribed such a regime.

Increased antibiotic resistance is a recognised problem affecting the overall success rate of eradication of H.pylori. To minimise the clinical impact of antimicrobial resistance and eradication failure, several studies recommend antimicrobial susceptibility testing prior to initiating treatment. [19,20] Ideally antibiotic choice should be based on culture and sensitivity of each H.pylori strain cultured in the biopsy however this is not practiced at Mater Dei Hospital and the eradication therapy is chosen empirically.

The difficulties associated with performing culture and antibiotic sensitivity studies for H.pylori include both expense and the fastidiousness of the organism. Studies have shown that the use of a single antral biopsy for assessing efficacy of a particular treatment regimen may fail to detect resistant strains. [21] Thus resistance variability of H.pylori organisms at different gastric mucosal sites is a contributing factor to higher eradication rates and such

antibiotic resistance would require multiple gastric biopsies from different sites.

Limitations to this study which may have affected the positivity rate of the RUT and thus the outcome of the study is that there is no recorded data of how many biopsies and from where they were taken and used for each RUT, although it is standard practice to take at least one sample from the antrum of the stomach.

Another possible limitation to the study is that there is no available follow up on successful eradication of H. Pylori though this was not one of the intended aims of the study as this is not routine practice at Mater Dei Hospital but the choice of the respective endoscopist.

Conclusion

This study demonstrates that various regimens are used in clinical practice. However, we have to note the increasing antibiotic resistance. Various eradications rates have been ascribed to different regimens. In view of the relatively low prevalence of H.pylori, especially amongst young patients, antimicrobial resistance studies of H.pylori should be actively considered as to have better guidance with regards to antibiotic prescription. Furthermore, the significant increase in positivity post- 4 hours necessitate that if the test is still negative at 4 hours, it has to be re-analysed at 24 hours.

References

1. Manuel A, Peek, Richard MP Jr. "Pathobiology of Helicobacter pylori–Induced Gastric Cancer". *Gastroenterology*. 2016**;150 (1):** 64–78.
2. Infection with Helicobacter pylori. *IARC MonogrEvalCarcinog Risks Hum*. 1994**;61:**177–240.
3. Bytzer P, Dahlerup JF, Eriksen JR, Jarbøl DE, Rosenstock S, Wildt S, *et al.*"Diagnosis and treatment of Helicobacter pylori infection". *Dan Med Bull*. 2011**;58 (4):** C4271
4. Malfertheiner P, Megraud F, O'Morain C. "Guidelines for the management of Helicobacter pylori infection". *Business Briefing: European Gastroenterology Review.* 2005**.** Accessed October 2016 on: http://www.helicobacter.org/download/summary_guidelines_hp_infection%20_business_briefing.pdf
5. Stenström B, Mendis A, Marshall B. "*Helicobacter pylori*—The latest in diagnosis and treatment". *AustFam Physician*. 2008**;37 (8):** 608–12.
6. NICE (2014) Gastro-oesophageal reflux disease and dyspepsia in adults: investigation and management. Clinical guideline 184. Published September 2014, Last updated November 2014. Accessed October 2016 on: https://www.nice.org.uk/guidance/cg184/chapter/1-recommendations?unlid=7844170620162313724
7. Graham DY, Shiotani A. New concepts of resistance in the treatment of Helicobacter pylori infections. *Nat ClinPractGastroenterolHepatol*. 2008**;5:**321–331.
8. Bao-Zhu L, Diane Erin T, Ji-Yao W, Jian-Ming X, Jin-Qiu Y, Chao Z *et al.* Comparative effectiveness and tolerance of treatments for Helicobacter pylori : systematic review and network meta-analysis. *BMJ.* 2015**;**101hh21**.**
9. Ogihara A, Kikuchi S, Hasegawa A, Kurosawa M, Miki K, Kaneko E *et al.* Relationship between Hellicobacter pylori infection and smoking and drinking habits. *J GastroenterolHepatol.* 2000**;15(3):** 271-6.
10. Parasher G and Eastwood GL. Smoking and peptic ulcer in the Helicobacter pylori era. *Eur J GastroenterolHepatol.* 2000**;12(8):** 843-53.
11. Camargo MC, Piazuelo MB, Mera RM, Fontham ETH, Delgado AG, Yepez MC *et al.* Effect of smoking on failure of H.pylori therapy and gastric histology in a high gastric cancer risk area of Colombia. *ActaGastroenterolLatinoam***.** 2007**; 37(4):** 238-45.
12. Takeshi S, Keitaro M, Hidemi I,Sawaki A, Hirose K, Wakai K *et al.* Smoking Increases the Treatment Failure for *Helicobacter pylori* Eradication. *The American Journal of Medicine.* 2006; **119(3):** 217-224.
13. Borody T. Quadruple should be the first-line therapy for Helicobacter pylori infection. *Helicobacter pylori.* 2000; **26–29**: 623–9.
14. Mohie A AA K, Shabaan E K, Amal A A. Cigarette smoking status and Helicobacter pylori infection in non-ulcer dyspepsia patients. *Egyptian Journal of Chest Diseases and Tuberculosis.* 2014; **63(3):** 695-699.
15. Gisbert JP, Khorrami S, Carballo F,Calvet X, Gené E, Dominquez-Muñoz JE. H. pylori eradication therapy vs. antisecretory non-eradication therapy (with or without long-term maintenance antisecretory therapy) for the prevention of recurrent bleeding from peptic ulcer. *Cochrane Database Syst Rev.* 2004: CD004062.

16. Dye KD, Marshall MJ, Frierson HF, Barrett LJ, Guerrant RL, McCallum RW. Is CLO test* alone adequate to diagnose Campylobacter pyloridis. *Am J Gastroenterol.* 1988**; 83:**1032.
17. Marshall BJ, Warren JR, Francis GJ, Langton SR, Goodwin CS, Blincow ED. Rapid Urease test in the management of Campylobacter pyloridis- associated gastritis. *Am J Gastroenterol.* 1987; **82(3):** 200-210.
18. Sonnenberg, A. "Time trends of ulcer mortality in Europe". *Gastroenterology.* 2007*;* **132 (7):** 2320–7.
19. Toracchio S, Cellini L, Di Campli ED *et al.* Role of antimicrobial susceptibility testing on efficacy of triple therapy in Helicobacter pylori infection. *Aliment Pharmacol Ther*. 2000; **14**:1639–43.
20. Romana M, Ioyene MR, Montella F *et al.* Pre-treatment antimicrobial susceptibility testing in eradication of *H. pylori* infection. *Am J Gastroenterol*. 2000; **95**: 2317–18.
21. Jorgensen M, Daskalopoulos G, Warburton V, Mitchell H, Hazell S. Multiple strain colonisation and metronidazole resistance in Helicobacter pylori-infected patients: Identification from sequential and multiple biopsy specimens. *J Infect Dis*. 1996; **174**: 631–5.

Original Article

Thr300Ala ATG16L1 Polymorphisms and Bone Strength in Crohn's Disease Patients

Neville Azzopardi, Pierre Ellul, Christian Saliba, Neville Calleja, Godfrey LaFerla, Godfrey Grech

Abstract

Introduction: Studies on the effect of deletion of ATG5 and ATG7 proteins on bone cell function and bone strength in laboratory mice have revealed an association between autophagy and osteoporosis. The effect on bone strength of the Thr300Ala variant (rs2241880 polymorphism) of the ATG16l1 gene, a Crohn's disease susceptibility gene essential in macro-autophagy, has not yet been explored.

Methods: 101 Crohn's disease patients underwent DEXA bone density scanning. Real time PCR, high resolution melt (HRM) and restriction fragment length polymorphism (RFLP) were made use of as to assess for the rs2241880 polymorphism of the ATG16L1 gene in these patients.

Results: HRM and RFLP demonstrated that 39.6% had the wild type rs2241880 (Thr300Ala) polymorphism while 7.9% were homozygous and 52.5% were heterozygous for the polymorphism. Mean DEXA bone mineral density scores in these patients showed lower T scores at the hip (-1.74) among patients with the homozygous polymorphism than among patients with the heterozygous polymorphism (mean T score hip: -1.29). The highest mean T scores were found in patients with the wild type polymorphism (-1.04).

Discussion: This study demonstrates the first evidence that polymorphisms in the ATG16L1 gene may play a role in bone metabolism.

Neville Azzopardi MD, MRCP(UK), MPhil (Melit.)*
Gastroenterology Department,
Mater dei Hospital,
Msida, Malta
neville.azzopardi@gov.mt

Pierre Ellul, MD, PhD, FRCP(UK), MSc
Gastroenterology Department,
Mater dei Hospital,
Msida, Malta

Christian Saliba,
B.Pharm.(Hons)(Melit.),Ph.D.(Cagliari) Genetics Department, University of Malta

Neville Calleja, M.D., M.Sc. (Lond.), M.Sc., M.F.P.H., C.Stat., C.Sci.
Department of Public Health,
Malta

Godfrey LaFerla M.D.,Ph.D.(Glas.),M.R.C.S.(Eng.), L.R.C.P.(Lond.)
Dean
Faculty of Medicine & Surgery,
University of Malta
Msida, Malta

Godfrey Grech, B.Sc.,M.Phil.,Ph.D.(Erasmus)
Genetics Department,
University of Malta
Msida, Malta

**Corresponding Author*

Keywords

Osteoporosis; Autophagy; Crohn's Disease; ATG16L1

Introduction:

The ATG16l1 (autophagy-related 16-like) gene, essential in macro-autophagy, is located on chromosome 2q37.1.[1] The Thr300Ala variant (rs2241880 polymorphism) of this gene, located in the c-terminal WD40 domain,[1] is an important

Crohn's disease (CD) susceptibility gene. Osteoporosis is common among CD patients [2,3] being associated with an increased risk of fracture.

The autophagy pathway appears to play an important role in bone metabolism and bone diseases, by having a direct effect on osteoclast, osteoblast and osteocyte function.[4] Autophagy maintains cellular homeostasis and allows longer-surviving cells like osteocytes, to function normally. In conditions of increased stress, autophagy is increased, resulting in recycling of intracellular components like amino acids and prolonging cell survival.[5] Autophagy also allows osteocytes to adjust to a more hypoxic and nutrient poor environment. Osteocytes play a key role in bone remodelling by synthesizing sclerostin, receptor activator of nuclear factor kappa B-ligand (RANKL), fibroblast growth factor-23 and collagens. Osteocyte autophagy plays an important role in age-related bone loss.[6] Deletion of the autophagy protein Atg7 from osteocytes resulted in reduced bone mass in mice and suppressed autophagy in osteocytes appears to lead to age-related bone loss.

Autophagy is also important in osteoclasts, as has been demonstrated using conditional knockout mice. Deletion of key proteins involved in autophagosome formation, like Atg5, resulted in increased bone volume in vivo and protection from ovariectomy-induced bone loss. Deletion of ATG7 was associated with reduced resorptive capacity of osteoclasts. This data seems to suggest that inhibition of autophagy in osteoclasts may provide a potential therapeutic mechanism for various bone disorders. Osteoclast formation and size is increased by hypoxia [7-8] and upregulation of autophagy has been shown to increase this effect.[9] However, Rapamycin, an autophagy inducer, has been shown to decrease the number of osteoclasts and to be associated with reduced bone resorption in laboratory rats.[10] More in vivo and in vitro studies are needed to better understand the role of autophagy in regulating osteoclast function.

Osteoblasts are also under autophagic control.[11] Animal models have shown that impaired autophagy is associated with severe osteopoenia due to reduced bone formation.[12] Rapamycin appears to promote osteoblast differentiation therefore suggesting that autophagy increases bone formation while impaired autophagy may be associated with an increased risk of osteoporosis.[13]

Studies analysing the association between autophagy and osteoporosis have mainly investigated the effect of deletion of ATG5 and ATG7 proteins on bone cell function and bone strength in laboratory mice. No studies on the effect of impaired autophagy secondary to polymorphisms in the ATG16L1 gene on bone strength in humans have been carried out to date.

Impaired autophagy and CD have both been shown to be associated with an increased risk for osteoporosis and osteopenia. Impaired autophagy has also been shown to be one of the pathways involved in mucosal inflammation in CD, with a higher overall risk of CD in patients with the rs2241880 ATG16L1 polymorphism. However, this polymorphism has never been studied as a potential risk factor for osteoporosis. We therefore hypothesized that CD patients with impaired autophagy secondary to the rs2241880 ATG16L1 polymorphism have lower bone mineral density dual energy X-Ray absorptiometry (DEXA) scores than patients not exhibiting this polymorphism.

Methodology

Ethical approval was obtained through the University of Malta Research and Ethics Committee. Maltese CD patients diagnosed through standard clinical, histo-pathological and endoscopic findings were recruited prospectively through the gastroenterology clinic at Mater dei Hospital (MDH), Malta.[14] All CD patients seen at medical out-patients between September 2012 and June 2014 were invited to participate. Patients with indeterminate colitis and individuals who did not have Maltese ancestry were not included in the research. CD diagnosis was defined according to the Copenhagen Diagnostic Criteria.[15] Written informed consent was obtained from each patient. Each patient was asked questions related to his duration, type, location and severity of Crohn's disease, ongoing and previous treatments and the duration of this treatment, history of previous fractures and the aetiology behind such fractures. Where possible, this information was corroborated with information from the patients' files. All data was entered into a tailor-made database. All patients underwent a DEXA bone mineral density scan at the MDH using a Hologic DEXA scanner. T score (comparison of bone density with peak bone mass at around age 30) was used to assess risk of fracture with T scores <-2.5 being indicative of osteoporosis and T scores between -1 and -2.5 being suggestive of osteopenia. However, since a section of the population included young, premenopausal women and men younger than 50 years of age, the Z score was also used as a marker of bone density.[16]

Genotyping for the common coding variant rs2241880 (Thr300Ala) of the ATG16l1 gene was carried out on peripheral venous blood extracted from the CD patients. Three millilitres of whole blood was extracted from each patient and collected in an ethylenediamine tetraacetic acid (EDTA) tube. Deoxyribonucleic acid (DNA) extraction from whole blood of these CD patients was carried out using the DNA Mini Kit (Qiagen, Hilden, Germany).[17] Gradient polymerase chain reaction (PCR) was then carried out to establish the optimal annealing temperature for this variant. Real-time PCR and high resolution melt (HRM) were subsequently carried out at annealing temperatures of 54ºC (optimal temperature found at gradient PCR). The reaction mixture for real time-PCR and HRM consisted of 4.0µl of 5x Hot FirePol® EvaGreen® qPCR Mix Plus (*Solis BioDyne*™), 0.5µl of 10µM primers F and R, 14.0µl of distilled water and 1µl of template DNA. The samples were then run through qRT-PCR and HRM under the following conditions: initiation at 95ºC for 5 minutes, denaturation at 95ºC for 10 seconds, annealing at 54ºC for 30 seconds, extension at 72ºC for 10 seconds (denaturation, annealing and extension were repeated for 40 cycles) and HRM at 75–95 ºC. The results obtained using HRM were then compared with the results of restriction enzyme digest of the PCR product. Restriction fragment length polymorphism (RFLP) was carried out using the SfaNI/LweI enzyme (NEB *Inc*). The reagent mixture consisted of 2µL of NE Buffer 4 (X10), 1µL of the SfaNI/LweI enzyme (2,000 units/mL), 9µL of DNase and RNase free water, and 8µL of the DNA under study (0.5-1µg/µL). 5µl of this mixture was added to 2µl of 6X DNA loading dye buffer double blue and then loaded on 2.5% agarose gel. 5µl of 100 base pair DNA Ladder were also loaded on the same gel. The electrophoresis was run for 1 hour and 30 minutes at 100 Volts and then the gel was analysed to identify the

mutant and wild type samples.

Kruskall-Wallis test and χ^2 test were used to analyse for any statistical differences in the phenotypic characteristics of patients with the wild type, heterozygous and homozygous rs2241880 polymorphisms. Kruskall-Wallis test was also used to assess whether there were any statistically significant differences in bone mineral density T scores and Z scores in patients with the wild type, heterozygous and homozygous rs2241880 polymorphisms.

Results

Demographic and Phenotypic Characteristics

One hundred and one (101) patients with CD were recruited. This represents approximately 25% of the Maltese CD population and should therefore be a truly representative sample of the Maltese CD cohort. Table 1 demonstrates the phenotypic characteristics of these patients. The mean duration of CD was 8.2 years (range: 5 months to 32 years). Table 2 describes the relevant drug history of the CD patients in the study population.

Table 1*: Phenotypic characteristics of CD cohort*

Characteristics	
Current Age, mean, (*years)* [range]	39.9 [18-83]
Male	51 patients
Postmenopausal Women	13 %
Patients with family history of IBD	4 %
Current Smokers	20 %
ex- smokers	6 %
Documented fractures	7 patients
Hip	1 patient
Spine	1patient
Others	5 patients
Montreal Classification:	
A1	12.8%
A2	64.4%
A3	22.8%
L1	23.8%
L2	33.7%
L3	42.5%
B1	69.3%
B2	23.8%
B3	6.9%
Perianal disease	5%
Extra-intestinal manifestations :	21%
H/o IBD related abdominal surgery	25%

A1 - age at diagnosis <17 years; A2 – age at diagnosis 17-40 years; A3 – age at diagnosis >40 years; L1 – ileal disease only; L2 – colonic disease only; L3 – ileocolonic disease; B1 – non-stricturing, non-penetrating disease; B2 – stricturing disease; B3 – penetrating disease

***Table 2**: Medical Treatment*

Treatment	Number of patients (mean dose)
5-Amino- Salicylates	82 %
Thiopurines	55 %
Current steroid use	7 %
Previously or currently on steroids	69%
Previously on anti-TNF -alpha	7%
Currently on anti-TNF-alpha	37%
5 mg/kg every 8 weeks	31%
10 mg/kg every 8 weeks	5%
5 mg/kg every 4 weeks	1%
Dual Immunosuppressant Use (anti-TNF-alpha and Thiopurine)	28%
Previous Elemental Diet	3%
Methotrexate	6%
Calcium and Vitamin D replacement	11%
Bisphosphonates	2%

TNF : Tumour necrosis factor; mg – milligrams; kg – kilograms

***Table 3:** Mean T and Z scores at the Hip and Spine*

	Mean T Score (Normal: -1.0 to 1.0)	**Mean Z score**
Hip	-1.22 (Range:-5.2 to 1.2)	0.55 (Range:-3.6 to 1.68)
Spine	-0.80 (Range:-5.1 to 1.6)	-0.41 (Range:-2.2 to 1.9)

Bone Densitometry Results

Table 3 describes the mean T and Z scores at the hip and spine. Eleven percent (11%) of patients had osteoporosis at the hip (T score <-1.5) and 6% had osteoporosis at the spine while 46% had osteopenia at the hip (T score -1.0 to -2.5) and 34% had osteopenia at the spine. Seven patients had documented fractures. Two patients had rib fractures and humeral fractures. These were all related to major trauma (motor vehicle accidents). Another 3 individuals had Colles' fractures and rib fractures related to minor trauma (fall from low height).

The mean T score (hip) among these 5 patients was -1.0 (Z score: -0.5) and mean T score (spine) was -1.2 (Z score: -1.0). One patient had a history of vertebral fracture

with no documented trauma (DEXA bone mineral density T score spine: -2.86, Z score spine: -2.07) and one patient had a hip fracture following a fall from her own height (T score hip: -2.3, Z score hip: -1.7).

Two patients with a previous history of fractures (one had a hip fracture and the other patient had a vertebral fracture) were being administered oral bisphosphonates. Both patients had a T score less than -2.5 (mean T score: -3.6). All patients on Vitamin D and calcium replacement treatment (11%) had a T score less than -1.0 (mean T score -1.9).

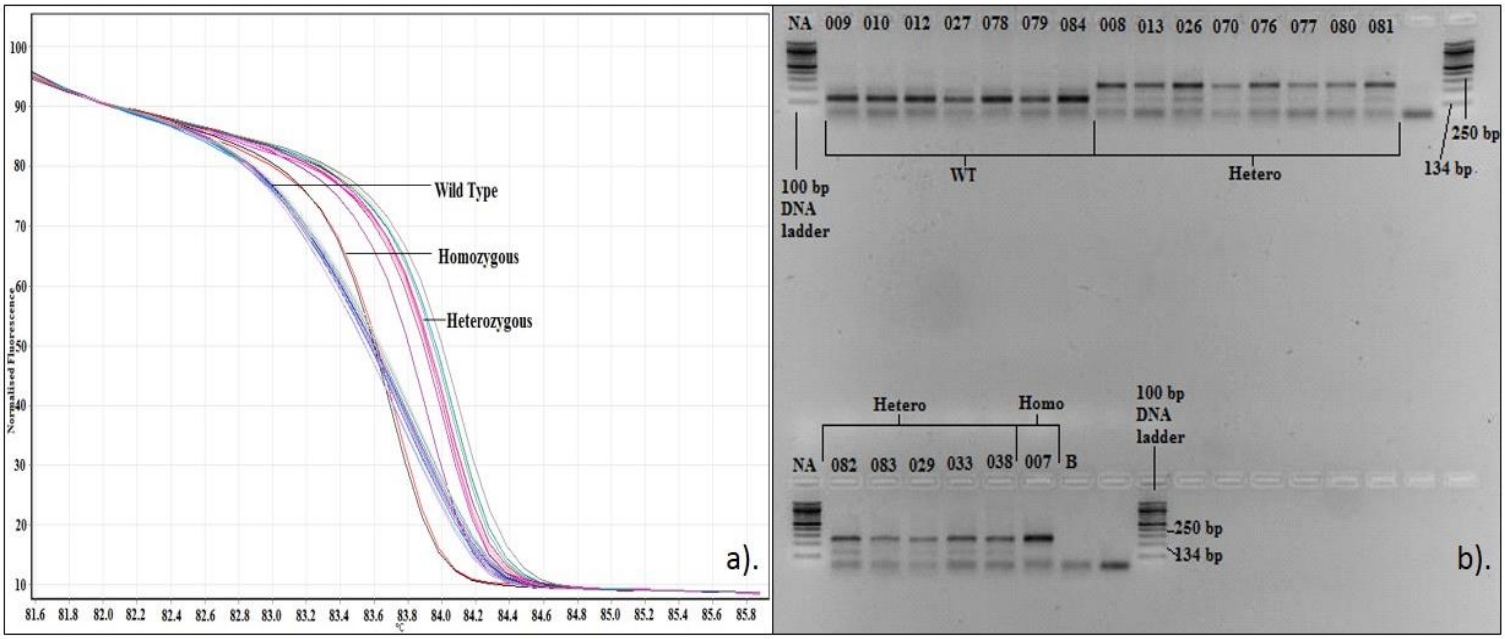
Genotype Analysis

HRM (sample on Figure 1a) on the 101 DNA samples from the study population revealed that 39.6% of the CD patients (40 patients) did not have the rs2241880 (Thr300Ala) polymorphism (wild type) while 7.9% (8 patients) were homozygous. The rest, 52.5% (53 patients) were heterozygous for the polymorphism. These results were confirmed by RFLP (Figure 1b).

Phenotypic differences between patients with wild type, homozygous and heterozygous rs2241880 polymorphisms were then analysed (Table 4). Using the chi-squared (χ^2) test, there were no significant differences in the age at diagnosis (*p*: 0.21) of CD, disease location (*p*: 0.73), disease behaviour (*p*: 0.36), history of surgical intervention (*p*: 0.46) and smoking history (*p*: 0.26) between patients with the wild type, heterozygous and homozygous rs2241880 polymorphism. There were also no significant differences in the use of thiopurine therapy (χ^2 test *p*: 0.12), corticosteroids (*p*: 0.83), anti-TNFα therapy (*p*: 0.78) and use of dual immunosuppression therapy (thiopurine and anti-TNF alpha) (*p*: 0.82) between patients with the wild type, heterozygous and homozygous rs2241880 polymorphism. There was no significant differences in disease duration (Kruskall –Wallis test *p*: 0.22) and body mass indices (*p*: 0.52) between patients with the wild type, heterozygous and homozygous rs2241880 polymorphism.

The mean T and Z scores at the hip and spine of patients with the wild type and mutant genotypes are shown in Table 1. There was no statistically significant difference in the T score at the hip between patients with the wild type, heterozygous and homozygous ATG16L1 rs2241880 variants (Kruskall-Wallis test *p*: 0.09). Similarly, there were no significant differences in the mean T score spine (*p*: 0.48), Z score hip (*p*: 0.23) and Z score spine (*p*: 0.73) between patients with the wild type and mutant ATG16L1 variants. However, a trend in DEXA T scores was observed with patients with the homozygous rs2241880 polymorphism (T300A variant) having lower T scores than patients with the heterozygous and wild type polymorphisms. Subgroup analysis on 13 postmenopausal CD women in our population revealed that 3 patients exhibited the wild type polymorphism, 2 had the homozygous and 8 had the heterozygous polymorphism. Mean T scores (hip) among postmenopausal women demonstrated a similar trend to that seen in the CD population (mean T score wild type: -0.1, heterozygotes: -1.19, homozygotes: -2.58). A similar trend was also seen at the spine (mean T score wild type: -0.8, heterozygotes: -0.96, homozygotes: -1.89).

Figure 1: a). High Resolution Melt using 5x Hot FirePol® EvaGreen® qPCR Mix Plus for Exon 9 (Thr300Ala) of the ATG16L1 gene with rs2241880 primers using DNA from Crohn's disease patient samples b). Agarose gel analysing Restriction Fragment Length Polymorphism for the Thr300Ala variant (rs2241880 polymorphism) using the SfaNI/LweI enzyme on DNA extracted from Crohn's disease study patient samples (bp: base pairs, B: blank sample, NA: code for Crohn's disease samples, WT: Wild Type allele, Hetero: Heterozygous allele, Homo: Homozygous allele for rs2241880 variant)



Discussion

An association between autophagy and bone metabolism has recently been established.[5-13, 18] However, the effect of impaired autophagy secondary to ATG16L1 polymorphisms on bone strength has never been studied. In this study, we have analysed whether individuals with impaired autophagy secondary to the rs2241880 ATG16L1 polymorphism have lower bone mineral densities than individuals not exhibiting this polymorphism. This analysis was carried out on a population of CD patients since this polymorphism has been shown to be an important CD susceptibility gene in several population studies.[19-20] In addition, CD is associated with lower bone mineral densities and higher risk of osteoporosis. While many phenotypic characteristics have been identified as possible risk factors for osteoporosis in CD (age at onset, history of surgical intervention, male gender, corticosteroid use), studies linking CD susceptibility genes with risk for osteoporosis have not been carried out. Further evidence to the validity of our cohort is that the clinical characteristics of our cohort was very similar to that reported in other European countries.[21-22]

Genotyping of the CD study population was carried out using both RFLP and HRM. The results from both techniques were identical, allowing us to confirm the ATG16L1 variant genotypes of our population with two different techniques. Statistical analysis did not show any significant differences in the phenotypic and clinical characteristics of patients with wild type, heterozygous and homozygous

ATG16L1 polymorphisms. Statistical analysis also did not reveal any significant differences in DEXA bone mineral density T scores and Z scores between patients with the wild type, heterozygous and homozygous polymorphisms. However, a trend in the mean DEXA T scores at the hip and spine may be observed with lower T scores in patients with the heterozygous allele and with the lowest scores in patients with the homozygous allele (Table 4). A significant value might have been obtained if a larger cohort was studied.

Table 4: *Phenotypic characteristics and mean DEXA bone mineral density scores of patients with wild type, heterozygous and homozygous rs2241880 polymorphism*

	Wild Type (n=40)	Heterozygous (n=53)	Homozygous (n=8)
Gender (male:female)	22:18	26:27	3:5
Smoking	28%)	28%)	0
Mean body mass index (kg/m^2)	25.6	25.9	24.5
Montreal Classification			
A1	17.5%	11%	0
A2	67.5%	64%	50%
A3	15%	25%	50%
L1	27.5%	21%	25%
L2	32.5%	32%	50%
L3	40%	47%	25%
B1	67.5%	36 (68%	87.5%
B2	22.5%)	15 (28%)	0
B3	4 (10%)	2 (4%)	1 (12.5%)
Perianal Disease	1 (2.5%)	4 (7.5%)	-
Medical and Surgical Management			
Thiopurine)	50%	66%	7.5%
anti-TNF-alpha	42.5%	36%	25%
Dual immunosuppression (thiopurine + anti-TNF alpha)	27.5%	28%	25%
Surgical Intervention	27.5%	17%	25%
Mean DEXA Score			
T Score Hip	-1.04	-1.29	-1.74
T Score Spine	-0.72	-0.86	-0.88
Z Score Hip	-0.35	-0.68	-0.58
Z score Spine	-0.40	-0.46	-0.02

Age of onset(A) A1: <17 years, A2: 17-40 years, A3: >40 years; Disease location (L) L1: ileal disease, L2: colonic disease, L3: ileocolonic disease, Behaviour (B) : B1: non-stricturing, non-penetrating, B2: stricturing disease, B3: penetrating disease.

Studies on osteoclasts, osteocytes and osteoblasts have shown that impaired autophagy results in impaired bone mass while the autophagy inducer Rapamycin increases bone formation.[5-13] These studies suggest that impaired autophagy, including impaired autophagy secondary to genetic variations, results in impaired bone formation. Our findings demonstrate that gene polymorphisms in the autophagy ATG16L1 gene may be linked to lower bone mineral density scores. Larger prospective studies are however required before this link may be put into clinical practice. While this study was not powered enough to show a statistically significant association between bone density results and impaired autophagy, the trend in decreasing T scores in patients with the rs2241880 polymorphism should encourage functional studies on osteocyte, osteoblast and osteoclast activity in patients with this polymorphism.

References

1. Billmann-Born S, Lipinski S, Bock J, Till A, Rosenstiel P, Schreiber S. The complex interplay of NOD-like receptors and the autophagy machinery in the pathophysiology of Crohn disease. *Eur J Cell Biol*. 2011;90(6-7):593-602.
2. Van Schaik FD, Verhagen MA, Siersema PD, Oldernburg B. High prevalence of low bone mineral density in patients with Inflammatory Bowel Disease in the setting of a peripheral Dutch hospital. *J Crohns Colitis*. 2008;2(3):208-13.
3. Cravo M, Guerreiro CS, dos Santos PM, Brito M, Ferreira P, Fidalgo C et al. Risk factors for metabolic bone disease in Crohn's disease patients. *Inflamm Bowel Dis*. 2010;16(12):2117-24.
4. Hocking LJ, Whitehouse C, Helfrich MH. Autophagy: a new player in skeletal maintenance? *J Bone Miner Res*. 2012;27(7):1439-47.
5. Mizushima N, Levine B. Autophagy in mammalian development and differentiation. Nature cell biology. 2010;12(9):823-30.
6. Onal M, Piemontese M, Xiong J, Wang Y, Han L, Ye S et al. Suppression of autophagy in osteocytes mimics skeletal aging. *J Biol Chem*. 2013;288 (24):17432-40.
7. Arnett TR. Acidosis, hypoxia and bone. *Arch Biochem Biophys*. 2010;503(1):103-9.
8. Bozec A, Bakiri L, Hoebertz A, Eferl R, Schilling AF, Komnenovic V et al. Osteoclast size is controlled by Fra-2 through LIF/LIF-receptor signalling and hypoxia. *Nature.* 2008;454(7201):221-5.
9. Zhao Y, Chen G, Zhang W, Xu N, Zhu JY, Jia J et al. Autophagy regulates hypoxia-induced osteoclastogenesis through the HIF-1alpha/BNIP3 signaling pathway. *J Cell Phys*. 2012;227(2):639-48.
10. Cejka D, Hayer S, Niederreiter B, Siehgart W, Fuereder T, Zwerina J et al. Mammalian target of rapamycin signaling is crucial for joint destruction in experimental arthritis and is activated in osteoclasts from patients with rheumatoid arthritis. *Arthritis Rheum*. 2010;62(8):2294-302.
11. Whitehouse CA, Waters S, Marchbank K, Horner A, McGowan NW, Jovanovic JV et al. Neighbor of Brca1 gene (Nbr1) functions as a negative regulator of postnatal osteoblastic bone formation and p38 MAPK activity. Proc Natl Acad Sci U S A. 2010;107(29):12913-8.
12. Liu F, Fang F, Yuan H, Yang D, Chen Y, Williams L et al. Osteoblast targeted deletion of FIP200, an essential component of mammalian autophagy, leads to osteopenia in mice. *J Bone Miner Res*. 2013;28(11):2414-30.
13. Darcy A, Meltzer M, Miller J, Lee S, Chappell S, Ver Donck K et al. A novel library screen identifies immunosuppressors that promote osteoblast differentiation. *Bone.* 2012;50(6):1294-303.
14. Lennard-Jones JE. Classification of inflammatory bowel disease. *Scand J Gastroenterol* Suppl. 1989;170:2-6.
15. Langholz E. UC. An epidemiological study based on a regional inception cohort, with special reference to disease course and prognosis. Dan Med Bull 1999; 46(5): 400-15.
16. Leslie WD, Adler RA, El-Hajj Fuleihan G, Hodsman AB, Kendler DL, McClung M et al. Application of the 1994 WHO classification to populations other than postmenopausal Caucasian women: the 2005 ISCD Official Positions. *J Clin Densitom*. 2006:9(1).
17. Qiagen. DNeasy® Blood & tissue Handbook. 2006:25-7.
18. DeSelm CJ, Miller BC, Zou W, Beatty WL, van Meel E, Takahata Y et al. Autophagy proteins regulate the secretory component of osteoclastic bone resorption. *Dev Cell.* 2011;21:966– 74.
19. Buning C, Durmus T, Molnar T, de Jong DJ, Drenth JP, Fiedler T et al. A study in three European IBD cohorts confirms that the ATG16L1 c.898A>G (p.Thr300Ala) variant is a susceptibility factor for Crohn's disease. *J Crohns Colitis*. 2007;1(2):70-6.
20. Marquez A, Nunez C, Martinez A, Mendoza JL, Taxonera C, Fernandez-Arguero M et al. Role of ATG16L1 Thr300Ala polymorphism in inflammatory bowel disease: a Study in the Spanish population and a meta-analysis. *Inflamm Bowel Dis*. 2009;15(11):1697-704.

21. Burisch J, Pedersen N, Cukovic-Cavka S, Brinar M, Kaimakliotis I, Duricova D et al. East-West gradient in the incidence of inflammatory bowel disease in Europe: the ECCO-EpiCom inception cohort. *Gut* 2014;63(4):588-97.
22. Cleynen I, Boucher G, Jostins L, Schumm LP, Zeissig S, Ahmad T et al. Inherited determinants of Crohn's disease and ulcerative colitis phenotype: a genetic association study. *Lancet* 2016;387:156-67.

Original Article

The difficulties in identifying and grafting an intramuscular coronary artery

Andre' Navarro, David Sladden, Aaron Casha, Alexander Manche'

Abstract

Myocardial bridging involves tunnelling of one of the coronary arteries through the myocardium, resulting in what are known as intramyocardial coronaries. While most patients with intramyocardial coronary vessels are asymptomatic, there is evidence that myocardial bridging may be the cause of sudden death. Given the low detection rate on coronary angiography, myocardial bridging may complicate coronary artery bypass grafting (CABG). This case report discusses a 72-year-old gentleman who underwent CABG, during which an undiagnosed intramuscular left anterior descending (LAD) coronary artery was found.

Andre' Navarro MD, MRCSEd, MSc*
Mater Dei Hospital
Msida, Malta
navarro.andre@outlook.com

David Sladden MD, MRCSEd, MSc*
Mater Dei Hospital
Msida, Malta

Aaron Casha MD, MPhil, PhD, FRCS(Gen. Surgery), FETCS, FRCS(CTh)
Mater Dei Hospital
Msida, Malta
University of Malta
Msida, Malta

Alexander Manche' MD, MPhil, FRCS(CTh), FECTS
Mater Dei Hospital
Msida, Malta

**Corresponding Author*

With only the tapering end of the LAD visible at the apex of the heart, a small incision was made at this site and a 1mm probe inserted. At the uppermost reach of the probe the tip was felt to point superficial and therefore a second more proximal incision was performed. The left internal thoracic artery (LITA) was than successfully anastomosed with the proximal arteriotomy and a length of saphenous vein was used for anastomosis with the distal arteriotomy where the probe was originally inserted. The patient was discharged home 5 days post operatively.

Introduction

Myocardial bridging occurs when a segment of epicardial coronary artery is situated beneath a band of myocardium, resulting in intramyocardial coronary vessels. It was first described in 1737 at autopsy and first detected on angiography in 1960.[1-2] The prevalence quoted in the literature varies greatly and an exhaustive anatomical study of over 1000 cadavers identified intramuscular coronaries in 26%.[3] The detection rates using conventional coronary angiography is significantly lower, at just 12%.[4] CT angiographic studies have identified myocardial bridges in up to 60% of people.[5-6] These rates are significantly higher than the detection rates on coronary angiography.[7] This is likely due to the fact that the features on angiography are more difficult to identify.

The importance of this anatomical variation is widely debated. Since the

majority of coronary blood flow occurs in diastole the systolic compression rarely causes ischaemia. However there is some equivocal evidence that atherosclerosis is accelerated immediately proximal to the segment being compressed by the bridge, whilst the intramyocardial and distal portions are usually disease free.[8-9]

The commonest artery to be compressed by myocardial bridges is the LAD at 62.5%, with the marginal branch of the right coronary artery coming in second at 16.7%.[10] The length of artery covered by myocardium varies considerably from around 3mm to 40mm.[7]

Characteristically the intramyocardial coronary vessel is seen to buckle inwards during systole and in 7% of these is also seen to temporarily narrow during systole.[11-12] These angiographic findings were described by Portmann in 1960 and currently still remain the most common method of diagnosing myocardial bridging. However more advanced technologies such as coronary CT, intravascular ultrasound, intracoronary Doppler and fractional flow reserve, can help quantify the degree of compression.[10]

While most agree that these anomalies are harmless when in isolation, there is no data on the prognosis of myocardial bridges in the context of coronary artery disease. There is anecdotal evidence of myocardial bridging potentially causing sudden cardiac death, with several studies noting them present in healthy young people who died suddenly with no other cause found at autopsy.[13] The LAD seems to be a culprit artery in these cases of sudden death, as is the length of artery covered and the depth of muscle it lies within.[14]

Case Presentation

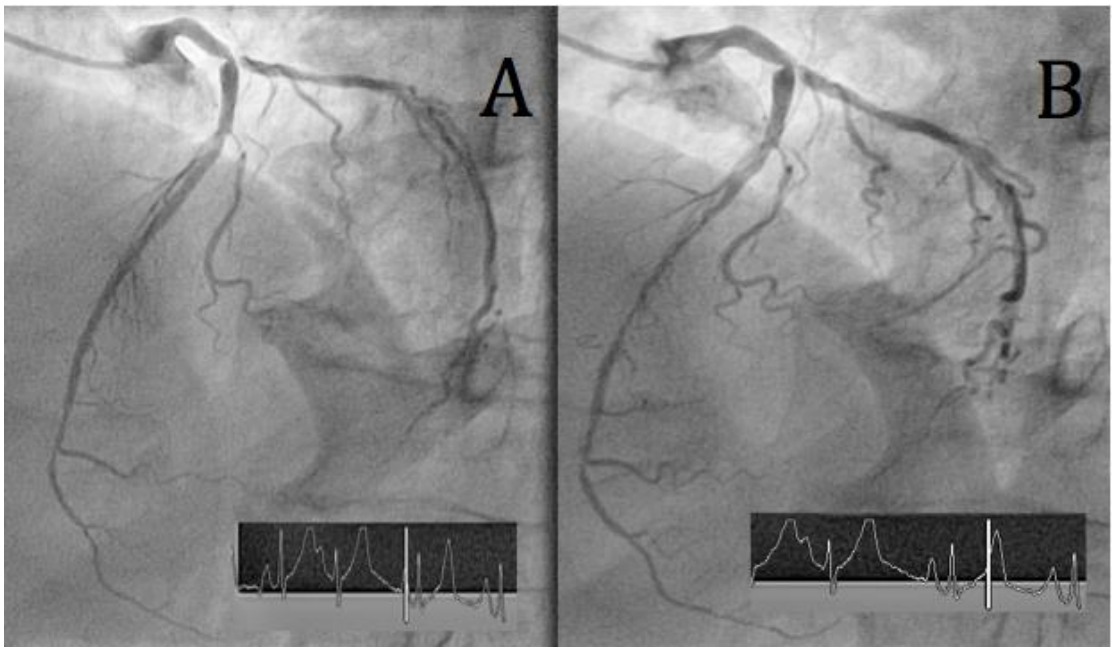
A 72 year-old male presented with angina. He was not a smoker and not diabetic, and presented with features suggestive of Parkinsonism. An exercise stress test was positive and a coronary angiogram was performed. This showed a tight stenosis of the distal left main coronary artery, systolic compression of a long-segment of the mid-LAD, with further stenotic disease at the origin of this segment (figure 1). His ejection fraction on echocardiography was 82%, Parsonnet score of 15 and logistic Euroscore of 3.26%. The patient was prepared for a CABG, with planned grafts to the LAD and the obtuse marginal (OM) branch of the circumflex artery.

Standard median sternotomy was performed with harvest of the LITA and simultaneous minimally-invasive long saphenous vein harvest from the thigh. Surgery was performed with conventional atrio-aortic cardiopulmonary bypass at normothermia and myocardial protection was with antegrade cold blood cardioplegia. The native vessels were inspected and while the OM was clearly visible the LAD was not. On closer inspection the tapered end of the LAD was visible at the apex of the heart. This was considered an unsuitable site for grafting as the LITA was not long enough and the calibre of the distal LAD very small.

Following the OM anastomosis a limited opening was made in the LAD at the apex and a 1mm metal probe was inserted retrogradely. The proximal LAD was then exposed proximally by cutting deep into the myocardium, using the probe as a guide. The LITA was anastomosed at this site and a further length of saphenous vein anastomosed to the distal LAD arteriotomy.

The patient was discharged home after 5 days without complications. At six months follow-up he remains well with good exercise tolerance and no recurrence of angina.

Figure 1*: Left anterior oblique (LAO) 45 degrees and Cranial 20 degrees angiographic view showing the LAD artery in diastole (A) and in systole (B). The ECG tracings show at which point of the cardiac cycle the image was taken. Note the change in vessel calibre between the two images.*



Discussion

This case highlights two key points in the process of grafting an intramuscular coronary artery. The first is the pre-operative identification of such an anomaly, which may be missed on angiography. Localised bridging is usually identified with a short segment that takes a deeper course and may exhibit contraction during systole.[15] When the bridging is long and covers an extensive segment of the intramuscular coronary artery the angiographic signs may be subtle. Our case highlights one of the subtle signs on standard coronary angiography, which is the compression of the coronary artery along its intramuscular length during systole. This is also known as the 'milking effect' and is very often missed especially if the recording speed is too low (<25 frames/second).[13] Computed Tomography has been shown to be an excellent method of visualising the intramuscular coronary artery preoperatively, and may be used in cases where this situation is suspected.[16]

The second important topic regards the intraoperative proceedings. Intramuscular coronary arteries are often discovered at operation. Locating and exposing the intramuscular artery is a key step in performing the operation. With short myocardial bridges the coronary artery is usually visible proximal and distal to the buried segment, which may be de-roofed by dividing the muscular fibres. When the entire artery is intramuscular it is only visible as a small vessel at the heart apex.[7] Several alternative techniques have been described. Aydin U. et al utilised intraoperative fluoroscopic identification of the left anterior descending artery using a radio-opaque graded marker on the myocardial surface and a antegrade root injection of contrast via the cardioplegia line.[17] High frequency ultrasound probes were first used to detect coronary arteries embedded in fat in 1986. More recently, linear ultrasound transducers have been put

to use in detection of intramyocardial LAD arteries.[18-19] The sterile epicardial ultrasound probe is used on doppler setting to identify the mid-portion of the intramyocardial coronary artery and using a sterile surgical marker a mark is made to guide the incision to expose the artery.[11]

On-table coronary angiography and the use of a specially made radio-opaque marker strip on the surface of the heart has also been described.[17] These techniques all involve imaging and add to the complexity of the operating room setup.

Oz M.C. et al describe a method when the great cardiac vein overlies the anterior descending artery. A deep elastic traction stitch mounted on a blunt needle is placed around both structures and traction is applied to mobilise the artery to the side and superficially, thereby facilitating its dissection.[20] This technique carries the risk of damaging the artery and also entering the right ventricle.

The method we here describe is similar to that outlined by Robinson in 1973 and requires no additional equipment.[21] A small arteriotomy is performed distally, allowing the retrograde insertion of a probe to identify the proximal LAD. Depending on the calibre of the vessel and the proximity to the apex the surgeon may opt to close the arteriotomy or anastomose a graft to this segment, thereby revascularising an important territory distal to the intramuscular segment. This method relies on a rigid probe to identify a proximal portion of LAD and is, in our opinion, easier than relying on imaging techniques. Both our method as well as imaging methods may be used to de-roof a long segment of the buried LAD. However, the risk of entering the right ventricle is increased and we prefer, instead to apply a second, distal graft where indicated.

References

1. Reymann HC. Disertatio de vasis cordis propriis. Bibl Anat. 1737;2:359–75.
2. Portmann WC, Iwig J. Die intramurale koronarie im angiogramm. Fortschr Rontgenstr. 1960;92:129–32.
3. Risse M, Weiler G. [Coronary muscle bridge and its relations to local coronary sclerosis, regional myocardial ischemia and coronary spasm. A morphometric study]. ZKardiol. 1985;74:700–5.
4. Soran O, Pamir G, Erol C, Kocakavak C, Sabah I. The incidence and significance of myocardial bridge in a prospectively defined population of patients undergoing coronary angiography for chest pain. Tokai J Exp Clin Med [Internet]. 2000;25:57–60. Available from: http://www.ncbi.nlm.nih.gov/pubmed/11127508
5. Zeina AR, Odeh M, Blinder J, Rosenschein U, Barmeir E. Myocardial bridge: Evaluation on MDCT. Am J Roentgenol. 2007;188(April):1069–73.
6. Poláček P. Relation of myocardial bridges and loops on the coronary arteries to coronary occlusions. Am Heart J [Internet]. 1961;61:44–52. Available from: http://www.scopus.com/inward/record.url?eid=2-s2.0-0001773691&partnerID=tZOtx3y1
7. Lujinović A, Kulenović A, Kapur E, Gojak R. Morphological aspects of myocardial bridges. 2013;13(4):212–7.
8. Erbel R, Ge J, Möhlenkamp S. Myocardial bridging: A congenital variant as an anatomic risk factor for myocardial infarction? Circulation. 2009;120:357–9.
9. Chatzizisis YS, Giannoglou GD. Myocardial bridges spared from atherosclerosis: overview of the underlying mechanisms. Can J Cardiol [Internet]. 2009;25(4):219–22. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2706759&tool=pmcentrez&rendertype=abstract
10. Lee MS, Chen CH. Myocardial Bridging: An Up-to-Date Review. J Invasive Cardiol [Internet]. 2015;27:521–8. Available from: http://www.ncbi.nlm.nih.gov/pubmed/25999138
11. Olearchyk AS, Nayar AP. Use of a handheld epicardial ultrasonic Doppler flow detector to locate an intramyocardial coronary artery encased in inflamed neoplastic pericardium. Tex Hear Inst J [Internet]. 2004;31:425–8. Available from: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=15745297
12. Olearchyk AS. Intramyocardial Coronary Arteries: Dissection During Coronary Artery Bypass Surgery in 70 Patients. Vasc Endovascular Surg [Internet]. 1992;26:649–55. Available from: http://ves.sagepub.com/cgi/doi/10.1177/153857449202600808
13. Bruschke AVG, Veltman CE, de Graaf MA, Vliegen HW. Myocardial bridging: What have we learned in the past and will new diagnostic modalities provide new insights? Vol. 21, Netherlands Heart Journal. 2013. p. 6–13.
14. Morales AR, Romanelli R, Tate LG, Boucek RJ, de Marchena E. Intramural left anterior descending coronary artery: Significance of the depth of the muscular tunnel. Hum Pathol. 1993;24:693–701.

15. Endo M, Woo Lee Y, Hayashi H, Wada J. Angiographic evidence of myocardial squeezing accompanying tachyarrhythmia as a possible cause of myocardial infarction. Chest. 1978;73:431–3.
16. Donkol RH, Saad Z. Myocardial bridging analysis by coronary computed tomographic angiography in a Saudi population. World J Cardiol [Internet]. 2013;5(11):434–41. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3857236&tool=pmcentrez&rendertype=abstract
17. Aydin U, Kocogullari CU. A method for locating embedded left anterior descending coronary arteries. Ann Thorac Surg [Internet]. 2013;95:360–1. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23272867
18. Hiratzka LF, McPherson DD, Brandt B, Lamberth WC, Marcus ML, Kerber RE. Intraoperative high-frequency epicardial echocardiography in coronary revascularization: Locating deeply embedded coronary arteries. Ann Thorac Surg. 1986;42.
19. Miwa S, Nishina T, Ueyama K, Kameyama T, Ikeda T, Nishimura K, et al. Visualization of intramuscular left anterior descending coronary arteries during off-pump bypass surgery. Ann Thorac Surg. 2004;77:344–6.
20. Oz MC, Cooper MM, Hickey TJ, Rose EA. Exposure of the intramyocardial left anterior descending artery. Ann Thorac Surg [Internet]. 1994;58:1194–5. Available from: http://dx.doi.org/10.1016/0003-4975(94)90495-2
21. Robinson G. Location of the proximal left anterior descending coronary artery. The Annals of thoracic surgery. 1973 Mar 31;15(3):299-300.

Review Article

The role of kisspeptin signalling in the hypothalamic-pituitary-gonadal axis

Carol Cardona Attard

Abstract

Kisspeptin is a hypothalamic peptide hormone, which plays a crucial role in puberty and fertility control by stimulating the release of gonadotrophin-releasing hormone, which in turn stimulates the release of luteinizing hormone and follicle stimulating hormone. It also interacts with neuropeptides neurokinin B and dynorphin A, and is under negative and positive feedback influences relayed by gonadal sex steroids. Loss of kisspeptin signalling results in hypogonadotrophic hypogonadism and impaired puberty. Kisspeptin expression and secretion is also affected by metabolic status and stress. Several studies have indicated a potential role for kisspeptin in the treatment of disorders causing hypogonadotrophic hypogonadism. This review aims to summarize the importance of kisspeptin and its role in the hypothalamic-pituitary-gonadal axis.

Keywords

kisspeptin, gonadotrophin-releasing hormone, gonadotrophins, fertility, puberty

Carol Cardona Attard MD, MRCP (UK)
Diabetes and Endocrine Centre
Mater Dei Hospital
Msida, Malta
Department of Medicine
University of Malta
Medical School
Msida, Malta
carol-diane.attard@gov.mt

**Corresponding Author*

Introduction

Kisspeptin is a hypothalamic peptide encoded by the KISS1 gene, which is found on chromosome 1q32, and is a powerful stimulator of the hypothalamic-pituitary-gonadal (HPG) axis. It is involved in puberty onset and fertility control,[1] and appears to be important for sexual development and differentiation in the early postnatal period, possibly through regulation of postnatal testosterone secretion.[2] It acts upstream to gonadotrophin-releasing hormone (GnRH) and is cleaved from a 145-amino acid precursor peptide into a 54-amino acid peptide, which can be further cleaved into 14, 13 and 10-amino acid peptides. These carboxy-terminal RF-amide peptides are collectively called kisspeptins.[1]

Kisspeptin binds to the G-protein coupled receptor 54 or KISS1 receptor (KISS1R in humans/Kiss1r in rodents), expressed on GnRH neurons (also widely expressed within both cortical and subcortical regions and peripherally). It stimulates the pulsatile secretion of GnRH from GnRH neurons into the portal circulation, which will then stimulate the release of luteinizing hormone (LH) and follicle stimulating hormone (FSH) from the gonadotrophs in the anterior pituitary.[1]

Kisspeptin neurons and sexual dimorphism

In humans, kisspeptin neurons reside in the hypothalamic rostral preoptic area, in the infudibular (arcuate) nucleus, in the anterior parvocellular and magnocellular

subdivisions of the paraventricular nucleus and in the ventral and rostral periventricular hypothalamic nucleus.[3] Infudibular and rostral kisspeptin neurons are in close apposition with GnRH neurons. Kisspeptin neurons in the infudibular nucleus also express neurokinin B and dynorphin A, and are thus called KNDy neurons. Neurokinin B and dynorphin A autosynaptically control kisspeptin pulsatile secretion and thus GnRH release, by binding to the neurokinin B receptor (stimulatory) and kappa opioid peptide receptor (inhibitory) respectively, which are found on KNDy cells. GnRH neurons do not express the oestrogen, progesterone or androgen receptors, which are however found on KNDy neurons. This suggests that KNDy neurons are responsible for relaying sex hormone negative and positive feedback, and thus regulating pulsatile kisspeptin release.[1] The oestrogen receptor alpha (ERα) is fundamental for positive feedback, but was not found to be important for negative feedback on GnRH/LH secretion in adult female mice.[4]

Females have appreciably more kisspeptin fibres and cell bodies in the infudibular nucleus as well as a significantly greater number of kisspeptin fibres in the ventral periventricular area compared to men. In addition, expression of kisspeptin cell bodies in the rostral periventricular zone is seen in females only.[1] This greater number of kisspeptin neurons in females will allow them to secrete more kisspeptin, which will enable them to produce the LH surge.[2] Kisspeptin can reset the GnRH pulse generator in men i.e. kisspeptin can induce an LH pulse regardless of the timing of previous endogenous pulses.[1] This is not seen in women across the different menstrual cycle phases, which could be due to changes in the sex hormone levels along the cycle. Different kisspeptin responses depending on the menstrual cycle phase were noted in the study by Chan *et al*, and it appeared that kisspeptin tone was greater in the early follicular phase in contrast to the rest of the cycle phases.[5]

Kisspeptin and the reproductive axis

Kisspeptin's effect on LH secretion is greater, often causing a 2 to 3-fold rise in most circumstances with a robust increase in both pulse frequency and amplitude, while its effect on FSH release is of a lower magnitude and less consistent.[6-9] The gonadotrophin secretory pattern difference in response to kisspeptin could be due to variations in the secretory pattern between LH and FSH, being more constitutive for FSH; differences in the effect of kisspeptins on the GnRH secretory pattern, where induction of high frequency GnRH pulses will preferentially stimulate LH; and differences in regulatory influences of gonadal peptides such as inhibins, which regulate FSH release.[10]

Kisspeptin neurons in the infudibular nucleus were found to become hypertrophied and harboured more KISS1 messenger ribonucleic acid (mRNA) in post-menopausal women compared to pre-menopausal women,[11] with increased expression of neurokinin B[12] and lack of dynorphin A signalling.[1] In a recent study, the number of kisspeptin-immunoreactive neurons within the infudibular nucleus of humans was found to be significantly higher in the infant/prepubertal and elderly (58-90 year olds) periods rather than during the adult period (22-44 year olds).[13] This means that oestrogen (or testosterone in males) suppresses kisspeptin and neurokinin B expression and release, whereas dynorphin A would be upregulated through negative feedback on the KNDy neurons, leading to a reduction in tonic GnRH and gonadotrophin

release, as occurs during the pre-ovulatory follicular phase.[1] Only a partial inhibition of KISS1 mRNA was noted in sheep following progesterone replacement.[10]

A change to positive feedback, which is associated with an increase in the oestrogen levels, occurs in the late follicular phase to induce the LH surge and ovulation.[1] Many studies have shown that kisspeptin is essential for the LH surge. KISS1 mRNA increased in the anteroventral periventricular (AVPV) nucleus of ovariectomized rats at the time of the oestrogen and progesterone induced LH surge.[14] Exogenous kisspeptin induced oocyte maturation and an LH surge during an FSH/GnRH antagonist *in-vitro* fertilisation protocol.[15] Moreover, KISS1 and Kiss1r knock-out (KO) mice fail to mount this pre-ovulatory LH surge.[16] An intrinsic circadian Kiss-clock in the hypothalamic AVPV nucleus of female mice acting in combination with the suprachiasmatic nucleus may be leading to a circadian pattern of kisspeptin gene expression and neuronal activation in females.[17] However, this circadian activation of kisspeptin neurons was found to rely on the presence of oestrogen, indicating that the LH surge is oestrogen dependent.[2,17] Changes in neurokinin B and dynorphin A levels may also contribute to the kisspeptin mediated LH surge.[1] A recent study has demonstrated that the positive feedback of progesterone is likewise required for the kisspeptin neuronal activation and induction of the LH surge.[18]

KISS1 and KISS1R genes were noted to be expressed in pituitary gonadotrophs, while gonadotrophins (LH more than FSH) were secreted from pituitary explants on treatment with kisspeptin, indicating that kisspeptin may directly stimulate the release of LH and FSH from gonadotrophs.[19-20] However, the principal mechanism of gonadotrophin secretion still appears to be through stimulation of GnRH.[21]

Kisspeptin may also have a role in direct signalling on the ovary. This is suggested by a study done in mice where haplo-insufficiency of the Kiss1r resulted in a premature deterioration of the ovulatory rate as well as progressive loss of pre-antral and antral follicles and oocytes, resulting in a decline in fertility, atrophic ovaries and a state of premature ovarian failure. A decrease in ovarian Kiss1r mRNA expression was noted in the absence of a decline in gonadotrophins. FSH actually increased due to follicular loss. On the other hand, Kiss1r null mice, which have arrested follicular development and are anovulatory, lacked normal ovulatory responses on standard gonadotrophin priming.[22] Moreover, KISS1 and KISS1R mRNA have been found to be expressed in human gonads. Some studies in rats have shown that ovarian KISS1 expression increases during puberty and prior to ovulation under the influence of gonadotrophins, with a possible role of locally produced ovarian kisspeptin in ovulatory regulation.[10] Kisspeptin has also been shown to potentiate the effect of human chorionic gonadotrophin on testosterone release from the testes, and it can increase spermatozoa motility and fertilization capacity.[23]

Role of kisspeptin in puberty

Inactivating mutations or KO of kisspeptin or its receptor result in hypogonadotrophic hypogonadism (HH) as well as impaired puberty/sexual maturation and infertility,[2,24-25] while activating mutations or administration of exogenous kisspeptin result in precocious puberty.[26-28] Loss of kisspeptin (or its receptor) is responsible for approximately 2% of HH cases in humans.[29] In a study done in mice,

there is an increase in the amount of GnRH neurons which depolarise in response to kisspeptin, from 27% in juvenile, to 44% in prepubertal, to >90% in adult mice, which means that GnRH neurons become more sensitive to kisspeptin during puberty. KISS1 mRNA increased from juvenile to adult mice in the AVPV nucleus, suggesting an increase in kisspeptin tone.[30]

Kiss1r mRNA expression is also increased,[31] which may play an important role in puberty by increasing the sensitivity of GnRH neurons to kisspeptin. Increase in Kiss1r expression appears to occur earlier in female than in male rats, providing a possible explanation for earlier puberty in females.[2] Furthermore, during puberty, there is an increase in the number of kisspeptin neurons and synaptic contacts with GnRH neurons.[10, 32] In mice, the activation of kisspeptin expression during puberty appears to be driven by oestrogen; therefore, it appears that kisspeptin may require some degree of ovarian activation, which however, may not be the case in humans.[10] In a study by Guerriero *et al*, the response of GnRH to kisspeptin was noted to switch from ovarian steroid independent (pre-pubertal) to dependent during puberty in female rhesus monkeys.[33] A recent study has shown that the leptin - alpha-melanocyte stimulating hormone - kisspeptin - GnRH neuronal pathway in rodent models is involved in the metabolic control of puberty.[34] Because of the greater amount of adipose tissue present close to puberty, more leptin would be secreted leading to kisspeptin release.[35]

Kisspeptin - link between reproduction and metabolic status

Reproduction requires sufficient amount of energy stores as it is highly metabolically demanding.[32] Kisspeptin is regulated by metabolic signals (e.g leptin, ghrelin and neuropeptide Y) and may sense energy stores, which then influences the pulsatile release of GnRH, thus providing a connection between nutritional/metabolic status and reproduction. Fasting and chronic undernutrition are associated with reduced kisspeptin and neurokinin B expression.[1] In contrast, prepubertal rats given a high fat diet, showed increased kisspeptin and neurokinin B expression as well as LH pulsatility, leading to precocious puberty. Kisspeptin expression is decreased in leptin deficiency or leptin receptor ablation, where gonadotrophin levels improve upon leptin or kisspeptin administration respectively. This suggests that leptin positively regulates kisspeptin expression.[36] Reduced kisspeptin expression and secretion may also be responsible for HH seen in patients with obesity and type 2 diabetes.[1] This is suggested by reduced hypothalamic KISS1 mRNA expression in a rat model of diabetes, with resultant low levels in gonadotrophins and sex hormones, which were corrected with kisspeptin administration.[37] Possible mechanisms for reduced kisspeptin signalling in obesity and diabetes include: a rise in oestrogen levels in obesity, which then feeds back negatively on kisspeptin secretion, leptin resistance, insulin resistance, hyperglycaemia as well as increased inflammation seen in diabetes.[1]

Stress and its effect on kisspeptin

During stress and inflammation there is reduced expression of kisspeptin and Kiss1r as well as a reduction in kisspeptin responsiveness, leading to reduced gonadotrophin levels. This is partially mediated by the rise in corticotrophin releasing hormone and glucocorticoids. Moreover, stress during neonatal period/early stages of reproductive

development has been found to lead to reduced KISS1 mRNA levels during puberty with pubertal delay in rats, indicating that the developing kisspeptin system is vulnerable to immune and metabolic stressors.[10]

Kisspeptin in pregnancy and lactation

The levels of kisspeptin, secreted from syncytiothrophoblast placental cells, are elevated in pregnancy by 7000-fold. These persistently high circulating levels can possibly cause desensitization to the kisspeptin stimulatory effect on gonadotrophin secretion, resulting in partially suppressed gonadotrophin levels during pregnancy.[10] Kisspeptin in gestation may also be important for trophoblast invasion, embryo implantation and maintenance of pregnancy.[3] Moreover, a reduction in responsiveness to kisspeptin and repression of its expression was noted during lactation, leading to an overall suppression of the HPG axis in this phase.[10]

Therapeutic potential of kisspeptin

Given the effects of kisspeptin on the HPG axis, it may potentially be considered for the treatment of some conditions which induce HH. When given in hypothalamic amenorrhoea, kisspeptin can induce and increase LH pulsatility, with an increase in frequency and mass per pulse,[38] though it has a lower effect on FSH.[8] Exogenous kisspeptin induced an increase in LH and testosterone in type 2 diabetes patients suffering from HH.[9] It also stimulated LH secretion by 2.5-fold in patients with neurokinin B system defects (who also suffer from HH due to failure to stimulate kisspeptin).[7] Kisspeptin plays a crucial role in hyperprolactinaemic anovulation and the resultant HH. This is because kisspeptin neurons express prolactin receptors, leading to reduced kisspeptin expression in hyperprolactinaemia. Gonadotrophin secretion and ovarian cyclicity is restored on administration of kisspeptin.[39] It can also be used to stimulate oocyte maturation in women at high risk of developing ovarian hyperstimulation syndrome during *in-vitro* fertilization therapy.[40] On the other hand, kisspeptin can be used as antagonistic therapy (high doses and continuous infusions can cause KISS1R desensitisation) to decrease GnRH and LH pulsatility in polycystic ovary syndrome, early puberty and menopause.[1]

Conclusion

It is evident that kisspeptin-KISS1R signalling is crucial to promote normal pulsatile GnRH and gonadotrophin secretion. This is important for sexual maturation and puberty as well as normal reproductive function and fertility.[2, 41] It may also have a potential role in the treatment of certain disorders causing HH as described above.

References:

1. Skorupskaite K, George JT, Anderson RA. The kisspeptin-GnRH pathway in human reproductive health and disease. Hum Reprod Update. 20. England: The Author 2014. Published by Oxford University Press on behalf of the European Society of Human Reproduction and Embryology.; 2014. p. 485-500.
2. Kauffman AS. Coming of age in the kisspeptin era: sex differences, development, and puberty. Mol Cell Endocrinol. 324. Ireland: 2010 Elsevier Ireland Ltd; 2010. p. 51-63.
3. Beymer M, Henningsen J, Bahougne T, Simonneaux V. The role of kisspeptin and RFRP in the circadian control of female reproduction. Mol Cell Endocrinol. 2016;438:89-99.
4. Dubois SL, Acosta-Martinez M, DeJoseph MR, Wolfe A, Radovick S, Boehm U, et al. Positive, but not negative feedback actions of estradiol in adult female mice require estrogen receptor alpha in kisspeptin neurons. Endocrinology. 2015;156(3):1111-20.

5. Chan YM, Butler JP, Sidhoum VF, Pinnell NE, Seminara SB. Kisspeptin administration to women: a window into endogenous kisspeptin secretion and GnRH responsiveness across the menstrual cycle. J Clin Endocrinol Metab. 97. United States2012. p. E1458-67.
6. George JT, Seminara SB. Kisspeptin and the hypothalamic control of reproduction: lessons from the human. Endocrinology. 153. United States2012. p. 5130-6.
7. Young J, George JT, Tello JA, Francou B, Bouligand J, Guiochon-Mantel A, et al. Kisspeptin restores pulsatile LH secretion in patients with neurokinin B signaling deficiencies: physiological, pathophysiological and therapeutic implications. Neuroendocrinology. 97. Switzerland: Basel.; 2013. p. 193-202.
8. Jayasena CN, Nijher GM, Chaudhri OB, Murphy KG, Ranger A, Lim A, et al. Subcutaneous injection of kisspeptin-54 acutely stimulates gonadotropin secretion in women with hypothalamic amenorrhea, but chronic administration causes tachyphylaxis. J Clin Endocrinol Metab. 94. United States2009. p. 4315-23.
9. George JT, Veldhuis JD, Tena-Sempere M, Millar RP, Anderson RA. Exploring the pathophysiology of hypogonadism in men with type 2 diabetes: kisspeptin-10 stimulates serum testosterone and LH secretion in men with type 2 diabetes and mild biochemical hypogonadism. Clin Endocrinol (Oxf). 2013;79(1):100-4.
10. Pinilla L, Aguilar E, Dieguez C, Millar RP, Tena-Sempere M. Kisspeptins and reproduction: physiological roles and regulatory mechanisms. Physiol Rev. 92. United States2012. p. 1235-316.
11. Rometo AM, Krajewski SJ, Voytko ML, Rance NE. Hypertrophy and increased kisspeptin gene expression in the hypothalamic infundibular nucleus of postmenopausal women and ovariectomized monkeys. J Clin Endocrinol Metab. 92. United States2007. p. 2744-50.
12. Rance NE, Young WS, 3rd. Hypertrophy and increased gene expression of neurons containing neurokinin-B and substance-P messenger ribonucleic acids in the hypothalami of postmenopausal women. Endocrinology. 1991;128(5):2239-47.
13. Taziaux M, Staphorsius AS, Ghatei MA, Bloom SR, Swaab DF, Bakker J. Kisspeptin Expression in the Human Infundibular Nucleus in Relation to Sex, Gender Identity, and Sexual Orientation. J Clin Endocrinol Metab. 2016;101(6):2380-9.
14. Smith JT, Popa SM, Clifton DK, Hoffman GE, Steiner RA. Kiss1 neurons in the forebrain as central processors for generating the preovulatory luteinizing hormone surge. J Neurosci. 26. United States2006. p. 6687-94.
15. Abbara A, Jayasena C, Comninos A, Nijher M, Christopoulos G, Izzi-Engbeaya C, et al. Kisspeptin: a novel physiological trigger for oocyte maturation in in-vitro fertilisation treatment. The Lancet. 2014;383.
16. Clarkson J, d'Anglemont de Tassigny X, Moreno AS, Colledge WH, Herbison AE. Kisspeptin-GPR54 signaling is essential for preovulatory gonadotropin-releasing hormone neuron activation and the luteinizing hormone surge. J Neurosci. 28. United States2008. p. 8691-7.
17. Chassard D, Bur I, Poirel VJ, Mendoza J, Simonneaux V. Evidence for a Putative Circadian Kiss-Clock in the Hypothalamic AVPV in Female Mice. Endocrinology. 2015;156(8):2999-3011.
18. Stephens SB, Tolson KP, Rouse ML, Jr., Poling MC, Hashimoto-Partyka MK, Mellon PL, et al. Absent Progesterone Signaling in Kisspeptin Neurons Disrupts the LH Surge and Impairs Fertility in Female Mice. Endocrinology. 2015;156(9):3091-7.
19. Gutierrez-Pascual E, Martinez-Fuentes AJ, Pinilla L, Tena-Sempere M, Malagon MM, Castano JP. Direct pituitary effects of kisspeptin: activation of gonadotrophs and somatotrophs and stimulation of luteinising hormone and growth hormone secretion. J Neuroendocrinol. 19. England2007. p. 521-30.
20. Navarro VM, Castellano JM, Fernandez-Fernandez R, Tovar S, Roa J, Mayen A, et al. Characterization of the potent luteinizing hormone-releasing activity of KiSS-1 peptide, the natural ligand of GPR54. Endocrinology. 146. United States2005. p. 156-63.
21. Smith JT, Rao A, Pereira A, Caraty A, Millar RP, Clarke IJ. Kisspeptin is present in ovine hypophysial portal blood but does not increase during the preovulatory luteinizing hormone surge: evidence that gonadotropes are not direct targets of kisspeptin in vivo. Endocrinology. 149. United States2008. p. 1951-9.
22. Gaytan F, Garcia-Galiano D, Dorfman MD, Manfredi-Lozano M, Castellano JM, Dissen GA, et al. Kisspeptin receptor haplo-insufficiency causes premature ovarian failure despite preserved gonadotropin secretion. Endocrinology. 2014;155(8):3088-97.
23. Clarke H, Dhillo WS, Jayasena CN. Comprehensive Review on Kisspeptin and Its Role in Reproductive Disorders. Endocrinol Metab (Seoul). 2015;30(2):124-41.
24. de Roux N, Genin E, Carel JC, Matsuda F, Chaussain JL, Milgrom E. Hypogonadotropic hypogonadism due to loss of function of the KiSS1-derived peptide receptor GPR54. Proc Natl Acad Sci U S A. 100. United States2003. p. 10972-6.
25. Seminara SB, Messager S, Chatzidaki EE, Thresher RR, Acierno JS, Jr., Shagoury JK, et al. The GPR54 gene as a regulator of puberty. N Engl J Med. 349. United States: 2003 Massachusetts Medical Society; 2003. p. 1614-27.
26. Silveira LG, Noel SD, Silveira-Neto AP, Abreu AP, Brito VN, Santos MG, et al. Mutations of the KISS1 gene in disorders of puberty. J Clin Endocrinol Metab. 95. United States2010. p. 2276-80.
27. Teles MG, Bianco SD, Brito VN, Trarbach EB, Kuohung W, Xu S, et al. A GPR54-activating mutation in a patient with central precocious puberty. N Engl J Med. 358. United States: 2008 Massachusetts Medical Society.; 2008. p. 709-15.

28. Navarro VM, Fernandez-Fernandez R, Castellano JM, Roa J, Mayen A, Barreiro ML, et al. Advanced vaginal opening and precocious activation of the reproductive axis by KiSS-1 peptide, the endogenous ligand of GPR54. J Physiol. 561. England2004. p. 379-86.
29. Francou B, Paul C, Amazit L, Cartes A, Bouvattier C, Albarel F, et al. Prevalence of KISS1 Receptor mutations in a series of 603 patients with normosmic congenital hypogonadotrophic hypogonadism and characterization of novel mutations: a single-centre study. Hum Reprod. 2016;31(6):1363-74.
30. Han SK, Gottsch ML, Lee KJ, Popa SM, Smith JT, Jakawich SK, et al. Activation of gonadotropin-releasing hormone neurons by kisspeptin as a neuroendocrine switch for the onset of puberty. J Neurosci. 25. United States2005. p. 11349-56.
31. Navarro VM, Castellano JM, Fernandez-Fernandez R, Barreiro ML, Roa J, Sanchez-Criado JE, et al. Developmental and hormonally regulated messenger ribonucleic acid expression of KiSS-1 and its putative receptor, GPR54, in rat hypothalamus and potent luteinizing hormone-releasing activity of KiSS-1 peptide. Endocrinology. 145. United States2004. p. 4565-74.
32. Kaur KK, Allahbadia G, Singh M. Kisspeptins in human reproduction-future therapeutic potential. J Assist Reprod Genet. 2012;29(10):999-1011.
33. Guerriero KA, Keen KL, Millar RP, Terasawa E. Developmental changes in GnRH release in response to kisspeptin agonist and antagonist in female rhesus monkeys (Macaca mulatta): implication for the mechanism of puberty. Endocrinology. 153. United States2012. p. 825-36.
34. Manfredi-Lozano M, Roa J, Ruiz-Pino F, Piet R, Garcia-Galiano D, Pineda R, et al. Defining a novel leptin-melanocortin-kisspeptin pathway involved in the metabolic control of puberty. Mol Metab. 2016;5(10):844-57.
35. Cortes ME, Carrera B, Rioseco H, Pablo del Rio J, Vigil P. The Role of Kisspeptin in the Onset of Puberty and in the Ovulatory Mechanism: A Mini-review. J Pediatr Adolesc Gynecol. 2015;28(5):286-91.
36. Navarro VM, Kaiser UB. Metabolic influences on neuroendocrine regulation of reproduction. Curr Opin Endocrinol Diabetes Obes. 20. England2013. p. 335-41.
37. Castellano JM, Navarro VM, Fernandez-Fernandez R, Roa J, Vigo E, Pineda R, et al. Expression of hypothalamic KiSS-1 system and rescue of defective gonadotropic responses by kisspeptin in streptozotocin-induced diabetic male rats. Diabetes. 55. United States2006. p. 2602-10.
38. Jayasena CN, Abbara A, Veldhuis JD, Comninos AN, Ratnasabapathy R, De Silva A, et al. Increasing LH pulsatility in women with hypothalamic amenorrhoea using intravenous infusion of Kisspeptin-54. J Clin Endocrinol Metab. 2014;99(6):E953-61.
39. Sonigo C, Bouilly J, Carre N, Tolle V, Caraty A, Tello J, et al. Hyperprolactinemia-induced ovarian acyclicity is reversed by kisspeptin administration. J Clin Invest. 2012;122(10):3791-5.
40. Abbara A, Jayasena CN, Christopoulos G, Narayanaswamy S, Izzi-Engbeaya C, Nijher GM, et al. Efficacy of Kisspeptin-54 to Trigger Oocyte Maturation in Women at High Risk of Ovarian Hyperstimulation Syndrome (OHSS) During In Vitro Fertilization (IVF) Therapy. J Clin Endocrinol Metab. 2015;100(9):3322-31.
41. Novaira HJ, Sonko ML, Hoffman G, Koo Y, Ko C, Wolfe A, et al. Disrupted kisspeptin signaling in GnRH neurons leads to hypogonadotrophic hypogonadism. Mol Endocrinol. 2014;28(2):225-38.

Transfusion-Related Acute Lung Injury – Case Report and Literature Review

Stephanie Attard, Denise Borg, John Mamo, Sandro Vella

Abstract

Blood transfusion is a common procedure that usually goes without complications. However, adverse transfusion reactions should not be overlooked. Transfusion-related acute lung injury (TRALI) is the leading cause of transfusion-related fatalities and is characterized by the onset of acute respiratory distress in the form of non-cardiogenic pulmonary oedema. We hereby report the case of a 28 year old Jamaican lady who developed acute onset dyspnoea, tachycardia and hypoxaemia following transfusion of fresh frozen plasma and red cell concentrates.

Stephanie Attard MD, MRCP (Edin)*
Department of Medicine
Mater Dei Hospital
Msida, Malta
Stephanie.b.camilleri@gov.mt

Denise Borg MD, MRCP (UK)
Department of Medicine
Mater Dei Hospital
Msida, Malta

John Mamo FRCOG (UK) MB, ChB (Manc) MRCOG
Department of Obstetrics and Gynaecology
Mater Dei Hospital
Msida, Malta

Sandro Vella MD (Melit), MSc (Roehampton), MD (Dund), FRCP (Edin)
Department of Medicine
Mater Dei Hospital
Msida, Malta

**Corresponding Author*

Introduction

TRALI is the leading cause of transfusion-related mortality and it often remains unrecognized clinically.[1] The true incidence of TRALI is unknown because of the difficulty in making the diagnosis and under-reporting especially in the intensive care setting where the development of symptoms may be attributed to multiple other disease processes or therapeutic interventions rather than transfusion. It is estimated to occur in 1:1300 to 1:5000 transfusions of plasma-containing blood products.[2] Though uncertainty remains with regards to the pathophysiology of TRALI, its development is influenced by both transfusion-related and patient-related risk factors.

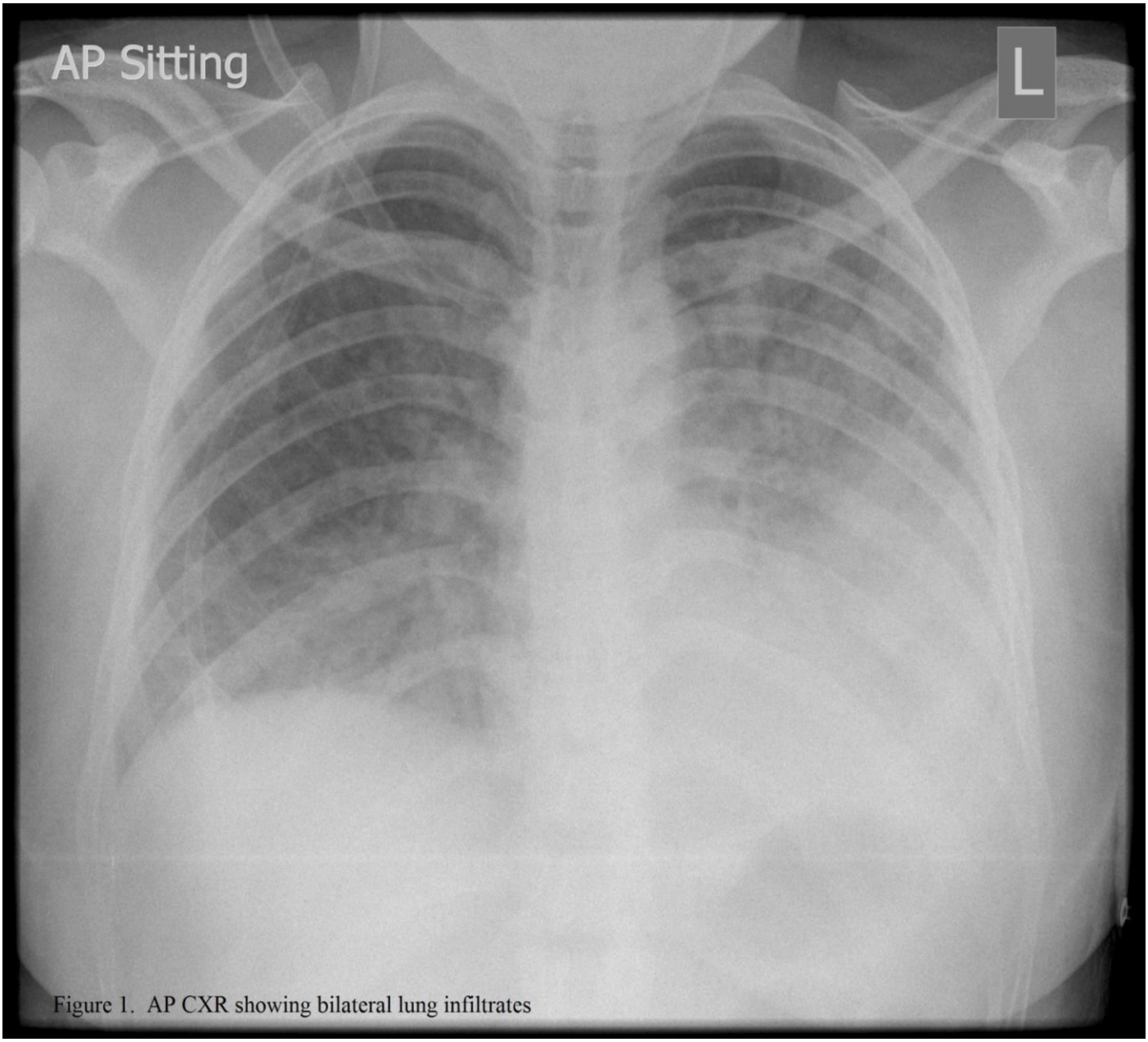
Case Report

A 28-yr old, previously healthy woman, presented to the Accident and Emergency department complaining of lower abdominal pain. A Focused Assessment with Sonography in Trauma (FAST) scan showed free fluid in the pelvis and she was subsequently diagnosed with a ruptured ovarian cyst. Laboratory investigations on presentation showed a white cell count of 6.3 x 10^9/L, haemoglobin of 7.8g/dL (normal mean corpuscular volume and mean corpuscular haemoglobin) and a platelet count of 166 x 10^9/L. Liver and renal function, glucose and coagulation screen were within normal limits. She underwent urgent laparoscopic ovarian cystectomy and was transfused four units of packed red cells

(PRCs) intra-operatively. In view of significant intra-operative bleeding she required further transfusion with fresh frozen plasma (FFP). However, whilst receiving her fourth unit of FFP, she became acutely dyspnoeic. On examination she had low-grade fever, tachycardia (145bpm) and tachypnoea with a respiratory rate of 25 breaths/minute and an oxygen saturation of 77% on room air. On auscultation of the chest, there was decreased air entry both bases with left basal crepitations. The rest of her physical examination was unremarkable.

CXR showed bilateral patchy infiltration particularly in the left lung field (Figure 1).

Figure 1*: AP CXP showing bilateral lung infiltrates*



The patient was diagnosed with transfusion-related acute lung injury and required admission to intensive care for ventilatory and haemodynamic support. The patient did not require intubation as she improved significantly with non-invasive ventilation.

Repeat laboratory investigations showed a transient leukopenia (white cell count of 3.4 x 10^9/L) followed by a leucocytosis (white cell count of 12.3 x 10^9/L) within 24 hours of her transfusion.

In view of leukopenia and the presence of infiltrates on chest x-ray, blood cultures were taken and she was also started on piperacillin/tazobactam to cover for any potential concomitant infectious pathology. The patient stabilised and made an uneventful recovery within a few days.

Definition of TRALI

TRALI has been defined by both the National Heart, Lung, and Blood Institute (NHLBI) working group as well as a Canadian Consensus Conference, as new acute lung injury (ALI)/acute respiratory distress syndrome (ARDS) occurring during or within six hours after blood product administration characterised by acute hypoxaemia, bilateral infiltrates on frontal chest radiograph and no evidence of circulatory overload/left atrial hypertension or pre-existing ALI/ARDS before transfusion.[1,3]

Pathogenesis

TRALI is postulated to develop as the result of two separate clinical events. The first or priming event is due to the patient's primary disease or condition, which results in activation of the pulmonary endothelium and the accumulation of primed, adherent neutrophils in the lung. The second event is the subsequent blood transfusion, whereby the primed neutrophils are activated by either a leucocyte antibody or biological response modifiers (BRM) present in the transfused blood product. Activation of the primed neutrophils results in augmented release of their microbicidal arsenal, which causes collateral injury to the pulmonary endothelium that manifests as capillary leak, and clinically as TRALI. Thus, the two-event mechanism proposes that both recipient and blood product factors contribute to TRALI pathogenesis.[4]

Risk Factors

All blood components have been implicated in TRALI; however, plasma containing blood components are most commonly implicated, with FFP and whole blood-derived platelet concentrates (WB-PLTs) having caused the largest number of reported cases. In most centres, the plasma is platelet-rich plasma. This use of platelet-rich plasma is significant for it allows for the infusion of platelet fragments and all endogenous growth factors and other mediators which are platelet-derived. Many of these compounds are effective activators of polymorphonuclear cells and innate immunity resulting in acute lung injury.

Certain patient risk factors have been identified as increasing the risk of TRALI.[5] These include:

- Higher IL-8 levels – may prime neutrophils and the lung endothelium[6]
- Shock – results in tissue injury possibly predisposing to TRALI through priming of the recipient's endothelium and immune cells[7]
- Liver surgery[5]
- Positive fluid balance – more likely to manifest pulmonary oedema when there is ALI[8]

- Peak airway pressure greater than 30cm of H_2O if mechanically ventilated before transfusion – increases the risk of ALI[9-10]
- Chronic alcohol abuse – results in lower levels of glutathione antioxidant in the lung[11-12]
- Current smoking[9-10]

Pearl Toy et al reported that receipt of plasma (including whole blood) from female donors is a strong risk factor, and reduction of this risk factor was concurrent with a decrease in TRALI incidence from approximately 1:4000 units to approximately 1:12000 units.[5,13] Furthermore, there is evidence that TRALI is commoner in recipients of blood products from multiparous female donors who are more likely to possess anti-HLA antibodies and anti-neutrophil-specific antibodies with increasing number of pregnancies.[14]

Following a case analysis, it was established that our patient had received blood products from ten different donors, three of which were females. Out of the female donors, one was nulliparous whilst the other two donors had a history of three or more gestations. Antibody testing of recipient and donor blood concluded that the donor with HLA antibodies with the same specificity as that of our patient was one of the multiparous females. This is in keeping with the literature data that suggests a higher risk of TRALI when receiving blood products from multiparous females.

The association between the risk of TRALI and blood product storage time has been debated for long. Whether longer RBC storage is associated with increased risk for lung injury and mortality is considered the most critical issue currently facing transfusion medicine. In a prospective case-control study by Toy et al (2012), evidence against longer storage of leuko-reduced RBC units being an important risk for TRALI was documented.[5,15] There is also conflicting data regarding the risk of TRALI with multiple transfusions. Results from the same case-control study by Toy et al had initially suggested increased risk for TRALI with increasing numbers of transfusions. However, when a multivariate analysis was carried out, no statistically significant correlation was found.[5]

Diagnosis and Management

TRALI occurs within 6 hours of transfusion with the majority of cases presenting during the transfusion or within the first 2 hours. TRALI is the insidious onset of acute pulmonary insufficiency presenting as tachypnoea, cyanosis, and dyspnoea with acute hypoxaemia, $PaO_2/FiO_2<300$ mmHg, and decreased pulmonary compliance, despite normal cardiac function.

Although the diagnosis is mainly based on clinical grounds, certain laboratory and radiographic investigations can facilitate the diagnostic challenge posed by TRALI.[14] Radiographic examination reveals diffuse, fluffy infiltrates consistent with pulmonary oedema.

Transient leukopenia has been temporally associated with the onset of TRALI (as in our case report), and serial measurements of white cell count may reveal this finding.[16,17]

Echocardiography, measurement of BNP levels and pulmonary oedema fluid protein analysis are complementary tests in helping to exclude cardiac dysfunction and volume overload as the cause of the acute symptomatology.[5,16]

As previously discussed, the precise mechanism responsible for TRALI is unknown, but the syndrome has been associated with passive transfer of leukocyte

antibodies and biologically active lipids in blood components. The majority of cases of TRALI (65-80%) are thought to be triggered by passive transfer of HLA Class I and/or neutrophil-specific antibodies[18-22] or HLA Class II antibodies[23,24] present in the plasma of the transfused blood product. In a minority of cases, the causative antibodies are present in the recipient, and react with transfused cellular material. Testing plasma samples from the implicated donor and patient for leukocyte antibodies can be very helpful in evaluation of a suspected TRALI case, however these are not found in all cases of TRALI.[5]

Toy et al found no evidence of TRALI after transfusion of blood products from one donor with multiple HLA antibodies into 103 recipients, 25% of whom had ≥1 HLA antigen that matched the donor antibody.[25] On the other hand, Kopko et al reported the presence of a mild to severe respiratory syndrome in 35% of recipients receiving blood products from a donor with anti-human-neutrophil-antigen (HNA)-3a antibody. Therefore, a case of TRALI may represent an isolated event, but donor granulocyte antibodies rather than HLA antibodies seem likely to cause multiple cases of TRALI.[18]

As with other forms of ALI/ARDS, there is no significant treatment for TRALI. If the patient is still being transfused when the diagnosis is first suspected, the transfusion should be stopped immediately. The treatment for TRALI is supportive and consists of aggressive respiratory support with supplemental oxygen and mechanical ventilation, if required, at low enough pressure and tidal volume to not induce barotraumas.[26-27] In rare cases, the hypoxemia resulting from TRALI can be so severe that extracorporeal oxygenation may be required as a temporizing measure while the lungs heal.[28,29] It is important to report any case of TRALI to the blood service so that an implicated donor can be contacted and, if appropriate, taken off the donor panel.[5]

Risk reduction strategies:

Different centres have adopted different strategies ranging from testing of allo-exposed donors for leucocyte antibodies to the exclusion of all females from donating high plasma volume products. Another strategy involves dilution of antibodies present by pooling of plasma donations of multiple donors. From a bedside view, the most important measure to prevent TRALI is to limit patients' exposure to allogenic blood products. Furthermore, recognition and awareness of the syndrome need to be heightened among clinicians.[8]

Conclusion

TRALI has been reported by haemovigilance programs to be the most frequent cause of transfusion-related mortality in the US and a leading cause of transfusion-related morbidity and mortality elsewhere. TRALI is thought to be under-diagnosed and under-reported, particularly in critical care setting where the development of symptoms may be attributed to multiple other disease processes or therapeutic interventions rather than transfusion. Thus, maintaining a high index of suspicion is crucial in making the correct diagnosis, especially when transfusing patients using fresh frozen plasma and whole blood-derived platelet concentrates.

References

1. Toy P, Popovsky MA, Abraham E, Ambruso DR, Holness LG, Kopko PM, McFarland JG, Nathens AB, Silliman CC, Stroncek D. Transfusion-related acute lung injury: definition and review. National Heart, Lung and Blood Institute Working Group on TRALI. Crit Care Med. 2005;33(4):721.
2. Christopher C Silliman, Yoke Lin Fung, J Bradley Ball, Samina Y Khan. Transfusion-related acute lung injury (TRALI): Current Concepts and Misconceptions. Blood Rev. 2009; 23(6): 245–255.
3. Kleinman S, Caulfield T, Chan P, Davenport R, McFarland J, McPhedran S, Meade M, Morrison D, Pinsent T, Robillard P, Slinger P. Toward an understanding of transfusion-related acute lung injury: statement of a consensus panel. Kleinman Biomedical Research, Victoria, British Columbia, Canada. Transfusion. 2004;44(12):1774.
4. John-Paul Tung, John F Fraser, Maria Nataatmadja, Kathryn I Colebourne, Adrian G Barnett, Kristen M Glenister et al. Age of blood and recipient factors determine the severity of transfusion-related acute lung injury (TRALI). Crit Care. 2012; 16(1): R19
5. Pearl Toy, Ognjen Gajic, Peter Bacchetti, Mark R. Looney, Michael A. Gropper, Rolf Hubmayr et al. Transfusion-related acute lung injury: incidence and risk factors. Blood. 2012; 119(7): 1757–1767
6. Parsons PE, Eisner MD, Thompson BT, Matthay MA, Ancukiewicz M, Bernard GR et al. Lower tidal volume ventilation and plasma cytokine markers of inflammation in patients with acute lung injury. Crit Care Med. 2005 Jan; 33(1): 1-6. Discussion 230-232
7. Blennerhassett JB. Shock lung and diffuse alveolar damage pathological and pathogenetic considerations. Pathology. 1985 Mar; 17(2):239–247. 1085
8. Wiedemann HP, Wheeler AP, Bernard GR, Thompson BT, Hayden D, deBoisblanc B et al. Comparison of two fluid-management strategies in acute lung injury. N Engl J Med. 2006 Jun 15;354(24):2564–75
9. Iribarren C, Jacobs DR, Jr, Sidney S, Gross MD, Eisner MD. Cigarette smoking, alcohol consumption, and risk of ARDS: a 15-year cohort study in a managed care setting. Chest. 2000 Jan;117(1):163–8
10. Carolyn S. Calfee, Michael A. Matthay, Mark D. Eisner, Neal Benowitz, Mariah Call, Jean-François Pittet et al. Active and passive cigarette smoking and acute lung injury after severe blunt trauma. Am J Respir Crit Care Med. 2011 Jun 15;183(12):1660–5.
11. Moss M, Guidot DM, Wong-Lambertina M, Ten Hoor T, Perez RL, Brown LA. The effects of chronic alcohol abuse on pulmonary glutathione homeostasis. Am J Respir Crit Care Med. 2000 Feb; 161(2 Pt 1):414–9.
12. Darren M. Boé, Tiffany R. Richens, Sarah A. Horstmann, Ellen L. Burnham, William J. Janssen, Peter M. Henson et al. Acute and Chronic Alcohol Exposure Impair the Phagocytosis of Apoptotic Cells and Enhance the Pulmonary Inflammatory Response. Alcohol Clin Exp Res. 2010 October; 34(10): 1723–32
13. Eder AF, Herron RM Jr, Strupp A, Dy B, White J, Notari EP et al. Effective reduction of transfusion-related acute lung injury risk with male-predominant plasma strategy in the American Red Cross. Transfusion. 2010 August; 50(8): 1732-42.
14. G Llango, S Senthilkumar, N Sambanthan. TRALI in peri-operative period – a case report. Indian J Anaesth. 2009 April; 53(2): 209-213.
15. Ness PM. Does transfusion of stored red blood cells cause clinically important adverse effects? A critical question in search of an answer and a plan. Transfusion. 2011; 51(4): 666–7.
16. Looney MR, Gropper MA, Matthay MA. Transfusion-related acute lung injury: a review. Chest. 2004 July; 126(1): 249-58.
17. Magali J. Fontaine, James Malone, Franklin M. Mullins, F. Carl Grumet. Diagnosis of transfusion-related acute lung injury: TRALI or not TRALI? Annals of Clinical and Laboratory Science. 2006; vol 36(1): 53-8.
18. *Kopko PM, Marshall CS, MacKenzie MR, Holland PV, Popovsky MA. Transfusion-related acute lung injury: report of a clinical look-back investigation. JAMA 2002; 287:1968–1971.*
19. Popovsky MA, Moore SB. Diagnostic and pathogenetic considerations in transfusion-related acute lung injury. Transfusion 1985;25(6):573-7.
20. Transfusion-related acute lung injury (letter). Rockville, MD: Center for Biologics Evaluation and Research, FDA; 2001. Available from: URL: www.fda.gov/cber/ltr/trali101901.htm.
21. Popovsky MA, Abel MD, Moore SB. Transfusion-related acute lung injury associated with passive transfer of anti-leukocyte antibodies. Am Rev Respir Dis 1983;128:185-9.
22. Davoren A, Curtis BR, Shulman AF, et al. TRALI due to granulocyte-agglutinating human neutrophil antigen-3a (5b) alloantibodies in donor plasma: a report of 2 fatalities. Transfusion 2003;43:641-45.
23. Kopko PM, Popovsky MA, Mackenzie MR, Paglieroni TG, Muto KN, Holland PV. HLA class II antibodies in transfusion-related acute lung injury. Transfusion 2001;41:1244-48
24. Flesch BK, Neppert J. Transfusion-related acute lung injury caused by human leukocyte antigen class II antibody. Br J Haematol 2002;116:673-6.
25. *Toy P, Hollis-Perry KM, Jun J, Nakagawa M. Recipients of blood from a donor with multiple HLA antibodies: a lookback study of transfusion-related acute lung injury. Transfusion 2004;44:1683–1688.*
26. Kleinman S, Caulfield T, Chan P, Davenport R, McFarland J, McPhedran S et al. Toward an understanding of transfusion-related acute lung injury: statement of a consensus panel. Transfusion. 2004 December; 44912) 1774-89.
27. Top P, Popovsky MA, Abraham E, Ambruso DR, Holness LG, Kopko PM et al. Transfusion-related acute lung injury: definition and review. Crit Care Med. 2005 April; 33(4): 721-6.

28. Lee AJ, Koyyalamudi PL, Martinez-Ruiz R. Severe transfusion-related acute lung injury managed with extracorporeal membrane oxygenation (ECMO) in an obstetric patient. J Clin Anesth. 2008 November; 20(7): 549-552.
29. Kuroda J, Masuda Y, Imaizumi H, Kozuka Y, Asai Y, Namiki A. Successful extracorporeal membranous oxygenation for a patient with life-threatening transfusion-related acute lung injury. J Anesth. 2009; 23(3): 424-6.