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REVIEW

Risk stratification for coronary artery disease in multi-ethnic populations: Are there broader considerations for cost efficiency?

Pupalan Iyngkaran, William Chan, Danny Liew, Jalal Zamani, John D Horowitz, Michael Jelinek, David L Hare, James A Shaw

ORCID number: Pupalan Iyngkaran (0000-0003-3169-3762).

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Pupalan Iyngkaran, Department of Cardiology, Flinders University, NT Medical School, Darwin 0810, Australia

William Chan, Department of Cardiology Alfred and Western Health, University of Melbourne, Victoria 3004, Australia

Danny Liew, Clinical Outcomes Research, School of Public Health and Preventive Medicine, Monash University, Melbourne VIC 3004, Australia

Jalal Zamani, Department of Interventional Cardiology, Feris Shiraz University, Shiraz University of Medical Sciences, Shiraz 71348-14336, Iran

John D Horowitz, Department of Cardiology and Clinical Pharmacology, the Queen Elizabeth Hospital, University of Adelaide, Adelaide 5011, Australia

Michael Jelinek, Department of Cardiology, Vincent's Hospital, Melbourne, Victoria 3065, Australia

David L Hare, Cardiovascular Research, University of Melbourne, Melbourne, Victoria 3084, Australia

James A Shaw, Department of Cardiology, The Alfred Hospital, Baker IDI Heart and Diabetes Institute, Melbourne, Vic 3004, Australia

Corresponding author: Pupalan Iyngkaran, FRACP, MBBS, Doctor, Department of Cardiology, Flinders University, NT Medical School, Darwin 0810, Australia. balaniyngkaran@hotmail.com
Telephone: +61-03-97487377
Fax: +61-03-97487377

Abstract

Coronary artery disease (CAD) screening and diagnosis are core cardiac specialty services. From symptoms, autopsy correlations supported reductions in coronary blood flow and dynamic epicardial and microcirculatory coronaries artery disease as etiologies. While angina remains a clinical diagnosis, most cases require correlation with a diagnostic modality. At the onset of the evidence building process much research, now factored into guidelines were conducted among population and demographics that were homogenous and often prior to newer technologies being available. Today we see a more diverse multi-ethnic population whose characteristics and risks may not consistently match the populations from which guideline evidence is derived. While it would seem very

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unlikely that for the majority, scientific arguments against guidelines would differ, however from a translational perspective, there will be populations who differ and importantly there are cost-efficacy questions, *e.g.*, the most suitable first-line tests or what parameters equate to an adequate test. This article reviews non-invasive diagnosis of CAD within the context of multi-ethnic patient populations.

Key words: Cost efficacy; Coronary artery disease; Coronary heart disease; Ethnicity; Outcomes; Risk stratification

Core tip: Coronary artery disease (CAD) is a leading cause of morbidity and mortality worldwide. Globalisation has seen an epidemiological shift in demographics and risk for populations. In planning a cost-effective health service it is important to understand demographic risk and variables in interpretating and managing CAD.

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INTRODUCTION

The lay term "Heart Attack", implying coronary heart disease (CHD) is associated with significant anxiety among all communities. The first descriptions for ischemic heart disease (IHD) can be traced back to Homer and many other ancient civilizations. In Western medicine "Angina Pectoris" as first described by Heberden was derived from Latin "infection of the throat", Greek "strangling" and Latin "pectus or chest", took a century to consolidate its nature as a syndrome, by Osler and focus on the coronary arteries[1-5]. As clinical medicine, physiology and pathology, three pillars of health sciences coalesced, clinicopathophysiology of diseases became the norm in clinical discussions. Further collaborations between cardiologists, epidemiologists and biostatistician in the 1940's paved the way for the Framingham Study, the first to put a numerical face to coronary artery disease (CAD) by objectively quantifying risk factors[5]. Subsequent evolutions were in diagnosis, therapies and now cost efficacy.

Australian cardiology practice today is fundamentally different, with new clinical paradigms in CAD emerging while traditional scientific collaborations have somewhat diminished. Examples include variations in symptoms as with angina equivalent symptoms, clinical impact of risk factors and epidemiology for pathological dynamism of coronary vascular tone. This manifests as presentations and rates of progression outside traditional models. These differences can be seen within racial or socioeconomic groups and more importantly between groups who have never been formally enrolled into studies but are now mainstay in cardiology clinics from migration and other factors.

When CAD is suspected referral for cardiac services is made for risk stratification. From the history, patients acquire a risk score that guides conformation *via* a physiological and anatomical test of the coronary arteries. Presently there are no conclusive guidelines that address the cost efficacy of risk stratifying multi-ethnic patients from presentation to diagnosing CAD should they not be represented in guideline derived studies. This review is focused on exploring the paradigm that may exist when treating a diverse population for CAD from the constraints of traditional evidence. The critical question is whether a one shoe fits all approach is sufficient to answer the questions in a multiethnic, broad aged and demographically diverse populations . We focus on the Health system in the Western suburbs of Melbourne.

DIVERGENT CORONARY ARTERY DISEASE EPIDEMIOLOGY IN DEVELOPED NATIONS

CAD the largest contributor to cardiovascular diseases (CVD) with 46% male and 38% female global CV deaths, is also the most prevalent non-communicable disease and greatest cause for morbidity and mortality worldwide; has common denominators in diet, obesity and physical inactivity which if eliminated can reduce risk by > 80%, including those with diabetes mellitus and stroke. The chronology of CAD from early observational studies has revealed temporal associations in its natural history, associated risk factors and with interventions ("primary", "secondary" and even "primordial"strategies). Consequences of CAD rest with the burden of IHD and its sequelae, measured with years of life lost from death and years of disability lived with nonfatal acute myocardial infarction (MI), angina pectoris, or ischemic heart failure. Globally total age standardized IHD incidence and mortality rates have reduced, increases in the global burden of IHD has developed predominately from population growth, aging and discrepancies in regional socioeconomic development[6,7].

The greatest trend in the West today is regional divergence where prevalence peaked in the last century, but with increases in both prevalence and burden in Asia, Middle East and lower socioeconomic regions. Since 1990 there was 35% increase in CAD related deaths worldwide to 7 million (Figure 1). Geography, ethnicity and gender are factors that influence incidence, prevalence of risk factors and overall risk. Death rates (per 100000) vary 20 times from 35 to > 733 between South Korean and Ukrainian males; and around 30 times from 11 to 313 between French and Ukrainian women[8-15]. Community and global studies have gradually built on the evidence from Framingham Health Study (FHS) showing the "epidemiologic transition" of mortality from middle age (stage 3) to elderly (stage 4) in developed nations. The pattern of epidemiological transition can be trends, a rise and fall, continued rise or plateau[13]. As many developed nations have absorbed a diverse ethnic and sociodemographic population it is unclear which trend could eventuate in future. There is evidence on the one hand the rise and fall pattern is stalling among young adults, but uncertain trends for Aboriginal and new migrants.

Australia has seen a national rise and fall trend for IHD deaths, for example 29637 deaths (23% of all deaths) *vs* 22983 (17% of all deaths) were reported in 1996 and 2006 respectively[16]. Table 1 and Heart Foundation report summarizes the epidemiology in context[17]. A divergent pattern does emerge when data is broken down into states and regions within states. Remoteness, certain ethnics groups and socioeconomic demographics do not fit into the rise and fall trend when data is looked at in totally or if mortality is reduced, IHD burden remains high, *e.g.*, Aboriginal and Torrens Strait Islanders, lower socioeconomic status, younger and advanced ages[18]. This then raises the question about fixed screening protocols for diagnostics and preventive strategies and its cost-efficacy.

EVALUATION OF CORONARY ARTERY DISEASE IN MULTIETHNIC COMMUNITIES

Medicare billing specialist reviews mandates prior primary care work-up. The volume of work leads to early triaging by general practitioners or emergency departments for first specialist encounter, where medicare funding regulations provide some basic framework. Figure 2 highlights the common pathway seen across medicare funded systems. Using a "three phase screening perspective", this is however not a foolproof system for optimal cost-efficacy or outcomes as many aspects of each phase are not explored through a specialty lens. Let's explore:

Clinical variables in CAD work-up

Clinical presentation: Firstly is documenting typical angina symptoms or equivalence, secondly eliciting markers of silent ischemia (discussed in risk scoring) and thirdly clinicopathophysiological correlations *e.g.*, CAD in women. Regards symptoms, chronic stable angina is the first presentation in 50% of cases, sudden cardiac death unstable angina or infarctions in 30%. The typical presentation of anginal chest pain or "anginal equivalents" include variations in pain referred between jaw and umbilicus, shortness of breath and constitutional symptoms such as nausea, fatigue, sweating or dizziness[6,19,20]. Diamond proposed a classification of typical, atypical or non-angina, based on number of descriptors *e.g.*, substernal location, exertional and response to rest or nitroglycerin, found good angiographic correlation between men of all ages and in older women[21]. Secondly, is silent ischemia which remains a controversial topic. On the third point, pathophysiological variations: in women for *e.g.*, plaque burden is more diffuse and less calcific, epicardial disease less likely among < 65 years of age and smaller diameter arteries with more vascular dysfunction. Clinically we see a biphasic presentation with

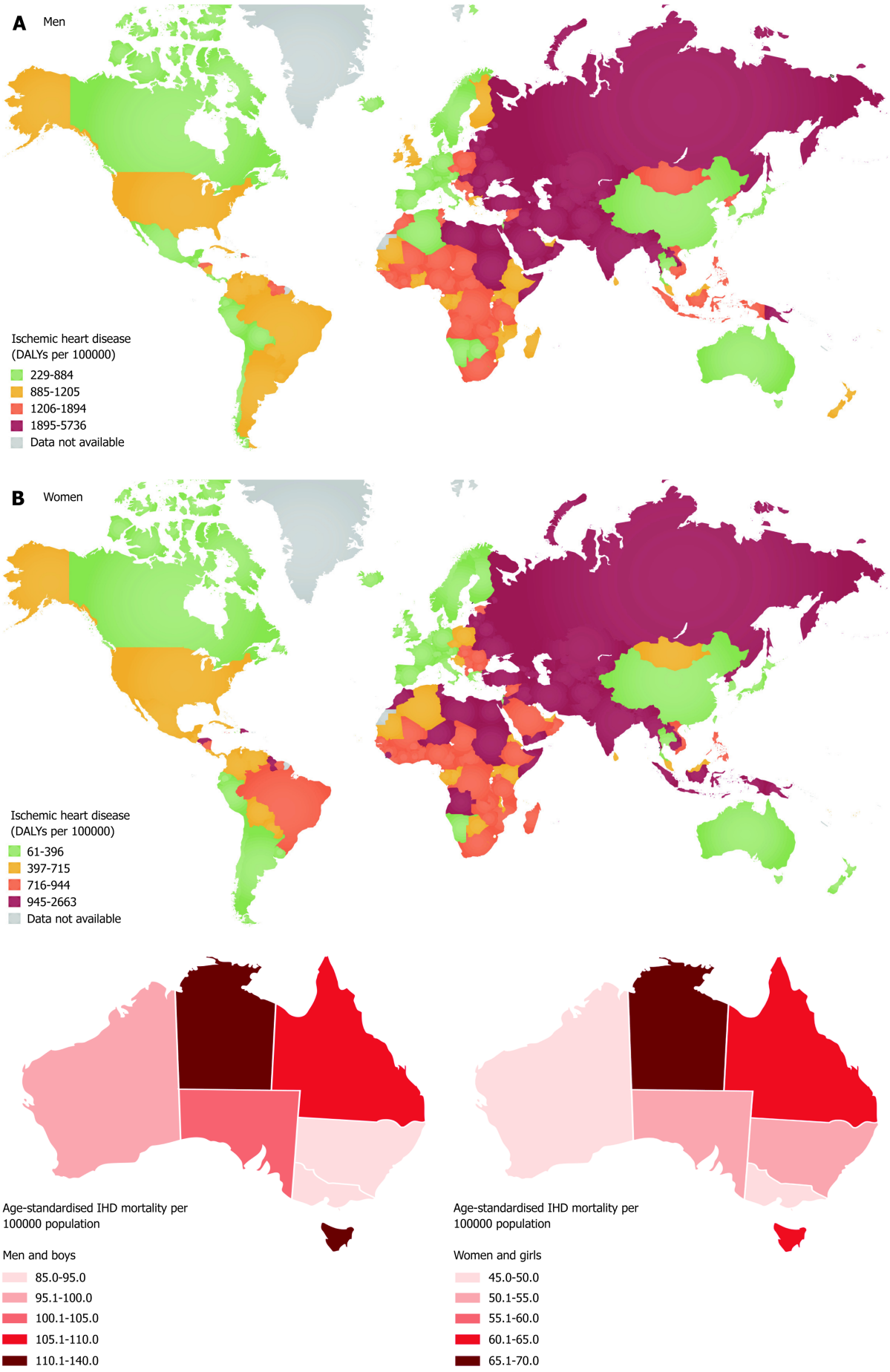

Figure 1 Ethnicity, epidemiological transition and coronary artery disease. A: The global distribution of CAD deaths. B: Mean national data may underrepresent variations within health system clusters of CAD risk, prevalence and incidences. Within developing nations, traditional and non-traditional factors contribute to risk and

may alter baseline risks associated with ethnicity and gender. When such groups' transition to developed nations such has Australia, excellent mean health care outcomes data could mask greater heterogeneity of outcomes data. These variations in epidemiological transition areas are also seen in some remote and Aboriginal populations. As demographic variations are represented variably within various health clusters this has to be factored in risk stratification. Centralized fixed guidelines and health funding models could thus be suboptimal for optimal health efficacy. (Geographical map from reference 6 and 17).

microvascular CAD at younger ages and higher associations of stress and mood changes from the history[22]; similarly a gradient of risk is seen from Asian to African American and Indigenous patients in severity and earlier age of onset[23-31]. Factoring these variations with language barriers can be challenging, as demonstrated by Shafiq *et al*[32] with patient and physician discordance in symptom reporting. Such factors could even account for variations in observed management and outcomes[33].

Risk scoring and pretest probability (PTP): From the history an assessment is made of a person's incidence or future risk of CAD. Only patients at the lowest risk of both are discharged from surveillance. Higher scores are also documented after observation of atherosclerosis and treatments such as coronary artery bypass grafting surgery or percutaneous coronary intervention. Scores determine the choice of diagnostics investigations and predict hard CAD endpoints of nonfatal or fatal MI[6]. The FHS importantly demonstrated CVD risks with inverse relationship between various lipoproteins in cholesterol, diabetes, women and hypertension. These findings also highlighted genomic determinants and risk clusters as the foundations for multivariable or global risk assessments[6,34-36]. Additional prospective observational studies added in other risk factors including age, gender, ethnicity, diet, cigarette use, exercise, sedentary lifestyles, excess weight and family or past history of CHD, as among the more important to shape scoring systems. Such systems have been developed for clinics (*e.g.*, Framingham Heart Disease Risk Scores) emergency departments [*e.g.*, ADAPT (Protocol for Cardiac Event Risk), Emergency Department Assessment of Chest Pain Score] or exercise (*e.g.*, Duke Treadmill Score).

Jensen *et al*[37] explored 5 risk scores (Diamond–Forrester, Updated Diamond–Forrester, Duke, Morise, CORSCORE), all use age, sex and symptoms; Duke and Morise also use tobacco use, diabetes and hypercholesterolemia, while Duke uses MI and electrocardiogram (ECG) changes and Morise uses family history of CAD, body mass index, oestrogen and hypertension. The most efficient risk models in predicting CAD are the Duke, updated Diamond–Forrester, and CORSCORE among patients presenting with angina, while the most user friendly is updated Diamond–Forrester with the lowest number of clinical variables[37]. Integration of this model into clinical care is discussed subsequently.

Stress testing and coronary artery evaluation

The premise of physiological testing is the ischemic cascade and the mechanisms for detecting the varying events[38]. Stressors (mode of exercise or pharmacological agent) and diagnostic imaging modalities will contribute to the overall short or long term statistical binary classifications for the screening tests in this case sensitivity and specificity. Within this broad classification it has never actually been identified or tested if there are categories (sub-populations *e.g.*, demography) who require new observations prior to committing to the current stress guideline classification.

Ischemic cascade in physiological stress tests: The ischemic cascade describes "an assumption of linear" temporal sequence of pathophysiological changes where the preceding change triggers the subsequent in response to increasing myocardial supply-demand imbalance (Figure 3). Maznyczka *et al*[39] proposed a more individualized concept that places the patient holistically at the center of the sequence, coining the term "ischemic constellation". This paradigm proposes no test as gold standard, that different cascade events can be abnormal at any temporal stage based on factors that can interrupt cascade linearity. These include stenosis severity or dynamism in the vascular bed, extent of stress or nature of stimulus, prior treatments, comorbidities, ischemic preconditioning and other individual factors[39]. This would suggest that from our traditional model of PTP, symptoms, ECG and diagnostic imaging, where the latter carries greatest discriminatory capacity; in the new model under varying conditions any factor could assume greater importance.

With this in mind important points to consider: (1) PTP (a) adequacy of scoring prior to referrals; and (b) standardizing scores for fixed factors *e.g.*, age, ethnicity, family history, birthplace, *etc*; (2) stress modality (a) adequacy of stress duration and achieving > 85% mean predicted heart rates across age, gender and ethnicity. Achieving 10 METS is an accepted target, however the comfort and recovery periods

Table 1 Summary of landmark international and Australia coronary artery disease studies

Study geography	Clinical summary (Population data available on). Demography; epidemiology; morbidity; mortality; regional variations in RF and outcomes; epidemiological transition; gaps
*Major international studies[6-8]	**Year, study, participants, sex and ethnicity:** US studies: (1946) The minnesota businessmen study – C, 281 M, < 55 yr; (1948) Framingham heart study - ; (1984) CARDIA – AA/C, 5115 M/F, 18-30 yr; (1987) ARIC – AA/C, 15792 M/F, 45-64; (1989) Strong Heart – AI, 4549 M/F, 45-75 yr; (1989) Cardiovascular health study – AA/C, 5888 M/F, 65-102 yr; (2000) Jackson heart AA, 5302 M/F, 21-94 yr; (2000) MESA AA/C/Ch/H, 6814 M/F, 45-80; (2006) Hispanic community health study/study of latinos – H, 15079 M/F, 18-72. Global: 1958 The seven countries study – C, 12763 M, 40-59 yr; (1979) MONICA – ME; 15m M/F, 25-64 yr; (1999) INTERHEART – ME, 15152 M/F, age/sex matched; (2002) PURE – ME, 153996 M/F, 45-69 yr; Japanese: (1965) Ni–Hon–San study – J, 20k M, 45-69 yr Europe: UK: (1967, 1985)Whitehall, Whitehall II – C, 18403 M/10314 M-F, 40-64/35-55 yr; Iceland 1968, 2003) Rejkavik, AGES studies – C, 9141/2499 M, 34-79; Germany (1979) PROCAM – C, 4043M/1333F, 50-65 **Summary of epidemiology findings:** Caucasian male population are baseline comparator group for epidemiology data High income countries: International trends shown strong ↓ mortality in high-income countries since 1980 Mortality gaps exist with ethnic differences (probably genetics) either ↑ or ↓ risk or even protection. Globally: Age-standardized acute myocardial infarction incidence and angina prevalence have ↓ and ischemic heart failure prevalence has increased since 1990 (6) High age-standardized IHD mortality in Eastern Europe, Central Asia, and South Asia point to the need to prevent and control established risk factors in those regions and to research the unique behavioral and environmental determinants of higher IHD mortality.(7) Much of the dramatic CHD mortality increases in Beijing can be explained by rises in total cholesterol, reflecting an increasingly "Western" diet. Without cardiological treatments, increases would have been even greater.(6-4 Critchley J) **Gaps in knowledge:** Paucity of data in older > 75 yr Ethnic, family and true genetic contributions to CAD with improved modifiable risk factor control
Australia[17,18]	**Mortality, morbidity and cost:** Death rates >Japan but < other high-income countries *e.g.* UK, Germany USA ATSI Deaths 1.5-3 x and IHD burden.0 0Smoking ATSI Ischaemic heart disease results in more. Australian deaths than any other single cause for both men and women. Death rates from heart disease are substantially higher among ATSI Australians, ranging from 1.5 to 3 times higher than in non-Indigenous Australians. Of all Australians aged 2 yr and over, 5% report living with heart, stroke or vascular disease. Among people aged 85 yr and over, this proportion rises to two in every five people (40%). In 2012–2013 the Pharmaceutical benefits scheme paid approximately $1.8 billion for cardiovascular system medicines, representing 21% of total benefits paid in that year. **Risk factor:** ↓ Smoking M:F18:14%: ATSI > 2x double non-Indigenous (41% daily smokers). < 10% of all met the NHMRC guidelines for vegetable consumption. In a national secondary school survey, 24% met recommendations for consumption of vegetables and 42% met recommendations for fruit consumption.

	Most Australians (58%) were either sedentary or had low levels of activity. Australians spent an average of 38.8 only 30% of children met physical activity recommendations, and only 10% met both physical activity and screen-time recommendations.
	13% of men and 10% of women reported drinking alcohol at levels likely to present a risk to health. Total per capita alcohol consumption fell between the early 1970s and the early 1990s, but has been relatively steady since then.
	One-third of Australians had high blood cholesterol (above 5.5 mmol/L). Almost four in every five Australians with abnormal cholesterol or triglyceride levels were not receiving treatment for it.
	One in five Australians had high blood pressure and the prevalence was higher in men than women. One in four Aboriginal and Torres Strait Islander Australians had high blood pressure. The prevalence of high blood pressure rose substantially with age, from less than 10% in the 25 to 34-year age group to almost 50% in people aged 75 years and over.
	More than two-thirds of men were classified as overweight or obese, as were 55% of women. One-quarter of children aged 2 to 17 years were classified as overweight or obese.
	The overall prevalence of diabetes in the Australian public was more than 5%, with a further 5% at increased risk of developing diabetes.
	The prevalence of mental disorders in 2007 was 17.6% in men and 22.3% in women; anxiety disorders were the most prevalent mental disorders in both sexes. Cardiovascular disease was responsible for nearly 44000 deaths in Australia in 2012, including more than 20000 deaths from ischaemic heart disease.

Common denominators in risk exist. Socioeconomic status and ethnography can contribute to this risk and needs to be factored in future risk scores. ATSI: Aboriginal and Torres Strait Islander; ET: Epidemiological transition; RF: Risk factors. *FHS: Framingham Heart Studies; NHMRC: National Health and Medical Research Council.

are not factored as risks[106]; (b) the effects on the ischemic cascade with pharmacological stressors; and (c) Long term prognosticator of pharmacological stressors; (3) contextualizing ECG changes with risk and normal imaging: (a) strongly positive ECG change (> 2 mm) in low to intermediate risk females; and (b) marginally positive *e.g.*, > 1 mm but < 2 mm in males; (4) marginally positive imaging without symptom or ECG changes; (5) when to combine physiological test and coronary imaging modality to a risk monitoring plan at baseline; (6) lower limit criteria for classifying negative or low future risk (no cardiac follow-up required); and (7) long term planning for undifferentiated chest pains and dynamic coronary ischemia and future health services utilization.

Evidence for using the ischemic constellation in multiethnic communities: Among the six cascade events, baseline characteristics and stressor can determine which factor is more prominent with stress provoked ischemia. The most powerful test finding would be from individuals who have achieved greater that predicted exercise stress with some direct vascular atherosclerotic scoring. We cite from experience that some ethnicities especially when associated with lower/higher body mass and older may not meet the physical challenges of the treadmill to achieve generic targets. Body mass, ethnicity and older adults indexed charts are not available. Language barriers in this group could also alter the interpretation of symptoms. ECG changes could have variable importance among, troponin negative acute chest pain discharges [40-42]. In acute chest pain presentations negative troponins could miss significant CAD[40,43], while high sensitivity troponin assays are plagued with specificity issues[44-51]. In summary, circumstances associated with ethnicities in some health clusters can alter the PTP, for *e.g.* difficulties with interpreting symptoms, the thresholds to provoke ischemia and constellation factors influencing the measured diagnostic parameters; greater vigilance is needed with post test decisions[40,41,52], including those who have normal coronary angiography[53]. Broadening decision support algorithms, presently inadequately used for more diverse demographic, could be part of future planning[54-59].

Imaging modalities, Ca Score and computer tomography coronary angiography (CTCA): When deciding on the imaging modality several factors have to be considered, including: availability, accessibility, reproducibility, cost and safety (Table 2). ECG based exercise stress testing remains first line for younger males with low risk that are able to exercise. Direct referrals for stress echocardiography is debated presently, but evidence points to superior cost efficacy when using a patient centric approach[60,61]. Much of the exercise and imaging quality deficits can be overcome by pharmacological agents *e.g.*, dobutamine and contrast agents[60]. Appropriate

Figure 2 Triage and subsequent evaluation of suspected coronary artery disease referrals. CAD presentations to specialist are predominately referrals from general practitioners or following triage from emergency departments. Referrers either request a specialized test or a cardiac consultation. The choice between EST and ESE are not always clear. Direct specialist consultation may precipitate a more risk score guided approach. Moving down the pathway following complaints of typical symptom or positive stress test, following ascertaining anatomical information, gaps still exist for future health encounters subsequent from inconsistent classification of CAD burden probable vasospastic angina or undifferentiated cases. Direct diagnostic referrals run the highest risk of duplication for future representations. *Highlights points in the pathways where models of cost-efficacy and risk stratification can be examined further. EST: Exercise stress test; ESE: Exercise stress echocardiography; FU: Follow-up.

remuneration strategies for this have not been factored outside tertiary centers. The main advantages appear to be access, accuracy, reproducibility and cost. The access may also be the Achilles heel for cost efficacy as guidelines for repeat testing are not well regulated. SPECT has equivalent accuracy but falls short in other parameters. A main advantage could be in those patients with baseline resting wall motion abnormalities, to quantitate location and size of infarct and obtain a gated blood pool scan ejection fraction. Cardiac MRI is safe and accurate but has other access and cost issues of SPECT. With existing dilated cardiomyopathies we suggest a baseline ESE for functional assessment combined with a baseline MRI or GBPS ejection fraction. Should an MRI be available, combination with cardiac CTCA without additional functional assessment, could be considered[61].

Ca Score statements support its use as a one off, in selected patients of low to intermediate risk to complement an existing functional assessment or modify risk to a higher or lower grade. We cannot advocate a role as a standalone investigation for CAD[62,63]. The utility of CTCA is now clear, with ongoing research areas for risk stratification in acuity[64,65]. As we are still unclear as to the boundaries of complimentary or additive information with functional tests and traditional risk scores, the patient cost and pretest counselling required, the role for non-specialist referrals without patients driven intent is open for further review.

Once a baseline test is done, the choice of future tests must be reproducible complimentary and provide additive information. Inter and intratest reproducibility is best achieved with stress echocardiography. All limitations of baseline test must be adequately documented for future references. The most important factor in future tests is pretest planning, *i.e.*, access to previous test findings, what additional

A

B

Pretest Probability for CAD	**Typical Angina (%)**			**Atypical Angina (%)**			**Non Anginal Chest Pain (%)**
1 Male	59-69-77-84-89-93			29-38-20-59-69-78			18-25-12-44-54-65
2 Female	28-37-49-58-68-76			10-14-34-28-37-47			5-8-12-17-24-32
Demographic	**Epidemiological transition**	**oCAD**	**vCAD**	**Syntax**	**Exercise capacity**	**Risk score**	**Notes**
1 Caucasion	Unimodal	40-60	↔	Mild (+)	Normal	↔	Benchmark group useos comparator CAD in the elderly is epidemic and requires more study
2 Asian	Bimodal	> 1	↑	< 1	< 1	↓	Lowest risk ET still being defined (*e.g.* China, SEA)
3 Indo-African-Hispanic-middle Eastern	Bimodal	< 1-2	?	> 1 and 2	< 1 (except African)	↑	Ehtnicity alone may pose a risk ET rates can be highly variable
4 Indigenous	Bimodal	< 3	?	> 3	< 1	↑ ↑	Highest risk across all categories
5 Female	Bimodal	> 60	↑ ↑	< male	< 1 and male 2-4 females could be lower	↓	vCAD Asian could be highest Blunting of protection with gp 3 and 4 especially if more RF.

Figure 3 Ischemic Cascade and Constellation in clinical context. A: Stress creates myocardial energy imbalance leading to any of six manifestations in the ichemic cascade (red arrow) in a forward linear direction. Individual variations and combinations of workload, stenosis severity and ischemia duration (outer green arrows) that inlfuences the order for components in the cascde, creates more reaslistically an ischemic constellation' of events, where any order for observable events is possilbe (multicolour arrows); B: PTP guides diagnostic workup. The probability is never 0 or 100%. With < 15 and > 85% guiding low or high risk. The actual number are for age range 30-39 years, increasing by decades, to > 80 years. Variations from the mean are determined by demography. No 1-4 represent males. Females across all groups represent the same CAD risk as the males except are relatively protected during middle ages, delaying mean onset of significant CAD. Etiological variations exist in: (1) vasospasm, being more likely in women, especially Asian. The actual rates among other non-caucasion female races are nor well defined; (2) gender protection: from premature CAD in women may also be blunted in higher risk groups 2; (3) ethnicity - Aboriginal populations suffer from the greatest risk and severity of CAD, followed by 3, 1 and 2. Asians have the lowest risks; (4) developmental status - of any group also lowers mean age of onset of any ethnicity. Defining the coronary anatomy early could be one way of risk stratifying subgroups within braod ethnic based categories. oCAD: Obstructive coronary artery disease; ET: Epidemiological transition; PTP: Pre-test probablity; RF: Risk factors; SEA: South East Asia; vCAD: Vasospastic coronary artery disease. (Concepts modified from ref 38, 39, 82-101).

information is required and the targets to be met. Reassessment of prognosis and diagnosis (if required must) also be documented in test or consultations reports. Any changes to baselines risk must also be recalculated.

Confounders in CAD evaluation and posttest review - Syndrome X, silent ischemia, diastolic heart failure and cardiac risk assessment in comorbidity and demography

Table 2 Sensitivity and specificity non-invasive test

Test	Guideline indication	Sensitivity	Specificity	Stress modality	Advantage	Disadvantage
ECG	1st L-PTP	45-50	85-90	Physical	Simple and safe	Accuracy
	2nd I-PTP				Availability	ECG artifact
					Lower cost	False positives
Echo	1st L-PTP	80-85	80-88	Physical	Simple and safe	Suboptimal image quality *e.g.*, resting wall motion defects, lung disease, respiratory artifact,
	2nd L-PTP			Pharma*	Availability	Image capture within 90 sec of peak HR
					Lower cost	Cost of contrast
					No radiation	
					ECG independent	
					Mobility independent*	
					Ischemia: Quantify and localize; greater spatial resolution (subendocardial)	
Myocardial perfusion scintigraphy (SPECT, PET)	1st L-PTP	73-92	63-87	Physical	Accurate quantification ischemic area	Cost
	2nd L-PTP	90%	75-87	Pharma*	Ischemia: Quantify and localize; greater spatial resolution (subendocardial)	Availability
					↑ accuracy with septal defects	Radiation and retesting
						Ischemia: ↓spatial resolution *e.g.* for subendocardial ischemia
						Pharma: CI, SE, ↓ sensitivity for multivessel disease
						↑ acquisition time
						Artifacts: Lung motion, breast tissue, diaphragm attenuation
MRI						
Ischemia	1st I-PTP	79-88	81-91	Pharma*	Body habitus/lung window independent	Cost
Perfusion	2nd L-PTP	67-94	61-85		Accurate	Availability
					No radiation	Expertise
					Operator independence	↓ Gating: Rhythm and rate
					High spatial resolution	
					Can perform absolute quantification of perfusion	
CA Score	1st L-PTP	95-99	64-83	Direct visualization coronary artery	Availability	Radiation
CTCA	2nd I-PTP				Non-invasive	Cost
					Anatomical information	Ca score role
					FFR	No functional information
						Contrast

↓ Gating: Rhythm and rate

Guideline referral for diagnostic evaluation is guided by PTP. All males start with a PTP of > 15% and thus warrant at least a Stress ECG. Females between 30 and 39 years have PTP 10% and clinical judgement may suffice. When PTP is > 85%, *e.g.*, males > 60 years with typical symptoms, coronary imaging can be considered first line or complimentary based on clinical judgement. Females are not given a PTP > 85% and thus should at least receive a functional test. The choice of functional test is described in the body of the table. Non-imaging stress ECG has three information sources - PTP, subjective (test symptoms and hemodynamics) and solitary objective (ECG) marker of the ischemic cascade. Imaging stress testing adds baseline myocardial function to the PTP and additional objective components of the ischemic cascade improving accuracy. Greater advancement in addressing issues of access, cost and reimbursement, complementary (dual) modality testing, additional means to assess ischemic cascade components (*e.g.*, tissue strain), image quality issues (*e.g.*, contrast echo) could alter the test indication and its accuracy. PET - imaging has lower radiation, higher resolution and quantify blood flows (including microvasular), but cost and availability are issues. MRI identifies wall motion changes *via* dobutamine or myocardial perfusion by vasodilators. Exercise: treadmill; bicycle; right ventricular pacing. Pharmacological: dobutamine; adenosine; dypiridamole, ragadenoson. Imaging Sources: SPECT isotope: Technetium 99m, thallium 201. CA: Calcium; CI: Contraindication; CTCA: Computerized tomography coronary angiography; ECG: Electrocardiography; Echo: Echocardiography; FFR: Fractional flow reserve; H-PTP: High pretest probability; I-PTP: Intermediate pretest probability; L-PTP: Low pretest probability; PET: Positron emission tomography; PTP: Pretest probability; MRI: Myocardial resonance imaging; SE: Side-effects; SPECT: Single photon emission computerized tomography (Data partly synthesized from reference 38, 100, 101). *Physical exercise is the preferred stress modality. It provides higher physiological stress and workloads with greater opportunity for corroboration with patient's symptoms. Although studies have shown similar accuracy between physical and pharmacological stress test, we feel there is insufficient evidence to draw similar long term conclusions on the diverse etiologies and severity of CAD emanating from more diverse patient populations.

Typical presentations of angina or angina equivalents with or without classical findings on diagnostics can often be related to syndromes involving the coronary vasculature or heart muscle. On diastolic heart issues we refer to our recent publication[66]. Numerous authoritative publications have explored the dynamism of the large and small coronary arteries. Important points to consider are the low rates of community and primary care recognition of this syndrome, higher frequency of representation and resource utilization, higher morbidity and prognostic considerations and lack of formally labelling patients with the diagnosis. Many of these issues can be negated by increasing first presentation diagnosis or labeling of non-cardiac chest pains following specialist encounters. Formalizing a treatment or action plan is also critical[53,66-70].

Ambulatory silent myocardial ischemia is demonstrated in nearly 50% of stable CAD, 15% with either hypertension or 12 and 33 with diabetes alone or with another risk factor[71-74]. Functional diagnostics induced silent ischemia; with at least mild to moderate CAD shares similar risk to symptomatic patients. As silent ischemia can occur with or without CAD the boundaries with vasospastic angina require greater exploration. In patients with risk factors identifying further fixed risk, *e.g.*, family history of premature CAD (before 60 years) in first degree relative, modifiable risk, *e.g.*, sedentary lifestyle, end organ changes, *e.g.*, proteinuria or early large or small vessel atherosclerosis, *e.g.*, retinopathy, peripheral or carotid artery disease. The choice of test favors an imaging based functional test, while imaging of a vascular bed must also be considered complimentary early in the screening process[75-79], although improved outcomes may yet to be defined[80,81]. Among women who are more likely to have ECG changes, prognosis is unclear and more work is needed[82].

While the decisions for either functional or anatomical test are clear, the decisions for both are less clearly stated. Several important parameters guide the need for angiography. When PTP score approaches 85% *e.g.*, male > 60 years with typical angina. Similarly there are categories of risk at young age amongst some population with risk factor clusters *e.g.*, diabetics and chronic renal impairment[82-98]. Should risk scoring algorithms be strengthened, more evidence develop, clearer positions for warranting both types of information could be forthcoming.

HEALTH CLUSTERS AND COST EFFICACY

It is always premature to rest when a plateau in knowledge is assumed to have been achieved. But in all developed health systems it is premature to invest public resources to understand variations in established observations that do not meet robust requirements of need, especially cost-efficiency. We accept differentials exist in phase 1-3 trials that assume largely private capital, so this argument is largely for phase 4 trials or post translational observations[99-102]. The observations presently is "the cost efficiency of using unimodal risk stratification of CAD within health clusters that serve a multiethnic patient population with varying stages of epidemiological transition based on findings and guidelines from homogenous populations". Presently true costings can't be evaluated without further understanding of some care domains and processes within them. Broadly however, CAD assessment requires acknowledgement of four fundamental goals (Figure 4).

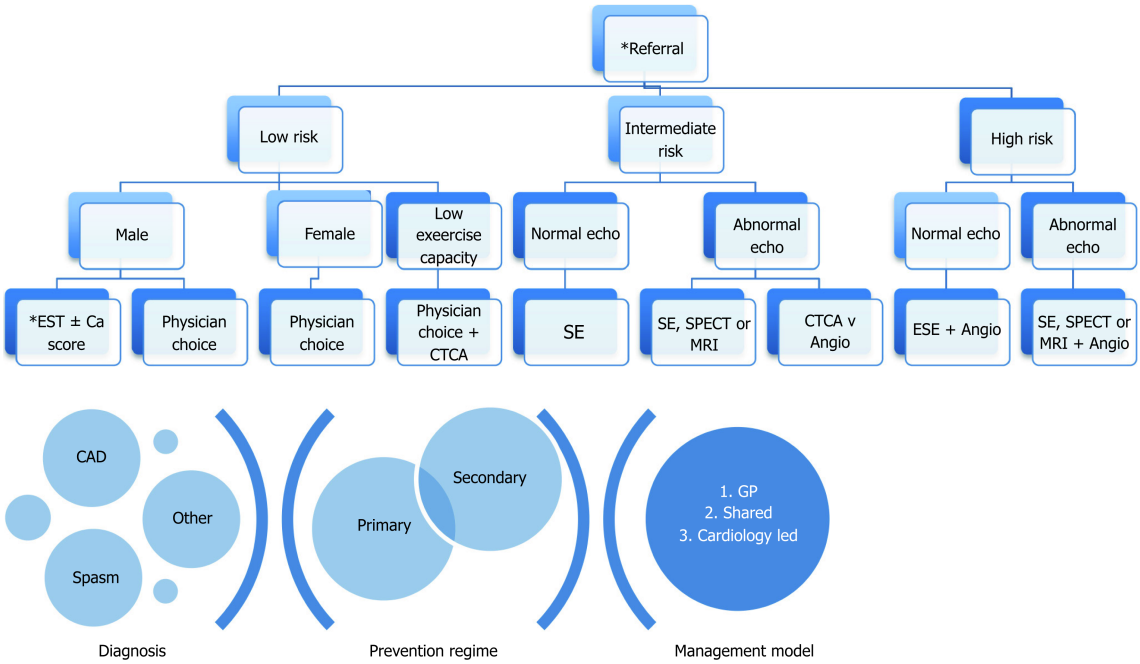

Figure 4 Theoretical Considerations for Future Cost-Efficiency. Three avenues for cost-efficacy analysis within a health cluster are identified. (1) Referral: the PTP gives a rough guide as to first choice of diagnostic test. High demand for evaluation raises an argument for observational studies for the best point of initial PTP work-up *e.g.* with GP supported education or early cardiology review; (2) Diagnostics: the combination of patient factors, cost and availability dictates stress echocardiography be the first choice of imaging modality, however more understanding of other modalities should also be encouraged; (3) Clinical Evaluation: this area is most likely to influence lifetime CAD cost-efficiency. Avenues to explore are: (a) patients and primary care preventive education after findings of minor CAD and/or with intermediate and high risk criteria factors; (b) patients at increased risk of recurrent chest pains and readmission *e.g.* vasospastic angina; (c) risk models for screening and follow-up of various ethnicities and demographies at different stages of epidemiological transition; (d) combinations of primary, specialist and tertiary care management models: the long term patient specific and health system goals once the work-up for CAD is initiated, aside from actual diagnosis of definite CAD, is prevention of disease progression or risk of future disease and preventing cost-ineffective utility of health resources especially unnecessary diagnostics and hospital beds. Physician Choice: includes any combination of investigation based on additional characteristics deemed to alter risk of accuracy of EST. Ca: Calcium; CAD: Coronary artery disease; CTCA: Computerized tomography coronary angiography; ESE/SE: Exercise stress echocardiography; PTP: Pretest probability; SPECT: Single photon emission computerized tomography.

Symptom description and risk scores the bed rock for CAD evaluation

Family history and ethnicity are examples of fixed risk that remains difficult to quantify. Clinical judgement is a difficult area to research, but plays a strong role in management. Further avenues to explore are: (A) Avenues to improve translated and cultural descriptors in history for anginal symptoms; (B) Standardizing multiethnic population level risk scores; (C) Factoring in the burden of disease in risk scores; (D) Referral forms with PTP screening tools or PTP done in waiting rooms prior to test; (E) What constitutes a baseline cardiac risk score? *e.g.*, biomarkers like hsTrop; and how often to do? Does one test supersede another? and (F) With higher risk scores do we need both functional and anatomical information?

Translating risk scores into satisfactory analysis of CAD

Without directly visualizing atherosclerosis, most conclusions are inferred. Gaps exist when CAD is not excluded and with non-critical CAD, greater risk factors or microvascular disease. (A) Redefining adequate physiological endpoints that account for the ischemic threshold, with varying grades of exercise; and (B) Visualizing alternate vascular beds as surrogate for coronary vasculature in selected cases.

Risk stratifying patients post stress testing

Once significant CAD is established management including rehabilitation and long term care pathways are well defined with solid health infrastructure in place. When patients are not cleared of CAD nor have nontraditional risks, grey areas emerge. Such areas include: (A) Factoring in the burden of disease and providing post-test risk scores; (B) Minimizing readmission and utilization of diagnostics for vasospastic CAD; (C) Licensing and return to work issues; and (D) Ensuring framework for longer term follow-up is documented, even when only a diagnostic test is requested.

Acknowledging the most cost efficacious method in achieving this goal

Ongoing health education and engagement of specialty, tertiary and regulatory bodies are important. The jurisdictions for many of these are still poorly defined. (A)

Health Clusters: (1) Jurisdictions: understanding of the boundaries, shared resources and negotiation of clinical scope could be better defined. State and federal funding tend to promote non-cooperative working relationships. Honorary acknowledgements of regional cardiologist in public institutions are examples to consider; and (2) Shared clinical and education models – research in health economics is largely a process of testing different working models. We suggest hybrid models which supports continuous information to and from all stakeholders; for providers' flexibility of management, with accountability; and public bodies the most effective resource sharing models as important areas to explore; (B) Audit and Research Importance of audits – while regulation from authorities create a blanket rule, it is preferable that physicians using public funds should provide evidentiary data to governing bodies. Several options include: firstly, continuous professional development – to include questionnaires on selected key performance indicators across a range of issues in that community. This way accumulation of knowledge by a specialist is also transferred back to governing bodies; secondly, a mandatory requirement for audit, *via* governing body attached institutes across a range of domains at stipulated intervals. Health trainees could be assigned to assist. This will ensure a robust standard in documentation and management. As an important source for variations are referral biases, while structures have to be in place to align outcomes, punitive approaches must be avoided, as would providing sufficient time alerts to align practices; (C) Terminology - standardizing the minimum information required and the terminology to assist inter-observer interpretation. For example, define: (1) Nature of CAD - *e.g*., atherosclerotic, vasospastic, undefined; (2) Symptom - *e.g*., angina, angina equivalent, silent; (3) Etiology - *e.g*., main cause, CAD in women; (4) Structure - *e.g*., associated myopathies and anomalies; (5) Risk - *e.g*., main etiology and future risks; and (6) Monitoring - *e.g*., define suitable chronology of reinvestigations; and (D) Conflicting evidence in medicine - translational research questions should focus on answering many broad questions rather than, "yes" and "no" question such as mortality. We must continue to reinforce while hard major adverse cardiovascular outcome are not produced for all ethnicities, this should be a secondary outcome in phase-4 studies. The primary focus are delivery models and cost-efficiency across established health service Taxonomy domains. Nesting this question within larger studies could also broaden the funding appeal[102-105].

CONCLUSION

CAD work-up in multiethnic populations requires a more considered approach. While it is unlikely significant CAD is missed further considerations could reveal opportunities to improve cost-efficiency through the entirety of a patient's health journey. Established pathways provide a good foundation to work from. Fine tuning this approach based on improving the translation of: ischemic symptom, "angina and equivalents" across language and cultures; risk scores when factoring ethnicity and country of birth; observable myocardial ischemia factoring individual variations and combinations of workload, stenosis severity and ischemia duration and its influence on the ischemic constellation. We have also highlighted potential options to explore. It is also becoming apparent that new paradigms in medical practice within well-defined disease processes such as CAD are developing. Such observations, calls for greater strengthening of collaboration within health clusters for education and research, while maintaining healthy competitive clinical service models to ensure uninterrupted and efficient care for patients, from all stakeholders in public and private sectors.

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MINIREVIEWS

Importance of the telemedicine network for neurosurgery in Slovenia

Tomaz Velnar, Tilen Zele, Roman Bosnjak

ORCID number: Tomaz Velnar (0000-0002-6283-4348); Tilen Zele (0000-0002-9355-8702); Roman Bosnjak (0000-0001-7565-0989).

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Tomaz Velnar, Tilen Zele, Roman Bosnjak, Department of Neurosurgery, University Medical Centre Ljubljana, Ljubljana 1000, Slovenia

Tomaz Velnar, AMEU-ECM, Maribor, Slovenia

Corresponding author: Tomaz Velnar, MD, PhD, Assistant Professor, Doctor, Department of Neurosurgery, University Medical Centre Ljubljana, Zaloska 7, Ljubljana 1000, Slovenia. tvelnar@hotmail.com
Telephone: +386-1-5223250
Fax: +386-1-5222218

Abstract

The number of invasive procedures in medicine is increasing, as is the employment of new technological achievements. In the era of information-communication technology, one such achievement is also the telemedicine network. In Slovenia, it is known as the Telekap (TeleStroke) network, which was primarily designed for fast and efficient management of stroke patients. In the neurosurgical community, the system is frequently used also for conveying vital information regarding subarachnoid haemorrhage and trauma. Especially in neurosurgical emergencies, this communication system offers thorough information about the extent and location of bleeding and facilitates the preoperative planning of neurosurgical interventions. From our experience so far, the system should be expanded to other neuro-centres as well to all neurosurgery departments in order to facilitate patient management, their acute hospital care, and inter-speciality collaboration.

Key words: Telemedicine; Neurosurgery; Neurosurgical planning; Neuroimaging; Slovenia

Core tip: The telemedicine network in Slovenia is known as the Telekap (TeleStroke) network, which was primarily designed for fast and efficient management of stroke. In the neurosurgical community, the system is frequently used for conveying information regarding subarachnoid haemorrhage and trauma. It offers thorough information about the extent and location of the pathology and facilitates the neurosurgical intervention.

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INTRODUCTION

The number of invasive procedures and the use of new technological achievements in medicine are increasing. Telemedical connections are one of such important acquisitions in the era of information-communication technology (ICT)[1,2]. Telemedicine is defined as the use of medical data, mostly in emergencies, which emergency services are sharing between themselves by using electronic communication and with the purpose to improve healthcare, health and educational services in outpatient and emergency situations[1,3]. Hospital and outpatient neurology is one of the emerging subspecialties in neurology that was developed to improve access to neurological care in areas without specialised neurology centres[3-6]. Telemedical network system is not used only by neurological professions, and it is also very useful for neurosurgery.

IMPLEMENTATION OF THE TELEKAP SYSTEM IN NEUROSURGERY

In Slovenia, the telemedicine network is called Telekap (TeleStroke) and it has been used since 2015. All hospitals in the country are connected in this network, which provides continuous access to the data and connections with consulting specialists in the tertiary centre. The main idea during establishing this system was that telemedicine may provide care for all neuropathology patients in the country with quick and easy accessibility. This telemedicine network was initially designed for fast and efficient treatment of patients with a stroke. The Telekap system enables clinical and radiological assessment of location and scope of stroke, tumour, hydrocephalus, or head and spine injuries, and fast decision-making about further diagnostic procedures. It also provides triage for patients and thus enables a suitable therapy. When the patient is admitted to a peripheral hospital, the images are forwarded directly to the telemedicine centre, where neurosurgeons may plan the optimal treatment strategy. According to the conveyed images, the resuscitation and surgical team may be properly prepared for the operation during the transport of the patient. Additionally, the postoperative accommodation and care of such a patient may be forecast and organised. Increased prevalence and mortality due to stroke is partially the consequence of slow treatment due to large distances to specialised neurology centres[7]. The time of treatment is therefore extremely important, and it affects the outcome of the treatment. The patient suffering from stroke must not only be transferred to an appropriate centre as soon as possible, and data about the disease that provide fast and expert assessment, planning, and correct action during admittance are also important. However, this is possible only if the data is promptly available. Similarity also applies for neurosurgical pathology where fast action is needed[8,9].

The Telekap system is being used in neurology principally in relation to haemorrhagic and ischemic strokes as it provides fast informing and transfer of data from remote hospitals to centres that are specialised in stroke treatment[5,9]. However, the telemedicine system is useful not only for informing and preparation for stroke treatment but also for other types of pathology. Beside neurology, the Telekap system is extremely valuable for neurosurgeons. They are using it frequently for acquiring significant information, mostly in relation to subarachnoid haemorrhage, tumours, and head injuries, being most frequent indications[10].

In Slovenia there are two referral tertiary neurosurgical centres for the 1.8 million population. Due to the specifics of neurosurgical pathology, the paucity of neurosurgery experts in peripheral emergency departments, and the lacking availability of a range of effective treatments there, the patients with corresponding pathology and in need of emergency treatment are transferred for the specialist management to the either centre always when possible. On some occasions, the worsening clinical condition would direct immediate action and preclude the transport. In these sceneries, the Telekap is particularly valuable. Numerous factors have all contributed to optimal conditions for Telekap implementation in Slovenia, including the opportunity to improve access and quality of care, narrow window of time frame and treatment efficacy, the resources required for ground and helicopter

medical transportation, and the expansions and improvements of the medical care dedicated ICT. As a result, the Telekap is being used extensively in the national health care and its use is still rising.

On some occasions, the surgeons in local hospitals are capable of exerting immediate neurosurgical treatment for the patients who need urgent action and where transport would deteriorate the worsening condition due to the time required for reaching neurosurgical department[11-13]. These are in the most cases the trauma settings with intracranial pressure (ICP) monitor placement and operations where evacuation of acute blood is needed from the brain in order to prevent a high rise of ICP and brain herniation. In such cases, only immediate surgical decompression can save the patient[14-16]. Mostly these emergency procedures in Slovenia are connected with acute subdural, epidural, and rare superficial intracerebral hematomas and decompressive craniectomies as well as when there are difficulties encountered with the subsequent postemergency neurosurgical care.

This communication system is especially important in neurosurgery cases as it provides complete information on the extent and location of haemorrhage, and it simplifies pre-operative planning of a neurosurgical procedure. Information that is available from images on the computer system and is important for the neurosurgeon includes the type and precise location of the intracranial pathological process (injury, bleeding, ischemia, tumour, and hydrocephalus), the extent of affected brain tissue due to oedema or adjoining ischemia, and combined injuries or pathological processes which may also affect extracranial parts, such as cranial and facial bones, as well as scalp and facial soft tissues[17,18]. Data on the optical nerve channel, eyeballs, pyramid bones, or sinuses are also extremely important, especially in the acute setting, such as head and face injuries. This information is crucial for planning surgical treatment where interdisciplinary operation is necessary, especially in patients with combined injuries. In such difficult medical emergencies, the help of maxillofacial surgeons, ophthalmologists, or otorhinolaryngologists is always welcome and leads to increased treatment quality and faster patient care[17-19].

The Telekap has been designed to provide any patient with symptoms and signs consistent with acute neurosurgical pathology with a quick expert clinical evaluation, a review of diagnostic finings, a diagnosis, decision making, emergency treatment recommendations, and postoperative advice. It is the most reasonable to perform the Telekap consultation in collaboration with the treating physicians and nurses, as they are the ones that know the patient's condition in detail.

When the patient is accepted to a general hospital, the system submits images directly to the telemedicine centre. According to the information, neurosurgeons can optimally plan the treatment, which depends on the type of pathology. Additionally, the reanimation and surgical team can suitably prepare for the surgical procedure during the transport of the patient. It is also possible to plan and organise postoperative management of such patients, often needing intensive care and prolonged hospitalisation[18-22].

Time is an important factor in brain injury, which may affect the final treatment outcome. The delays in providing adequate resuscitation and definitive care in a timely fashion and appropriate management are often detrimental for patients with brain injury[19]. Thus, the telemedicine network is indispensable for neurosurgeons for the treatment of patients with urgent surgical situations, especially for those who were transferred from remote centres where time plays an important role[22]. The Telekap can be used regardless of the hospital location, emergency department versus other unit, time of day, and proximity to the nearest neurosurgical centre. According to previous extremely positive experience, we propose that such a system should be expanded to other centres with neurological patients, and also to all neurosurgery departments in the country in order to facilitate preparation for procedures for such patients, their acute hospital treatment, and interdisciplinary collaboration. The process is still ongoing and we are expecting further expansion, modifications, and improvements of the system in the following years.

Decision making is a central function of a telestroke network. In hospitals without neurosurgical experience and neurocritical care units, the surgeon on call may perform the initial intervention. In such typical neurosurgical setting taking part in the Telekap service, medical consultations are delivered to local hospitals from specialists in the referral centre who are located at distant sites and they have unusually no connection with the patients and their treatments in the local hospitals. The referral centre is frequently an academic medical centre and provides the neurosurgical Telekap services to distant sites within its geographic region. The most numerous emergencies are those related to the high ICP for various reasons. It is essentially to release this high ICP in order to assure the best possible recovery for the patient. With the Telekap network, the operating surgeon in a distant hospital may sometime be guided with the audio-video support of a neurosurgeon. Later on, when

the decompression is achieved, the ICP rescued, and the patient stable, a transport into the tertiary centre may be organised in order to completely accomplish the surgical procedure. In case the patient requires a higher level of care following the Telestroke evaluation, a transfer to the corresponding neurosurgical canter is facilitated. When the transfer is indicated, the referral centre typically receives the patient from the local hospital and can provide continuity of care, having already observed the patient virtually. Alternatively, the neurosurgeon may be transferred to the local hospital. In our clinical practice, the latter is a frequent event, where the neurosurgeon may take over the operation in the local hospital in case of difficulties and advises during the immediate postoperative care.

OUR EXPERIENCE WITH THE TELEKAP

In the period of using the Telekap system in University Medical Centre Ljubljana, we surgically treated patients at the neurosurgery department for various pathologies. Those included cerebral vascular diseases (aneurysms, arteriovenous malformations, ischemic and haemorrhagic strokes), injuries (contusions, epidural and subdural haematomas, cranial and facial fractures, and soft tissue injuries), brain oedema of various aetiologies, and primary and secondary brain tumours where oedema or bleeding occurred and the neurological status of patients deteriorated due to a sudden increase of ICP. The larger share of patients included those with ischemic and haemorrhagic strokes where surgical intervention was required by inserting a sensor for measuring ICP, external ventricular drainage, evacuation of brain haemorrhage, or decompressive craniectomy for lowering refractory increased ICP.

Worldwide, the value of telemedicine has been confirmed clinically and scientifically. In 2009, the American Heart and American Stroke Association reported about the evidence of telemedicine significance in the stroke schemes of care and made recommendations for telestroke implementation[19,23]. The 2009 policy statement included guidelines with the recommendations that were based on class I evidence[23]. Key recommendations emphasized the value of telestroke system to support the assessment of acute stroke severity, the equivalence to that of a bedside evaluation, the review of imaging results by remotely located stroke specialists, and urgent decisions about further treatment[24-26]. In our experience, such evidence may directly mirror the neurosurgical practice. The Telekap proved to be an invaluable tool in the management and care of patients, as perceived by the treating surgeons and consulting neurosurgeons in the two Slovenian referral centres. As communicated in the current stroke guidelines, telestroke remains a standard care in hospitals that cannot provide an acute stroke team[24,27]. In recent years since implementation of the Telekap in the emergency neurology services, we have successfully broadened its applications also into neurosurgical practice. We may therefore state that in Slovenia the Telekap system has developed in the last three years into a standard and indispensable tool for communication, connection, and flow of medical information between the hospitals that cannot provide a neurosurgical team and the referral centres. It now remains a standard element of the treatment strategy for neurosurgical patients from distant locations.

The monitoring of the image transmission from primary diagnostic centre (*i.e.*, emergency or radiology centre in the local hospital) as well as the flow and quality of video, audio, and Internet connectivity should be a part of technology workflow. In order to perform the consultation, surgical and postoperative guidance soundly and to achieve good treatment results, a flawless operation of all systems involved and a god technical support are mandatory[28,29].

Considering the positive experience, we believe that it would be necessary to expand the use of Telekap also to other medical specialities in order to facilitate the flow of important medical and treatment related information. Especially, emergency medical services among the hospitals in the country may be connected, including prehospital units, interventional specialities, and traumatology, as well as paediatric units and internal medicine emergency services, in order to improve and speed up the management of urgent conditions and to improve treatment outcomes.

CONCLUSION

Progress in the treatment of intracranial pathology included the development and expansion of computer systems, networks, and applications for fast and simple informing and data transfer from the location of primary diagnostics to specialised centres. Beside technical support, which includes information communication

technology, a close cooperation of various experts is also important for the efficient operation of the entire treatment mechanism. Such a multidisciplinary team includes, in addition to neurologists and neuroradiologists, also interventional radiologists, intensive care specialists, anaesthesiologists, and neurosurgeons. It would be useful to provide such an information network, which would be intended for other specialists in medicine as well, to all hospitals and diagnostic centres in Slovenia in order to significantly improve the speed and quality of healthcare and thus improve the treatment efficacy for patients even more.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Treatment patterns of primary care physicians *vs* specialists prior to subspecialty urogynaecology referral for women suffering from pelvic floor disorders

Abigail Prentice, Ali Ahmad Bazzi, Muhammad Faisal Aslam

ORCID number: Abigail Prentice (0000-0003-1815-2347); Ali Ahmad Bazzi (0000-0003-4233-3113); Muhammad Faisal Aslam (0000-0001-7654-8441).

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Abigail Prentice, Muhammad Faisal Aslam, Department of Family Medicine, Marquette Health System, Marquette, MI 49855, United States

Ali Ahmad Bazzi, Department of Obstetrics and Gynaecology, St. John Hospital and Medical Center, MI 48236, United States

Corresponding author: Ali Ahmad Bazzi, BSc, MD, Doctor, Department of Obstetrics and Gynaecology, St. John Hospital and Medical Center, 22201 Morross Road, Detroit, MI 48236, United States. ali.bazzi3@ascension.org
Telephone: +1-313-5229762

Abstract

BACKGROUND

There are approximately 25% of women in the United States suffering from pelvic floor disorders (PFDs) and this number is predicted to rise. The potential complications and increasing healthcare costs that exist with an operation indicate the importance of conservative treatment options prior to attempting surgery. Considering the prevalence of PFDs, it is important for primary care physician and specialists (obstetricians and gynecologists) to be familiar with the initial work-up and the available conservative treatment options prior to subspecialist (urogynecologist) referral.

AIM

To assess the types of treatments that specialists attempted prior to subspecialty referral and determine the differences in referral patterns.

METHODS

This is a retrospective cohort study of 234 patients from a community teaching hospital referred to a single female pelvic medicine and reconstructive surgery (FPMRS) provider for PFD. Specialist *vs* primary care provider (PCP) referrals were compared. Number, length and treatment types were studied using descriptive statistics.

RESULTS

There were 184 referrals (78.6%) by specialists and 50 (21.4%) by PCP. Treatment (with Kegel exercises, pessary placements, and anticholinergic medications) was attempted on 51% (n = 26) of the PCP compared to 48% (n = 88) of the specialist referrals prior to FPMRS referral (P = 0.6). There was no significant difference in

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length of treatment prior to referral for PCPs *vs* specialists (14 mo *vs* 16 mo, respectively, $P = 0.88$). However, there was a significant difference in the patient's average time with the condition prior to referral (35 mo *vs* 58 mo for PCP compared to specialist referrals) ($P = 0.02$).

CONCLUSION
One half of the patients referred to FPMRS clinic received treatment prior to referral. Thus, specialists and generalists can benefit from education regarding therapies for PFD before subspecialty referral.

Key words: Pelvic floor disorders; Referral patterns; Female pelvic medicine and reconstructive surgery; Primary care provider

Core tip: The true value of this study highlights the finding that half of the patients sent for subspecialist (urogynecologic) evaluation did not receive any treatment from primary care physicians and specialists (obstetricians and gynecologists) prior to the referral. This suggests that there is a potential paucity of knowledge about non-invasive therapy options available for pelvic floor disorders. This leaves room for education about these disorders, whether during residency training or through certification examinations. This could result in decreased healthcare costs and morbidities associated with surgical procedures.

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INTRODUCTION

One in four women in the United States suffer from at least one pelvic floor disorder (PFD), which includes fecal incontinence, urinary incontinence or pelvic organ prolapse[1]. These disorders are more prevalent with increasing age and obesity; thus, will likely become even more pronounced in the future[1]. Consequently, this results in an overall increase in healthcare expenditure and potential complications that exist with an operation; especially when the lifetime risk of pelvic surgery is estimated to be between 11%-19%[2]. In 2005-2006, after adjusting for deductibles and co-payments, the average annual cost of ambulatory physician services for PFDs in the United States was $412 million and this number was expected to increase[3]. Factoring in expensive surgical procedures, this can be a huge burden on overall healthcare costs[4]. Surgical therapy, while normally minimally invasive for PFDs, is costly and has many possible side effects[1]. It is associated with increased healthcare costs, medical comorbidities, pain, and prolonged recovery time[5]. Therefore, conservative treatment options should be offered to a patient prior to attempting surgery.

Considering the prevalence of these disorders and, it is important that physicians are familiar with the initial work-up, and the available treatment options. To accomplish this, it is important to understand the types of treatment, if any, that patients receive prior to subspecialty referral. This assessment starts with the primary care providers (PCPs) and specialists. According to a previous study, PCP are familiar with overactive bladder and urinary incontinence, but less familiar with pelvic organ prolapse[6]. Obstetricians and gynaecologists see PFDs with higher frequency and may be more familiar with the treatments available for those disorders[7]. This allows them to partake in earlier interventions and this can have clinical and economic implications.

Female pelvic medicine and reconstructive surgery (FPMRS) providers specialize in PFDs, often offering additional care, which includes non-invasive treatment options and surgical interventions[4]. These non-invasive treatment modalities include dietary intervention, pelvic floor physical therapy, and medications; all which could be attempted at the primary care level[8]. This would allow for earlier intervention and

more efficient use of medical resources. A recent 2014 study found that using pelvic floor muscle training for urinary incontinence should be a recommended first line intervention[9]. With the appropriate training and minimal resources, PCPs and OBGYNs can counsel patients on this treatment to allow for earlier treatment and intervention. The purpose of this study was to assess which treatments were attempted by specialists and primary care physicians, and to determine if there was a significant difference in the treatments attempted prior to referral to a urogynecologist.

MATERIALS AND METHODS

This is a retrospective cohort study whose primary goal was to determine the types of treatments for PFDs, if any, that PCPs and OB/GYN physicians attempted prior to FPMRS referral. The secondary aim was to determine if there was a difference in referral patterns between providers. The participants were recruited from a community teaching hospital in Detroit between August 1, 2015 and August 31, 2017. The patient cases were identified by use of electronic medical record. All protected health information was de-identified. Only patients who were evaluated by the single FPMRS provider during the study period were included. The research protocol was approved by the relevant Institutional Review Board.

A total of 234 participants were included in the assessment. The inclusion criteria included female patients between the ages of 18-95 years referred to a single provider subspecialty FPMRS clinic for PFDs. These PFDs included urge, stress incontinence, and fecal incontinence as well as pelvic pain and pelvic organ prolapse during the study period. Patients under 18 and over 95 years of age were excluded. Certain patient information was abstracted, including demographics, specialty of the referring physician, type of PFD, and treatments utilized. Finally, patients that were referred to the urogynecology clinic by various specialists were compared to those referred by their primary care physicians.

Statistical analysis

Statistical analysis was performed using SPSS version 25 (manufacturer: IBM, location: Armonk, New York). A *P*-value of 0.05 or less was considered to indicate statistical significance. Descriptive statistics were generated to characterize the study population. This included the mean, standard of deviation, median and range. The number, length and types of treatments were compared. A univariate analysis was performed using Student's *t*-test to show differences between groups on continuos variables[10]. Categorical variables were described as frequency distributions. Chi-square was used to indicate associations between categorical variables. Fisher's exact test was used when assumptions for Chi-square distribution were violated.

RESULTS

The study included 234 referrals to the single FPMRS provider clinic from August 2015 through August 2017. Our population was predominately white (60.1%) compared to 35.3% African American, 1.3% Native American, 0.8% Asian, and 0.4% Hawaiian/Pacific Islander. Patient demographics are shown in Table 1.

We also looked at the referral pattern between PCPs and specialists by individual PFD category. Most patients were referred for multiple coincident disorders, with a total number of 474 disorders (234 patients referred, but many for multiple disorders). Overall, stress incontinence was the leading medical issue that was referred to the FPMRS specialty clinic. One hundred forty-three (30.2%) of the referrals were at least partially for stress incontinence. One hundred twenty-seven (26.8%) of referrals had urge incontinence as one of the reasons for referral. Forty-two (8.8%) of patients were referred for fecal incontinence, 22 (4.6%) for pelvic pain, and 140 (29.5%) were referred for pelvic organ prolapse. Referral patterns of PCPs *vs* specialists based on PFD is portrayed in Table 2.

There was no difference in the number of patients for whom treatment was attempted in either group prior to the referral. PCPs attempted treatment on 26 (51%) patients prior to urogynecology referral, compared to 88 (48%) of the patients that were referred by the specialists ($P = 0.6$).

There was also no significant difference in length of treatment prior to referral with a mean of 14 mo for primary care and 16 mo for specialist ($P = 0.88$). There was, however, a significant difference in the patient's average time with condition prior to referral. Average time with index condition was 35 mo for primary care referrals

Table 1 Patient race/ethnic background

Race	Frequency (*n*)	Percent	Valid (%)	Cumulative (%)
African American	84	35.3	35.9	35.9
American Indian/Alaskan Native	4	1.7	1.7	37.6
Asian	2	0.8	0.9	38.5
Hawaiian/Pacific Islander	1	0.4	0.4	38.9
White	143	60.1	61.1	100
Total	234	98.3	100	

compared to 58 mo for specialist referrals ($P = 0.02$).

The most common treatments attempted prior to FPMRS referral were Kegel exercises, pessary placement, and anticholinergic medications. Kegels were attempted by 27 (15.7%) of the patients referred by obstetricians, and in 6 (12%) of the patients referred by primary care doctors. Pessaries were attempted in 27 (15.7%) of the obstetric referrals as well, and 2 (4%) of the primary care referrals. Anticholinergics were attempted by 23 (13.4%) of the patients referred by obstetricians and 4 (8%) of the primary care referrals. Across the board, a similar number of women with different PFDs had treatments attempted at all prior to the referral. This includes 72 out of 143 (50.3%) women with stress incontinence, 68 out of 127 (53.5%) with urge incontinence, 65 out of 140 (46.4%) with pelvic organ prolapse and 15 out of 22 (68.2%) with pelvic pain. Overall, this shows that approximately half of the patients with each form of PFD received treatment prior to referral.

DISCUSSION

The results of this cohort study show that there was no significant difference in the number of treatments attempted by PCPs versus specialists. We theorized that obstetricians would be more familiar and better prepared to treat PFDs given their background with gynaecologic problems. For this small subset of providers in our study, this was not the case. However, it should be noted that 121 more patients were sent by obstetricians than primary care physicians, which may indicate that they are more comfortable and familiar with the role of urogynecologists.

Even though stress incontinence was the most often referred PFD, comprising 30.2% of the referrals, it was not the most often condition treated. By percentage treated, pelvic pain received treatment most often prior to referral (68.2%) versus stress incontinence which only received treatment 50.2% of the time. Stress urinary incontinence is estimated to affect between 4% and 35% of adult women[9,11]. Generally speaking, urinary incontinence is a very common problem affecting women, with more than half of the population over 20 years of age affected[11,12]. The prevalence of incontinence in females suggests it is a disorder that should be taken seriously, and the various treatment modalities should be better understood.

Our study showed no significant difference in the length of treatment prior to referral, but it did show a difference in the time with the condition prior to referral. Often the referred patients were impacted by the disorder for at least 1 year prior to referral. With only half of the patients receiving any documented treatment for their condition, this implies that many of the referred patients received no intervention for at least a year. This can be important for its implications, which includes healthcare costs, patient satisfaction, surgical complications, length of time prior to treatment. Our assessment included a limited time span of new patients referred to the clinic. Furthermore, we only studied patients referred to a single FPMRS specialist. A larger multicenter study with multi-providers may be useful to fully explore the treatments attempted prior to referral by multiple FPMRS specialists[13]. This study can serve as a branching point for further studies that can further explore this avenue. Furthermore, this study can be used to encourage further utilization of non-invasive therapies for PFDs by primary care and OB/GYN physicians. Another limitation is the retrospective nature of this study[14]. Future studies can be designed to follow the referral patterns over a longer period of time.

The true value of this study highlights the finding that half of the patients sent for urogynecologic evaluation did not receive any treatment prior to the referral. This suggests that there is a potential paucity of knowledge about non-invasive therapy options available for PFDs. This leaves room for education about these disorders, whether during residency training or through certification examinations. There is a

Table 2 Referral patterns categorized by pelvic floor disorder

	Primary care provider (*n*)	Specialist (*n*)	Total (*n*)
Stress Inc	32	111	143
Urge Inc	28	99	127
Fecal Inc	10	32	42
Pelvic pain	6	16	22
Prolapse	29	111	140

Inc: Incontinence.

plethora of reasons as to why physicians may not attempt treatments prior to referral. This could be due to lack of confidence with recommending and overseeing these treatments, lack of resources/time, or simply that they would prefer the disorder be managed by a specialist in PFDs given the possibility for surgical intervention. Future studies can be aimed at understanding this question by surveying a large pool of physicians amongst various fields of medicine.

ARTICLE HIGHLIGHTS

Research background

Approximately one half of the patients referred to a Female Pelvic Medicine and Reconstructive Surgeon for pelvic floor disorders (PFDs) did not receive any non-surgical treatment prior to referral. Rather than being managed conservatively, patients end up undergoing surgical procedures, which are associated with their own risks. Through education about these disorders, whether during residency training or through certification examinations, this may results in decreased healthcare costs and morbidities associated with surgical procedures.

Research motivation

The main topic of these articles revolves around female PFDs and conservative management prior to subspecialist referral. The key problem to be solved is determining the extent to which treatments are attempt prior to subspecialist referral and if education about PFDs would be beneficial. This could reduce the total number of surgical procedures performed, which would decrease the medical comorbidities associated with surgery. Furthermore, this would result in fewer healthcare costs associated with subspecialty referral and surgical procedures.

Research objectives

The objective of this study was to assess the types of treatments that primary care physicians and obstetricians and gynecologists (specialists) attempted prior to subspecialty female pelvic medicine and reconstructive surgery (subspecialists) referral. The secondary goal assessed the differences in referral patterns. Future studies can be aimed at understanding this question by surveying a large pool of physicians amongst various fields of medicine.

Research methods

A retrospective cohort study of 234 patients was included in the assessment after the inclusion and exclusion criteria were met. The PFDs included urge, stress incontinence, and fecal incontinence as well as pelvic pain and pelvic organ prolapse during the study period. Certain patient information was abstracted, including demographics, specialty of the referring physician, type of PFD, and treatments utilized. Finally, patients that were referred to the urogynecology clinic by various specialists were compared to those referred by their primary care physicians. Descriptive statistics were generated to characterize the study population. This included the mean, standard of deviation, median and range. The number, length and types of treatments were compared.

Research results

There were 78.6% of referral by specialists and 21.4% by primary care provider (PCP). Treatment (with Kegel exercises, pessary placements, and anticholinergic medications) was attempted on 51% (n = 26) of the PCP compared to 48% of the OB/GYN referrals prior to FPMRS referral (P = 0.6). There was no significant difference in length of treatment prior to referral for PCPs *vs* specialists (14 mo *vs* 16 mo, respectively, P = 0.88). However, there was a significant difference in the patient's average time with the condition prior to referral (35 mo *vs* 58 mo for PCP compared to specialist referrals) (P = 0.02).

Research conclusions

Our results showed that there was no significant difference in the number of treatments attempted by PCPs versus specialists. We theorized that obstetricians would be more familiar and better prepared to treat PFDs given their background with gynaecologic problems. For this small subset of providers in our study, this was not the case. However, it should be noted that 121 more patients were sent by obstetricians than primary care physicians, which may indicate

that they are more comfortable and familiar with the role of urogynecologists. Even though stress incontinence was the most often referred PFD, comprising 30.2% of the referrals, it was not the most often condition treated. By percentage treated, pelvic pain received treatment most often prior to referral (68.2%) versus stress incontinence which only received treatment 50.2% of the time. Our study showed no significant difference in the length of treatment prior to referral, but it did show a difference in the time with the condition prior to referral. Often the referred patients were impacted by the disorder for at least 1 year prior to referral. The true value of this study highlights the finding that half of the patients sent for urogynecologic evaluation did not receive any treatment prior to the referral. There is a plethora of reasons as to why physicians may not attempt treatments prior to referral. This could be due to lack of confidence with recommending and overseeing these treatments, lack of resources/time, or simply that they would prefer the disorder be managed by a specialist in PFDs given the possibility for surgical intervention.

Research perspectives

Our study suggests that there is a potential paucity of knowledge about non-invasive therapy options available for PFDs. Future studies can be aimed at understanding this question by surveying a large pool of physicians amongst various fields of medicine. This study could be done retrospectively or prospectively.

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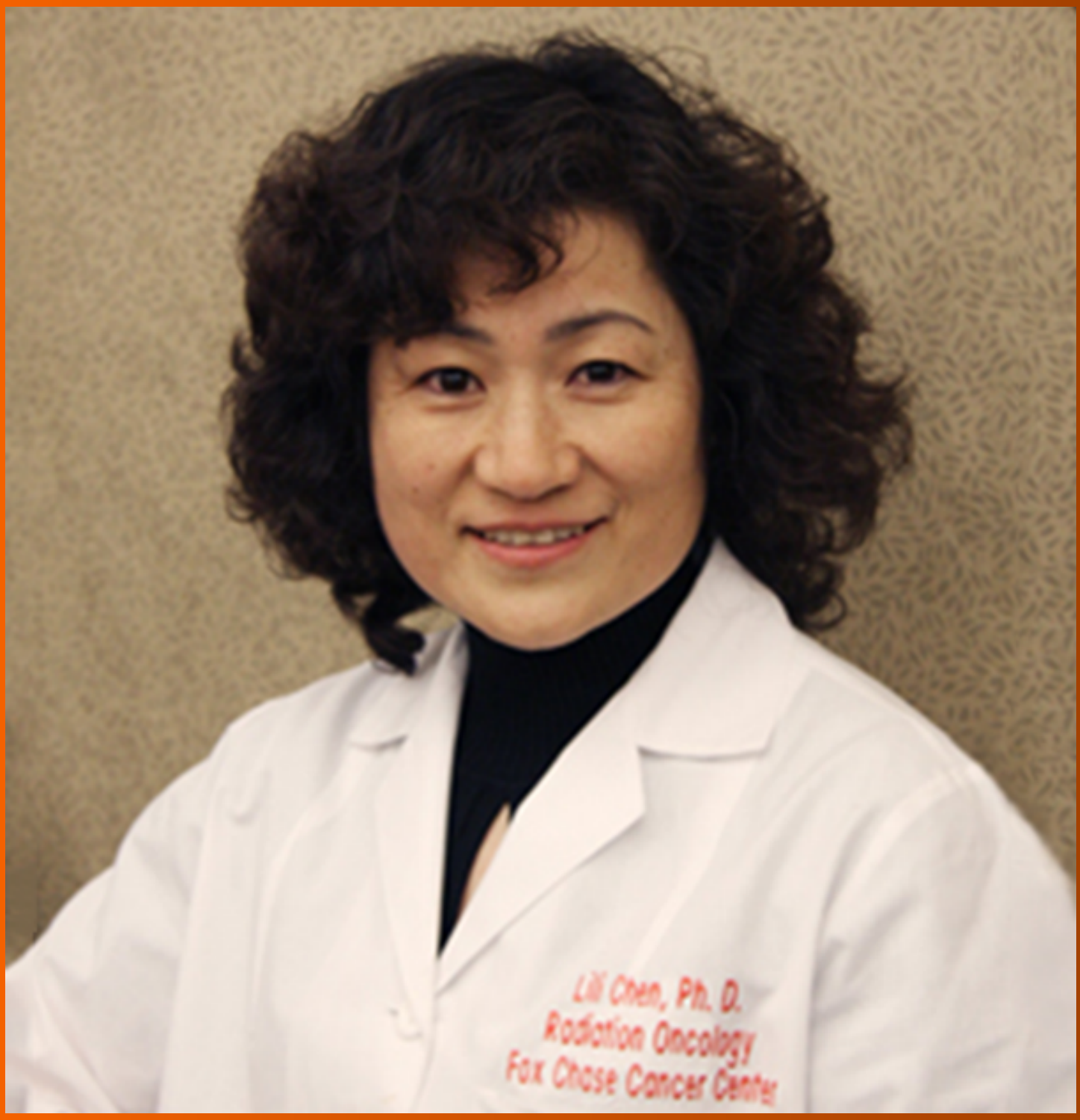

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Editorial Board Member of *World Journal of Methodology*, Lili Chen, PhD, Associate Professor, Department of Radiation Oncology, Fox Chase Cancer Center, Philadelphia, PA 19111, United States

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ORIGINAL ARTICLE

Basic Study

DNA extraction from archived hematoxylin and eosin-stained tissue slides for downstream molecular analysis

Pushkal Sinduvadi Ramesh, Venkatesh Madegowda, Suprith Kumar, Shailashree Narasimha, Devananda Devegowda, Parichay S R, Nandini Nandish Manoli, Devananda Devegowda

ORCID number: Pushkal Sinduvadi Ramesh (0000-0002-9238-7032); Venkatesh Madegowda (0000-0003-1916-5676); Suprith Kumar (0000-0001-5225-5273); Shailashree Narasimha (0000-0002-4227-3619); Parichay S R (0000-0002-5457-3352); Nandini Nandish Manoli (0000-0002-6207-7797); Devananda Devegowda (0000-0003-0577-4622).

Author contributions: Ramesh PS, S R P, and Devegowda D conceptualized the study; Ramesh PS, Madegowda V, Kumar S, and Reddy S performed all of the experiments; Devegowda D and Manoli NN aided in collecting samples and providing resources; Ramesh PS and S R P wrote the original draft; Devegowda D and Ramesh PS wrote the final draft after editing.

Institutional review board statement: The Institutional Ethical Committee of JSS Medical College and Hospital, JSS Academy of Higher Education and Research, Mysuru approved the study. All research was conducted in accordance with the approved submission.

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Pushkal Sinduvadi Ramesh, Venkatesh Madegowda, Suprith Kumar, Shailashree Narasimha, Devananda Devegowda, Center of Excellence in Molecular Biology and Regenerative Medicine, Department of Biochemistry, JSS Medical College, JSS Academy of Higher Education and Research, Mysuru 570015, India

Parichay S R, CIPHER Healthcare Pvt Ltd., Hyderabad 500034, India

Nandini Nandish Manoli, Department of Pathology, JSS Medical College, JSS Academy of Higher Education and Research, Mysuru 570015, India

Corresponding author: Devananda Devegowda, MSc, PhD, Assistant Professor, Center of Excellence in Molecular Biology and Regenerative Medicine, Department of Biochemistry, JSS Medical College, JSS Academy of Higher Education and Research, Mysuru 570015, India.
devanandd@jssuni.edu.in
Telephone: +91-961-1130458
Fax: +91-821-2548658

Abstract

BACKGROUND

Histopathologically stained archived tissue slides are stored in hospital archives for years to decades. They are the largest available source of biological materials and are a potentially useful resource that can be used for retrospective epidemiological studies. DNA recovered from the slides can be used for several downstream molecular processes including polymerase chain reaction, single nucleotide polymorphism analysis, and whole genome sequencing. The DNA from these slides can be utilized to compare gene signatures of normal and diseased tissues. However, extraction of high-quality DNA from archived stained hematoxylin and eosin (H&E) slides remains challenging.

AIM

To standardize a new protocol for extracting DNA from archived H&E-stained tissue slides for further molecular assays.

METHODS

A total of 100 archived H&E-stained cancer slides were subjected to a total of five methods of DNA extraction. Methods were varied in the deparaffinization step, tissue rehydration, duration of lysis, and presence or absence of proteinase K. The extracted DNA was quantified using a NanoDrop spectrophometer and the quality was analyzed by agarose gel electrophoresis. Then each sample was

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subjected to polymerase chain reaction (PCR) to amplify the internal control gene *GAPDH*, thereby confirming the DNA intactness, which could be further utilized for other downstream applications.

RESULTS
Of the five different methods tested, the third method wherein xylene was used for tissue deparaffinization followed by 72 h of digestion and without proteinase K inactivation yielded the highest amount of DNA with good purity. The yield was significantly higher when compared to other methods. In addition, 90% of the extracted DNA showed amplifiable *GAPDH* gene.

CONCLUSION
Here we present a step-by-step, cost-effective, and reproducible protocol for the extraction of PCR-friendly DNA from archived H&E-stained cancer tissue slides that can be used for further downstream molecular applications.

Key words: DNA extraction; Hematoxylin and eosin tissue slides; Molecular analysis; Polymerase chain reaction; Deparaffinization

Core tip: In our study, we discussed a step-by-step procedure and results for the extraction of PCR-friendly DNA from archived hematoxylin and eosin-stained tissue slides. Such extracted DNA has the potential to be used for further molecular analyses such as mutation studies, whole genome sequencing, and even the identification of differences in gene signatures between diseased and normal states. Our protocol is simple, cost-effective, and can be performed in a basic molecular biology lab with most common reagents.

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INTRODUCTION

Molecular diagnostics involves the analysis of DNA, RNA, and proteins or metabolites at the molecular level in order to detect genotypes, mutations, or biochemical changes in the body. The main objective is to test for specific states of health or to see if the disease exists. Analysis of DNA reveals a multitude of cellular processes and hence it is essential to look for alternate sources of DNA. Blood and saliva are among the most common sources of DNA[1], but they are not always available. Extraction of DNA from teeth, bone, and hair follicles has been followed routinely[2,3]. Even with all of these plethora of specimens, it warrants testing for new specimens that can be harnessed to obtain DNA. One such potential resource is archived hematoxylin and eosin (H&E)-stained slides.

Histopathologically stained archived tissue slides are stored in hospital archives for years to decades. They are the largest available source of biological materials [other than formalin fixed paraffin embedded (FFPE) tissue blocks] and are potentially useful resource that can be used for retrospective epidemiological studies. The increasing interest in the genetic basis of diseases has increased the value of available clinical samples, including archived tissue slides. Similar to the DNA recovered from FFPE blocks, DNA from slides can also be used for several downstream molecular processes such as polymerase chain reaction (PCR), real time PCR, single nucleotide polymorphism (SNP), and whole genome sequencing[4]. This can aid researchers in unlocking new genetic information that can answer questions related to the molecular basis of cancer and other diseases and also gaining an understanding of the response to specific therapeutic modalities. H&E-stained slides can also be used to acquire information such as the presence or absence of pathogens that would have been present at the time of sectioning. The high-quality DNA from these slides can be used

to compare gene signatures of normal and diseased tissues and also to detect mutations. Many studies have tried to optimize the extraction process, but a standard, reproducible, cost-effective protocol does not exist.

Challenges in the extraction of DNA from archived specimens

Even though molecular biology tools have seen a major evolutionary upgrade in recent years, extraction of DNA from archived tissue slides is problematic because formalin fixation induces protein-nucleic acid crosslinking. As a result of this cross linkage, it is difficult to separate DNA from histones and to obtain pure nucleic acids. These crosslinks are irreversible and not easily undone[5]. The incomplete reversal of crosslinks causes a negative impact on the quantity and quality of the recovered DNA. The fixation of tissues also leads to fragmentation of the nucleic acids that will affect the yield.

Removal of coverslip and deparaffinization is the biggest hurdle in the entire process. To extract DNA from the H&E-stained slide, first the coverslip must be removed and the tissue must be separated from the paraffin and then the tissue must be subjected to rehydration to allow for effective digestion[6]. Deparaffinization is usually performed using solvents such as xylene to dissolve the paraffin. However, researchers have found that using xylene can cause fragmentation of DNA and thus the loss of valuable specimens. A recent study suggested the use of mineral oil to melt away the paraffin[7]. After removal of the coverslip, it is essential to trim off the excess wax surrounding the tissue. However, some of the specimens are brittle due to age or poor storage conditions. This makes paraffin removal a little more difficult and can cause lower yields[8].

Molecular studies are highly dependent on the quality and quantity of the extracted nucleic acid, and hence it is quintessential to develop an extraction procedure that is suitable for downstream molecular processes. The goal of this study was to conduct a proof-of-principle study, as a new protocol for DNA extraction from H&E cancer slides based on the existing phenol chloroform isoamyl alcohol (PCI) method, which could be further used in molecular analysis.

MATERIALS AND METHODS

A successful DNA isolation requires four essential steps: effective disruption of cells or tissue; denaturation of nucleoprotein complexes; inactivation of nucleases; and maintaining the quality and integrity of the isolated DNA, which will directly affect the results of successful scientific research[9].

The three successfully employed DNA extraction techniques are: A salting out method using NaCl, a column-based method which the majority of commercially available kits use, and the PCI method. The PCI extraction method is by far the most commonly used DNA extraction method in the literature. Although newer procedures have been developed in recent years, the PCI method continues to be used routinely, possibly because it offers an economical advantage over commercially available kits[10]. For this reason, the PCI method was employed in our study but with a few modifications from the traditional one.

Tissue selection and processing

A total of 100 histopathologically confirmed H&E-stained slides were handpicked by a pathologist from JSS Medical College Hospital (Mysore, India). The age of the slides ranged from 1 to 2 years old. All slides were prepared from FFPE tissues that had been fixed in 10% buffered neutral formalin processed and embedded manually. The slides were carefully examined by a histopathologist and were confirmed cancer cases.

Other materials/reagents used in the study

Xylene (Product No. 35415; Thermo Fisher Scientific, Waltham, MA, United States); Scalpel/razor blade; ethanol (Cat No. 58051; Jebsen & Jessen GmbH & Co., Hamburg, Germany); Tris HCl (Cat No. 34969; Sisco Research Laboratories Pvt. Ltd., New Delhi, India); EDTA dipotassium salt extrapure (Cat No. 62196; Sisco Research Laboratories Pvt. Ltd.); NaCl (Cat No. 15915; Qualigens Fine Chemicals, Mumbai, India); sodium lauryl sulphate (Cat No. 32096; Sisco Research Laboratories Pvt. Ltd.); proteinase K (Cat No. RM2957; HiMedia Laboratories, LLC, Mumbai, India); phenol molecular biology grade (Cat No. 17286; Sisco Research Laboratories Pvt. Ltd.); chloroform molecular biology grade (Cat No. 96764; Sisco Research Laboratories Pvt. Ltd.); isoamyl alcohol extrapure (Cat No. 69931; Sisco Research Laboratories Pvt. Ltd.); sodium acetate anhydrous extrapure (Cat No. 40104 K05; SDFCL, Bengaluru, India); nuclease-free water (Cat No. ML024; HiMedia Laboratories); mineral oil molecular

biology grade (Cat No. MB161; HiMedia Laboratories); agarose low EEO (Cat No. MB002; HiMedia Laboratories); Taq polymerase (Cat No. MBT060A; HiMedia Laboratories); deoxynucleotriphosphates (Cat No. MBT078; HiMedia Laboratories); PCR tubes (Cat No. AB0620; Abgene, Portsmouth, NH, United States); microcentrifuge tubes (Cat No. 509-GRD-Q; QSP by Thermo Fisher Scientific).

Coverslip removal

Removal of coverslip is one of the biggest hurdles in the entire process. Generally for any further analysis on the slides, the coverslip has to be removed by immersing the slide in xylene for 4-6 d or until the coverslip falls off spontaneously. Recently, a study by Zhou *et al*[11] showed that coverslips could be rapidly removed by freezing in liquid nitrogen. However, not all laboratories have access to liquid nitrogen and it possesses a threat to the handler. So we came up with a less harmful idea, namely of using a -80°C freezer and a sterile razor blade. Slides used for the study were photographed and marked using a slide marker. The slides were placed inside a -80°C freezer for 2 h, after which a sterile sharp razor blade was slid under the edges of the coverslip to remove it (Note: Before placing the slides inside the freezer, we made sure the DPX merging the borders of the coverslip was carefully cut out). Sometimes, the coverslip tended to adhere more strongly to the slide; in such cases, the slide was immersed in xylene for 20 min and the coverslip fell off automatically or a spatula could be used to gently push it off. In another method, we also used the liquid nitrogen procedure described by Zhou *et al*[11], but the slides froze too much making it prone to breakage. Also, we experienced little hardship in using liquid nitrogen as we had a large tank that was not easily accessible to this procedure.

Tissue scraping

Once the coverslip was removed, the next step was to scrape out the tissue from the slide. It was important to remove the paraffin surrounding the tissue by cutting it out using a sharp sterile blade. Once the tissue was clear of the any residual paraffin, the tissue sections were scraped off by gently, pushing the blade against the slide and moving it from end to end. The scraped tissue was next transferred into a capped tube for further processing (Note: The size of the scraped tissues was not determined as the size varied greatly with each sample. All slides were photographed before processing for future reference).

Deparaffinization

The H&E-stained slides were prepared from FFPE tissue blocks. So when the sections were taken for the slides, the paraffin residues surrounding the tissue were also fixed to the slide. This paraffin can create problem with the DNA quality; hence it was essential to remove any residual paraffin or even the fixative used. After the tissues from the slides were scraped off, they were transferred into a capped eppendorf tube (2 mL) containing 800 μL 100% pre-warmed xylene (Thermo Fisher Scientific). The tubes were vortexed briefly for 20 s and centrifuged at maximum speed (13500 *g*) for 5 min. This step ensured that the residual fixative accumulated as supernatant that could be pipetted (Note: To confirm the complete removal of any residual material, the pellet was gently touched with a micropipette tip. If the tissue was free of residue, it would feel soft; otherwise, the xylene wash step was repeated again).

Destaining and ethanol rehydration

Treatment with solvents such as xylene eventually dehydrates the tissues. So the next step we followed was rehydration of the tissues using a series of different concentrations of ethanol. Ethanol wash in decreasing concentration ensures that the tissue is rehydrated slowly and also removes the stains added during tissue fixation on the slide. A study by Morikawa *et al*[12] showed that H&E staining does not interfere with DNA testing, so we did not perform any further steps to remove the stain.

The xylene-treated tissues were washed with 800 μL molecular grade ethanol of 100%, 75%, and 50% sequentially. In each step after addition of ethanol, the tubes were vortexed briefly and centrifuged for about 5 min at maximum speed (13500 *g*). At the end of centrifugation, the supernatant was completely removed. This step was employed as per Pikor *et al*[13] (Note: After each wash, the supernatant had a slight color. The color intensity decreased with each wash indicating removal of the stain). After the last ethanol wash, the pellet was air-dried for about 5 min. This step is essential as any ethanol residue can interfere with the composition of the lysis buffer.

Since our study evaluated new ways for DNA extraction, we looked into mineral oil as a potential candidate to carry out the deparaffinization. Even though the yield was good by this method, the purity was not up to the mark. Briefly, after coverslip removal, 250-500 μL molecular grade mineral oil was placed on the tissue surface on

the slide. Then the slide was placed on a thermoblock heater at 90°C for 20 min. After the prescribed duration of incubation, mineral oil was removed and the deparaffinized tissues were rehydrated in different grades of ethanol.

To examine whether this step of ethanol rehydration was necessary, we tested some slides without any ethanol wash and directly proceeded to lysis after xylene treatment. But this modification did not prove fruitful as a very low yield of DNA was obtained that was not at all amplifiable by PCR.

Tissue digestion

Tissue pellets obtained from the previous step, *i.e*. with or without ethanol rehydration, was digested with 500 μL lysis buffer (pH 7.8) and 5 μL proteinase K (200 μg/mL) incubated at 56°C in a shaker incubator for 6 h. The composition of the lysis buffer was 0.5 mol/L Tris HCl, 10 mmol/L EDTA, 100 mmol/L NaCl and 2% SDS. The tubes were agitated continuously at 25 g to ensure proper digestion. The following effects were examined by modifying the protocol as per our needs: effect of incubation time and effect of inactivating proteinase K.

To examine the effects of incubation time, the tubes with tissue pellet, lysis buffer, and proteinase K were incubated for different durations of time. The digestion was carried out for 6h, 12 h, overnight (24 h), 48 h, and 72 h. Tissues of similar sizes were used and the tubes were agitated all along and the temperature was maintained at 56°C (optimum temperature for proteinase K).

The function of proteinase K is to digest proteins and any harmful enzymes such as nucleases. But proteinase K itself being an enzyme may or may not hamper the quality and quantity of the extracted DNA. There is no evidence of proteinase K harming the quality or the quantity of the DNA. So in order to test this, we inactivated proteinase K after the complete incubation period in some of our samples. After the respective incubation period, the tubes were kept at 90°C in a block heater for about 20 min.

Nucleic acid purification

We employed the most common, cost-effective method of PCI for DNA purification. Using buffer saturated PCI mixture (24:24:1) and high-speed centrifugation (18500 *g* for 5 min) a biphasic phase was generated. DNA and RNA remained in the upper aqueous phase whereas proteins, lipids, and polysaccharides were sequestered in the interphase and organic phase. The aqueous phase was collected and subjected to further purification using only the chloroform-isoamyl alcohol mixture. This ensured removal of any phenol residues, which would affect the DNA integrity. After the high-speed centrifugation, the aqueous layer was collected again and was subjected to precipitation.

DNA precipitation

The aqueous layer obtained from the second phase separation was further subjected to precipitation. Ethanol precipitation is the most commonly used technique for concentrating and de-salting nucleic acid preparations in aqueous solution[14]. The basis of this technique is adding a salt and ethanol to the aqueous solution forces the precipitation of nucleic acids out of the solution. After precipitation, the DNA can be separated from the rest of the solution by centrifugation. The role of the salt is to neutralize the charges on the sugar phosphate backbone. A commonly used salt is sodium acetate. So in this protocol we used a 1/10 volume of sodium acetate-ethanol mixture to precipitate the DNA. After the addition of sodium acetate and ethanol mixture, the tubes were placed in a -20°C freezer for 30 min as the reduced temperature will aid in faster precipitation. The precipitated DNA was recovered by centrifugation at 13500 *g* for 10 min. The ethanol in the supernatant was discarded and the pellet was washed with 70% ethanol. Washing with ethanol made sure that none of the salt contaminants thrived. Again the tubes were subjected to ice-cold centrifuging to pellet the DNA. Ethanol was removed without disturbing the pellet and the tubes were air-dried. The removal of ethanol completely was essential as any leftovers could inhibit further PCR application. Once the tubes were completely free of ethanol, the pellets were re-suspended in nuclease-free water. The amount of nuclease free water added was directly proportional to the size of the pellet and ranged from 30 to 80 μL.

Assessing the quantity and purity of the recovered DNA

During several different stages of a molecular biology experiment, it is important to obtain a quick and accurate reading of DNA concentration and yield. The quantity and quality of genomic DNA isolated from these methods were compared using a NanoDrop spectrophotometer (DeNovix Inc., Wilmington, DE, United States). The purity of the extracted DNA was assessed as the ratio of absorbance at 260 nm and

280 nm (A260/280). The workflow of extraction of DNA from archived H&E-stained cancer tissue slides is summarized below (Figure 1).

Assessing the quality of the recovered DNA by agarose gel electrophoresis

The quality of the extracted DNA was assessed by running gel electrophoresis on a 1% agarose stained with ethidium bromide. Observation of a non-streaky band confirmed the presence of intact DNA.

Evaluating the integrity of the recovered DNA by PCR

It was essential to make sure that the extracted nucleic acid was intact before processing for downstream molecular assays. One of the goals of this study was to standardize a protocol to extract PCR-friendly DNA from the archived H&E-stained slides. So we subjected the recovered DNA for the amplification of housekeeping gene such as *GAPDH*. All of the DNA samples extracted from the slides were subjected to PCR, and only the ones confirmed to be intact were considered for the results.

The PCR was set up using commercially synthesized oligonucleotides for the *GAPDH* gene. A brief master mix was prepared containing 2.5 U Taq polymerase (Himedia), 2 μmol/L dNTPs, 10× buffer with 25 mmol/L $MgCl_2$ (working = 1× buffer), and 0.4 μmol/L of each of forward and reverse primers. DNA concentration ranging from 50 to 100 ng/reaction was added and the total reaction was made up to 30 μL using PCR-grade water. Amplification was performed in the automated Thermal cycler (Mastercycler gradient, eppendorf) at 95°C for 5 min, followed by 32 cycles at 94°C for 1 min, 58°C for 45 s, 72°C for 1 min and a final extension at 72°C for 2 min. DNA from a known cell line was used as a positive control and to check the adequacy of the program. PCR-grade water was used as a negative control. The primer sequences used were as follows: Forward 5′- GAA ATC CCA TCA CCA TCT TCC AGG-3′; reverse 5′- GAGCCCCAGCCTTCTCCATG-3′. The amplified PCR products were run on a 2% agarose gel stained with ethidium bromide and the gel image was captured on a gel documentation system.

Statistical analysis

All data were put together in GraphPad Prism 5.0 and the Student's *t*-test was performed to assess significance among various methods of DNA extraction.

RESULTS

Quantity and quality of recovered DNA from H&E-stained cancer tissues

Our overall strategy was to establish a new protocol to extract DNA from the archived H&E-stained slides. We compared the DNA quantity and purity (using NanoDrop in ng/μL) and quality (using agarose gel electrophoresis) that resulted from different methods of extraction from the slides having H&E-stained tissues. The results of the present study revealed that DNA from H&E slide tissues varied depending on the extraction method but were not degraded (Figure 2). Since our initial standardization showed that only longer duration of digestion improved the DNA yield, we used only a 24 and 72 h digestion protocol and then continued with the PCI extraction method throughout our experiment. Also, we observed that inactivation of proteinase K after digestion did not have much effect on the yield; hence we continued with the PCI extraction method without proteinase K inactivation.

Our data indicated that method 3 with xylene deparaffinization and 72 h digestion showed the highest yield of DNA and was also the best in terms of purity. The samples that were deparaffinized using pre-warmed xylene (about 50°C) produced greater DNA yields than concentrations of nucleic acids produced from the samples that were deparaffinized with mineral oil (molecular grade). The samples deparaffinized with mineral oil also yielded good amount of amplifiable DNA but the purity of the recovered DNA was not up to the mark. It also appears that digestion with proteinase K for duration longer than overnight improved the efficiency of DNA extraction from H&E-stained cancer tissues, and when the tissues were large, overnight digestion seemed to provide successful results (Table 1 and Figure 3).

The highest mean of DNA recovered and the best purity was observed in methods 3 and 4. So we performed the unpaired *t*-test to analyze if there was any significance between the results obtained. When we compared the results between the groups, groups 3 and 4 showed significance among all groups (Table 2).

DNA recovered from all groups was subjected to PCR to amplify the *GAPDH* gene, which was considered to be most stably expressed in almost all tissues and cells. More than 80% of our samples clearly demonstrated amplification of a 238 bp product of *GAPDH* gene, justifying the intactness of the DNA extracted (Figure 4).

Figure 1 Workflow for extraction of DNA from archived H&E-stained cancer tissue slides. A: Slides selected for the extraction of DNA; B: Coverslip removal and scraping of the tissues; C: Xylene treatment for deparaffinization; D: Ethanol rehydration and stain removal; E: Tissue digestion; F: Nucleic acid purification by phase separation followed by DNA precipitation; G: Quantification of DNA using Nanodrop; H: Assessment of quality of the extracted DNA.

DISCUSSION

This study was performed to standardize a cost-effective, reproducible protocol for DNA extraction from archived H&E-stained cancer slides and to show that they can be used for downstream molecular analysis. The results demonstrated the efficient amplification of DNA recovered from the archived H&E-stained cancer tissues. The noteworthy point in our protocol is that the samples that were deparaffinized with pre-warmed xylene, digested for up to 72 h, and extracted with the phenol-chloroform method produced the highest DNA yield (Table 1). Although, there are many studies evaluating the potential use of H&E slides for DNA extraction, none of them have assessed if they can be used for downstream molecular processes. Notably, no previous study has standardized a protocol with simplified coverslip removal and deparaffinization. Our data support the idea that archived cancer slides can be harnessed as a potential source for DNA extraction and routine molecular analysis.

Previous studies have evaluated potential extraction of DNA from archived FFPE tissue samples and shown that nucleic acids can be recovered and can be used for PCR and other downstream applications. In the recent past, many commercial kits have also been made available for the extraction of DNA/RNA/protein from the FFPE samples[15-18]. Historically, FFPE specimens have not been considered an ideal source for molecular analysis as nucleic acids may be heavily modified by crosslinking with proteins. However, the discovery of protease digestion releases

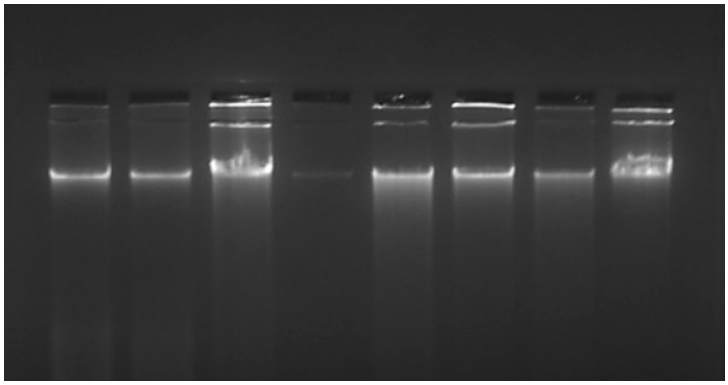

Figure 2 Representative image of DNA extracted from H&E-stained slides on a 1% agarose gel stained with ethidium bromide. The DNA extracted from archived H&E-stained slides were assessed for its quality by agarose gel electrophoresis. Only intact DNA as seen in the figure was considered fit for further analysis. H&E: Hematoxylin and eosin.

fragmented nucleic acids, making them suitable for downstream molecular analysis[19]. However, lack of standard protocol and high cost has always limited the use of FFPE specimens. H&E-stained slides, which are made of FFPE blocks, can also serve the same purpose and there are only a handful of studies that have actually looked into it.

As per the goal of our study, we evaluated five different methods of DNA extraction by modifying the coverslip removal, deparaffinization, and digestion steps. High-quality DNA was obtained with a mean yield of 20 ng/μL and more. A study by Sengüven *et al*[10] analyzed various methods of DNA extraction from archived FFPE specimens, and reported that proper deparaffinization and digestion leads to higher DNA yields. Another study by Snow *et al*[4] demonstrated the recovery of good yields of DNA from H&E-stained slides using an Qiagen column-based method and Pinpoint slide DNA isolation system with slight modifications. They also used the DNA for microsatellite instability allelic discrimination and variant detection using Sanger sequencing. Both methods described above used commercial kits and again the higher cost got in the way of frequent usage.

Xylene is the most conventional deparaffinization agent used worldwide, but xylene treatment takes time and requires the specimen to be dipped for at least 3-4 d[20]. Several investigators have successfully extracted high-quality DNA using xylene for many years. We optimized the deparaffinization step using pre-heated xylene and obtained significantly improved DNA yields. Shi *et al*[21] suggested that pre-heating FFPE specimens at higher temperatures in 0.1 mol/L NaOH solution highly increased the efficiency of DNA extraction. In order to avoid toxic chemicals such as xylene, Lin *et al*[22] demonstrated deparaffinization using mineral oil and then combining with DNA isolation protocol from a commercially available kit. They obtained high-quality DNA, which they tested in a genotyping experiment with 14 microsatellite markers. Contradictorily, in our study when we used mineral oil for deparaffinization, we obtained good DNA yields but the DNA was mostly impure. In addition, some of the samples could not be amplified even with quantitative PCR. That raised a few questions regarding the usage of mineral oil for deparaffinization and to what extent it can undo cross-linkage.

In conclusion, virtually every tissue removed from the body is fixed, paraffin-embedded, and then stored for years to decades. Given the established importance of DNA in molecular biology and its central role in determining fundamental operation of cellular processes, it is essential to look for alternate sources of DNA. Histopathologically stained archived tissue slides are stored in hospital archives for years to decades. They are the largest available source of biological materials and are a potentially useful resource, which can be used for retrospective epidemiological studies. The DNA extracted from fixed tissues can be used for diagnostic applications, such as the detection of mutations and viruses, when the need arises instead of requiring the collection of fresh tissue in anticipation of the need. One mundane but clinically important example is the identification of mislabeled specimens. However, the use of such samples for DNA analysis is limited due to chemical modification by formaldehyde and fragmentation of DNA during tissue processing and storage. Here, we discussed the results from our study for the extraction of PCR-friendly DNA from archived histopathologically stained tissue slides. Hopefully, this simple, cost-effective, and non-laborious protocol can facilitate the molecular analysis of a large number of archived specimens in retrospective studies. Also, similar kind of

Table 1 Yield and purity of the recovered DNA from various methods

Method	Sample size, *n*	Tissue deparaffinization	Digestion period	Proteinase K inactivation	DNA yield range in ng/μL	DNA purity range, A260/280
1	20	Xylene	24 h	No	7.65-45.23	1.42-2.01
2	20	Xylene	24 h	Yes	7.53-33.7	1.34-1.94
3	20	Xylene	72 h	No	23.68-208.31	1.68-1.89
4	20	Xylene	72 h	Yes	13-149.41	1.54-1.93
5	20	Mineral oil	72 h	No	11.74-86.74	1.41-1.74

methodology can be applied for tissues fixed with other fixatives and stained with dyes other than H&E, which may open up the field for future investigations.

Table 2 Statistical comparison of the DNA yield among various methods

Sl No.	Unpaired *t*-test	*P* value	*R* value	Significance
1	Method 1 *vs* Method 2	0.908	0.0003	NS
2	Method 1 *vs* Method 3	< 0.0001	0.401	c
3	Method 1 *vs* Method 4	0.0017	0.23	b
4	Method 1 *vs* Method 5	0.524	0.01	NS
5	Method 2 *vs* Method 3	< 0.0001	0.418	c
6	Method 2 *vs* Method 4	0.0011	0.248	b
7	Method 2 *vs* Method 5	0.393	0.019	NS
8	Method 3 *vs* Method 4	0.13	0.059	NS
9	Method 3 *vs* Method 5	< 0.0001	0.366	c
10	Method 4 *vs* Method 5	0.004	0.192	b

P value < 0.05 was considered significant; $^{b}P < 0.01$, $^{c}P < 0.001$. NS: Not significant.

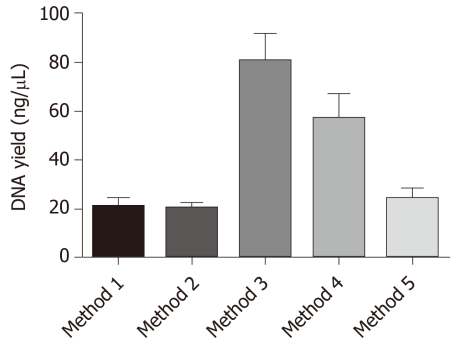

Figure 3 Graph showing DNA yield (ng/μL) from different DNA extraction methods. Data represented as mean ± standard error of the mean. It is clearly visible from the graph that method 3 wherein xylene was used for deparaffinization with 72 h of lysis duration showed the maximum yield of DNA. Similarly, method 4 with proteinase K inactivation also showed better DNA yield.

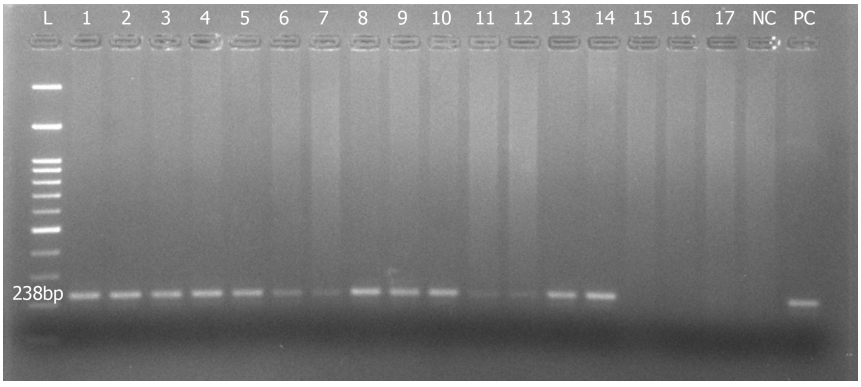

Figure 4 Representative image of 2% agarose gel showing amplified 238 bp product of the housekeeping *GAPDH* gene. Samples 1-5, 8-10, and 13-14 showed clear amplification bands, while samples 6-7 and 11-12 showed mild amplification. In contrast, samples 15-17 showed no amplification at all. L: 100 bp DNA ladder; PC: Positive control; NC: Negative control.

ARTICLE HIGHLIGHTS

Research background

Histopathologically stained archived tissue slides are stored in hospital archives for years to decades. They are the largest available source of biological materials and are a potentially useful resource that can be used for retrospective epidemiological studies. DNA recovered from the slides can be used for several downstream molecular processes including polymerase chain reaction, single nucleotide polymorphism analysis, and whole genome sequencing. The slides can also be used to acquire information such as the presence or absence of pathogens that would

have been present at the time of sectioning. The DNA from these slides can be utilized to compare gene signatures of normal and diseased tissues.

Research motivation

Generally, the extraction of high-quality DNA from archived stained hematoxylin and eosin (H&E) slides is challenging. Barring commercially available expensive kits, there is a drought of reproducible methods to extract nucleic acids from histopathologically stained tissue slides. The key problem to be addressed here was coming up with new methods for DNA extraction from archived tissue slides that can be easily implemented in molecular biology labs with low resource settings worldwide.

Research objectives

The objective of the study was to standardize a protocol for DNA extraction from archived H&E tissue slides that can be further used for downstream molecular analysis from basic PCR to whole genome sequencing. Also, our objective was to come up with a method that is not only reproducible but also cost-effective.

Research methods

A total of 100 archived H&E-stained cancer slides were subjected to a total of five methods of DNA extraction. Methods were varied in the deparaffinization step, tissue rehydration, duration of lysis, and presence or absence of proteinase K. The extracted DNA was quantified using a NanoDrop spectrometer and the quality was analyzed by agarose gel electrophoresis. Each sample was subjected to PCR to amplify the internal control gene *GAPDH*, thereby confirming the DNA intactness that could be further utilized for other downstream applications. Statistical analysis was performed to assess the different methods in terms of yield and purity of the DNA obtained.

Research results

Of the five different methods tested, the third method wherein xylene was used for tissue deparaffinization followed by 72 h of digestion and without proteinase K inactivation yielded the highest amount of DNA with good purity. The yield was significantly higher compared to other methods. Also, 90% of the extracted DNA showed amplifiable *GAPDH* gene indicating the intactness of the DNA, which in turn suggested that this DNA could be used for further molecular analysis.

Research conclusions

Our study explored the possible new methods for the extraction of PCR-friendly DNA from archived H&E-stained tissue slides for downstream molecular analysis. We tried and tested alternative methodologies for the removal of coverslip, deparaffinization of the tissues, rehydration and digestion by using simple facilities and common reagents in a basic molecular biology laboratory. We addressed the difficulties in removing the coverslip and deparaffinization of the tissues. Our data indicated that method 3 with xylene deparaffinization and 72 h digestion showed the highest yield of DNA and was also the best in terms of purity. The samples that were deparaffinized using pre-warmed xylene (about 50°C) produced greater DNA yields than concentrations of nucleic acids produced from the samples that were deparaffinized with mineral oil.

Research perspectives

In our study, we explored new possibilities of extracting DNA from archived specimens that can be used for molecular analysis. Similar to FFPE tissue blocks, quality H&E tissue slides can be critical in clinical studies and research. Since H&E slides are relatively inexpensive and easy to store, more work can be done with them. Thus recovered DNA can be utilized in the field of oncology for discriminating the mutational profile between the tumor and adjacent normal tissue or even in the field of hematology or immunology to understand the disease state, cause, and possible medication. Based on the preliminary evidence from our study, future research can focus on how to best utilize the discussed methods.

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