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World Heart Day serves as a reminder that cardiovascular disease remains the leading cause of global deaths. While most countries have experienced age-adjusted declines in cardiovascular disease-related mortality over the past 25 years, India’s rate has remained static\(^1\). Nearly 55 million Indians are estimated to have cardiovascular disease, and in 2016, 62.5 million years of life were prematurely lost in India due to cardiovascular diseases\(^1\). Substantial State-level variations exist in the cardiovascular disease burden in India, including a nine-fold variation in the burden of ischaemic heart disease (highest burden State: Punjab, lowest burden State: Mizoram)\(^1\). To help global, national and regional policymakers compare potential interventions, the third edition of the Disease Control Priorities Project identified evidence-based disease control priorities and priority interventions, including cardiovascular diseases, based on evidence synthesis of effectiveness, cost-effectiveness and extended cost-effectiveness, which includes the influence of interventions on financial risk protection\(^2\). Here we aim to (i) describe 14 evidence-based global cardiovascular, respiratory and related disease control priority interventions, (ii) identify opportunities and challenges for adaptation, implementation and scaling of priority interventions in India, and (iii) outline research to catalyze improvements in cardiovascular health in India.

**Evidence-based global cardiovascular disease control priority interventions**

The essential package of 14 priority interventions (Table) developed through the third edition of the Disease Control Priorities Project accounts for not only disease burden and effectiveness but also affordability and feasibility in a low- or lower-middle-income country context, such as India\(^2\). The interventions can be on the fiscal, inter-sectoral, public health, or personalized health service level, stratified by community, primary health centre, first-level hospital and referral or specialized hospital levels. There is substantial overlap with the World Health Organization’s Noncommunicable Diseases Best Buys\(^3\), but differences exist when cost-effectiveness data are limited (e.g. fruit and vegetable subsidies, medical management of atrial fibrillation). While these interventions are potentially cost-effective, they were not included as part of the essential package. Further, while these interventions are evidence based, governments will also consider relative affordability, political commitment, subnational or regional disease burden and long-term budget planning based on the optimistic premise of a growing, ageing population when determining which interventions would be most appropriate for implementation.

Fiscal and inter-sectoral interventions aim to reduce the burden of tobacco- and diet-related diseases, which are the leading causes of cardiovascular disease burden in India\(^1\). For example, tobacco taxes and regulation of advertising and labelling of tobacco products are components of the World Health Organization’s and Framework Convention on Tobacco Control’s MPOWER package. Further, reformulation of food products to reduce excessive sodium and eliminate artificial trans-fatty acids will be needed to reduce the burden of cardiovascular and related diseases. The May 2018 release of the World Health Organization’s REPLACE package\(^4\) to eliminate artificial trans-fatty acids in the global food supply is a step that puts this goal into the broader global health spotlight.

Personalized health service level interventions are largely focused on the primary health centre level and include diabetes screening; combination therapy for cardiovascular disease prevention among individuals with or at high-risk for cardiovascular

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\(^1\)This editorial is published on the occasion of World Heart Day - September 29, 2018.
disease events; acute treatment with aspirin for acute myocardial infarction; antibiotics for acute pharyngitis, acute rheumatic fever or rheumatic heart disease secondary prevention and inhaled corticosteroids and bronchodilators for individuals with asthma and some individuals with chronic obstructive pulmonary disease. In the first-level hospital context, tobacco cessation counselling and nicotine replacement therapy in certain circumstances are also recommended.

### Opportunities and challenges of priority interventions in India

In India, the opportunities and challenges for fiscal, inter-sectoral and personalized health service interventions exist in the wider socio-political context. For example, while tobacco remains the leading cause of preventable death in India\(^2\), the provision of large excise taxes requires political buy-in. Tobacco products sold in India are required to have pictorial warnings covering 85 per cent of the package based on the Cigarettes and Other Tobacco Products Act 2003\(^2\), yet this labelling regulation has faced and withstood repeated legal challenges with its final implementation in April 2018. The provision of tobacco cessation counselling requires not only infrastructure to deliver such services, including call centres staffed by trained professionals, but also of sufficient size and linguistic diversity to match the needs of India’s population. The availability and use of nicotine replacement therapy are limited by its exclusion from India’s national essential medicines list\(^6\), despite being on the World Health Organization’s Model List of Essential Medicines. Future expert panels may be receptive to adding nicotine replacement therapy to India’s list.

Dietary regulation offers another avenue for reducing the burden of cardiovascular and related diseases, yet technical and socio-political challenges remain. For example, salt reduction strategies require

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**Table.** Essential package of core, priority interventions to reduce the burden of cardiovascular, respiratory and related conditions outlined by the 3rd edition of the Disease Control Priorities Project

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ACE, angiotension converting enzyme

Source: Ref. 2
consideration of iodine supplementation, as well as historical considerations. Trans-fatty acid bans require upstream engagement and reformulation among not only food manufacturers but also oil manufacturers. In June 2018, the Food Safety and Standards Authority of India announced a target of less than two per cent trans-fatty acids in partially hydrogenated vegetable oils by 2022, and the draft regulations are under development. Dietary surveillance systems at the individual and food system levels, including related to sodium and trans-fatty acids, though remain in the development stage in India yet will be critically important for monitoring progress.

For treatment among individuals with or at high risk for developing atherosclerotic cardiovascular disease, combination therapy has been demonstrated to reduce the risk of cardiovascular events. When available, fixed-dose combination therapy is also recommended based on the improvements in adherence and health system efficiency gains. Despite the efficacy of medications including anti-platelets, statins and blood pressure lowering therapies in cardiovascular disease risk reduction, their long-term effectiveness can be limited by low persistence rates and relatively high costs. Individuals with heart failure face similar challenges though treatment with loop diuretics is commonly associated with symptomatic improvements that are more readily apparent to patients.

Treatment of acute myocardial infarction using aspirin is widely recommended and could even be administered at a primary health centre level. While modelling data demonstrate the cost-effectiveness of obtaining an electrocardiogram at the primary health centre level, rapid referral to a hospital setting for reperfusion therapy is preferred. Once patients with an acute cardiovascular condition reach a hospital setting, strategies to promote high-quality, safe care remain under study and are imperative to improve overall health system performance.

**Research to catalyze improvements in cardiovascular health**

While implementation of evidence-based strategies will be an important strategy to reduce burden of cardiovascular diseases in India in the years ahead, future research will also be needed to catalyze improvements in cardiovascular health. On a fiscal level, further research developing, implementing and evaluating strategies for universal health coverage will be needed to ensure financial risk protection given the high rates of catastrophic health spending and distress financing associated with acute cardiovascular diseases. On an inter-sectoral, policy-level, stronger evidence synthesis and health technology assessment research will improve evidence-informed policy-making. On a health system level, health management information system strengthening, coupled with robust risk factor and disease surveillance, will help India prioritize its interventions on national, State and local levels. Methods of task sharing, including through the use of traditional medical practitioners and practices, such as yoga, can also be studied. On a fundamental level, India can leverage the predilection for vascular disease that South Asians seem to face for discovery of new phenotypes and targets of intervention, though these will necessarily sit in the health system context. All areas will require a larger research workforce and sustainable funding mechanisms to build human resources and institutions in whom health research investments can be made. These pursuits should neither delay action, research, nor research training on broader levels yet should be considered mutually reinforcing.

India continues to face a disproportionate burden of cardiovascular disease compared with other countries around the world, yet priority targets and interventions have been developed to help improve the health and wellness of its people.

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The prevalence of obesity has been rapidly rising over the few decades globally and in India. Obesity also predisposes individuals to type 2 diabetes, hypertension and cardiovascular disease apart from osteoarthritis, sleep apnoea and even some forms of cancer. Obese individuals are also prone to psychological issues such as low self-esteem and depression. Medical intervention to manage obesity is very few, and most of them only work with a robust lifestyle intervention programme. This leads to frustration among individuals with obesity, leading them to resort to extreme dietary interventions to obtain quick weight loss. One such extreme dietary intervention which has gained popularity in recent years is the ketogenic diet.

**What are ketogenic diets?**

Ketogenic diets are characterized by a marked reduction in carbohydrates (usually to <50 g/day) and a relative increase in the proportions of protein and fat - usually extremely high percentages of fat because it is difficult to increase proteins beyond a point.

**Types of ketogenic diets**

**Standard ketogenic diet (SKD):** This is a very low-carbohydrate with moderate-protein and high-fat diet. It typically contains 70 per cent fat, 20 per cent protein and only 10 per cent carbohydrates.

**Cyclical ketogenic diet (CKD):** This diet involves periods of higher-carbohydrates in between the ketogenic diet cycles, for example, five ketogenic days followed by two high-carbohydrate days as a cycle.

**Targeted ketogenic diet (TKD):** This diet permits adding additional carbohydrates around the periods of the intensive physical workout.

**High-protein ketogenic diet (HPKD):** This diet includes more protein and the ratio around 60 per cent fat, 35 per cent protein and five per cent carbohydrates but as can be seen, it is still a very high fat diet.

The SKD and HPKD have been used extensively. The cyclical and targeted ketogenic diets are recent additions and mostly used by bodybuilders or athletes. The SKD is the most researched and recommended, and the rest of this article will deal with SKD.

**Physiological principles of ketogenic diets**

All ketogenic diets contain a very low carbohydrate percentage. After a few days with such drastically reduced carbohydrate consumption (below 50 g/day), glucose reserves become insufficient, both for normal fat oxidation through the supply of oxaloacetate in the Krebs cycle and for the supply of glucose to the central nervous system (CNS). The CNS cannot use fatty acids as a source of nutrition. Hence, after 3-4 days of carbohydrate restriction, the CNS is forced to find an alternative source of energy. This alternative source of energy is ketones. Ketone bodies are produced in the liver and are of two types: acetoacetate and β-hydroxybutyrate. As ketone bodies are produced by breakdown of fats, ketosis is the most reliable indicator of fat loss. Ketosis is a completely physiological mechanism. It was Hans Krebs who first differentiated physiological ketosis from pathological ketoacidosis seen in type 1 diabetes. In physiological ketosis (which occurs during very-low-calorie ketogenic diets), ketonaemia reaches maximum levels of 7-8 mmol/l (it does not go higher because the CNS efficiently uses these ketones) and also there is no lowering of blood pH. In diabetic ketoacidosis, it can exceed 20 mmol/l with a concomitant lowering of the pH.

**Benefits and adverse effects of ketogenic diets**

The ketogenic diet was originally developed in 1924 to treat epilepsy, but other, more recently discovered benefits include weight loss and reversal/control of type 2 diabetes. Use of ketogenic diets in weight management has gained tremendous popularity, but it has also generated several
controversies. Some researchers suggest that there are no metabolic advantages with low carbohydrate diets and that weight loss results simply from reduced caloric intake, probably due to the increased satiety effect of protein. However, the majority of *ad libitum* studies demonstrate that individuals who follow a low-carbohydrate diet lose more weight during the first 3-6 months compared with those who follow more balanced diets.

Besides a positive effect on weight loss, studies have shown that low-carbohydrate ketogenic diets also reduce serum triglycerides dramatically. Elevated serum triglycerides are common among Asian Indians, and this is one of the features of the so-called Asian Indian Phenotype. Reduction in total cholesterol and increase in high-density lipoprotein cholesterol have also been reported. A key enzyme in cholesterol biosynthesis is 3-hydroxy-3-methylglutaryl-CoA reductase, which is activated by insulin. This means that an increase in blood glucose and consequently of insulin levels will lead to increased endogenous cholesterol synthesis. A reduction in dietary carbohydrate will thus have the opposite effect and this, coupled with the additional inhibition by dietary cholesterol and fats on endogenous synthesis, is likely to be the mechanism by which physiological ketosis can improve lipid profiles.

Thus, low-carbohydrate ketogenic diets have been shown to have immense benefits in blood sugar control. There are some reported beneficial effects on cancer and neurological disorders such as Alzheimer’s disease and epilepsy, although these are not discussed further here as it is beyond the purview of this article.

However, there are also several adverse effects of ketogenic diets. These include muscle cramps, bad breath, changes in bowel habits, keto-flu and loss of energy. Hence, monitoring individuals on keto-diet closely once or twice a month for blood glucose, ketones cardiac and other parameters is essential.

**Should ketogenic diets be recommended?**

Indian diets are very high in carbohydrates. The STARCH study has shown that Indians with or without diabetes consume at least 65 per cent calories from carbohydrates. The Chennai Urban Rural Epidemiology Study (CURES) has also shown that carbohydrate constitutes the major source of calories in south India. We also know that India has a huge burden of type 2 diabetes and cardiovascular disease. Data from the PURE study showed that high carbohydrate intake (more than about 60% of calories) was associated with an adverse impact on total mortality and non-cardiovascular disease mortality. By contrast, higher fat intake was associated with lower risk of total mortality, non-cardiovascular disease mortality and stroke. In contrast, in a recent study on dietary carbohydrate and mortality, Seidelmann *et al.* showed that there existed a U-shaped relationship between carbohydrate intake and mortality. Both extremely high (60% and above) and low carbohydrate diets (<30% carbs) were shown to have higher mortality rates. The risk of dying was lowest when the carbohydrate intake was between 50 and 55 per cent. Moreover, mortality rates were lower when the dietary carbohydrates were replaced by plant-based proteins and fats but higher in those who were on animal-based proteins and fats.

One of the challenges of low-carbohydrate diets is that these have a lower intake of vegetables, fruits and grains and increased intakes of fat which can be detrimental. Long-term low-carbohydrate diets with increased fat consumption have been hypothesized to stimulate inflammatory pathways, oxidative stress and promote biological ageing.

The biggest problem with extreme diets like keto diets is their sustainability. In our experience, people are initially thrilled with the weight loss and the excellent diabetes control they get, after using keto diets. Slowly, however, they get bored with the diet. Furthermore, many feel weak and frustrated and start increasing the carbohydrate intake, and soon they are back to their original weight and diabetes control. Recent studies also suggest that ketogenic diets may, in fact, induce hepatic insulin resistance. There are also reports of micronutrient deficiency and cardiovascular safety. Hence, many more studies need to be done before these diets are widely recommended.

**So what is our final message?**

The dictum, ‘Moderation is the key’ should be used, while following any long-term diet plan. While low-carbohydrate ketogenic diet does, admittedly, show dramatic improvements in the short term, these can increase morbidity and mortality in the long run and are rarely sustainable. Instead of letting the pendulum of nutrients swing on either side, one must be vigilant of the balance and interplay of nutrients, and there should be a representation of all food groups on the plate.

For Indians, it appears that it would be most prudent to have a diet with about 50 per cent carbohydrate intake. This would provide the needed balance to avoid both the adverse effects of excessive carbohydrate and the detrimental effects of excessive fat consumption.
(using complex carbs and whole grains such as brown rice or whole wheat) about 20-25 per cent protein (preferably from vegetable proteins such as legumes and pulses) and the remaining 25-30 per cent from healthy fats like monounsaturated fats (e.g. groundnut oil or mustard oil and nuts and seeds) along with plenty of green leafy vegetables. Such a diet may not immediately give dramatic results as far as weight reduction is concerned. However, it will be sustainable in the long term and will be less risky and certainly more healthy and also help prevent non-communicable diseases such as diabetes, cardiovascular disease and certain cancers.

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The management of pancreatic ductal adenocarcinoma (PDAC) is a therapeutic challenge. Locally advanced/metastatic disease at presentation and significant genetic heterogeneity, a potential reason for resistance to cytotoxic chemotherapy are major barriers. Incidence (per 100,000) of PDAC is low in India, 1.4 in males and 1.0 among females, compared to North America where corresponding figures are 7.5 and 6.5, respectively. This burden of PDAC is likely to increase further in India with increase in longevity, changing lifestyle, increase in smoking/tobacco consumption, obesity and lack of physical activity/exercise - the key risk factors for this disease.

The current treatment approach for PDCA patients with advanced/metastatic disease is to use chemotherapy; the choice of the regimen is based on the patient’s fitness [Eastern Cooperative Oncology Group (ECOG) performance status], organ function, comorbidities, individual preference and psychosocial issues. Leucovorin plus short-term infusional fluorouracil plus oxaliplatin and irinotecan (FOLFIRINOX) is the preferred regimen for the young and fit patients [ECOG performance status (ECOG PS) 0, 1] with serum bilirubin <1.5 mg/dl. FOLFIRINOX was compared to single-agent gemcitabine in a randomized phase III trial, which included patients with metastatic pancreatic cancer with ECOG PS 0, 1 and serum bilirubin <1.5 mg/dl. FOLFIRINOX was associated with a better overall response rate (ORR) (32 vs. 9%), progression-free survival (PFS) (6.4 vs. 3.3 months) and overall survival (OS) (11.1 vs. 6.8 months). Patients in combination arm had higher Grade 3/4 toxicity including febrile neutropenia, thrombocytopenia, neuropathy and diarrhoea. It should be pointed out that only minorities of patients with metastatic pancreatic cancer are candidates for FOLFIRINOX due to older age and poor general condition. Patients who are candidates for intensive regimen and have serum bilirubin <1.5 mg/m² but not a candidate for FOLFIRINOX are treated with a combination of gemcitabine and nab (nanoparticle albumin-bound) paclitaxel. In a phase III randomized controlled trial, MPACT, the combination of nab-paclitaxel (125 mg/m²) and gemcitabine (1000 mg/m²) for 3 wk every 4 weekly was compared to single-agent gemcitabine (1000 mg/m² weekly 7 of 8 wk and then for 3 of 4 wk). The combination regimen was associated with a higher ORR (23 vs. 7%), PFS (5.5 vs. 3.7 months) and OS (8.5 vs. 6.7 months) and higher toxicity. This study included patients older than 75 yr and with ECOG PS 2 which are not represented in the studies evaluating FOLFIRINOX. It has been suggested that nab-paclitaxel may deplete the tumour stroma through the binding of albumin to fibroblasts containing secreted protein acidic and rich in cysteine.

In this issue Ostwal et al report a retrospective study evaluating the response and treatment outcome of patients with metastatic/locally advanced pancreatic cancer treated with a combination of gemcitabine and non-cremophor paclitaxel [abraxane (Abraxis BioScience, Inc., Los Angeles, CA, USA) or nanoxel (Dabur Pharma Ltd., India)]. The conventional paclitaxel is insoluble in water and hence cremophor; a castor oil-based surfactant is used to make it soluble. Cremophor contributes to the hypersensitivity reactions associated with paclitaxel infusion. The non-cremophor paclitaxel, abraxane is albumin-bound nanoparticles of paclitaxel while nanoxel is a polymeric-micelle formulation. The use of non-cremophor paclitaxel compared to conventional paclitaxel has an added advantage of lesser hypersensitivity reaction and lesser need for pre-medication. The authors report...
an ORR of 30.8 per cent, median PFS of 5.6 months [95% confidence interval (CI) 3.7-7.4] and a median OS of 11.6 months (95% CI 8.8-14.3) months. The OS and PFS in this study are comparable to that in the MPACT trial. There was no significant difference in the OS and PFS among patients treated with gemcitabine-abraxane and gemcitabine-nanoxel combination. There was no difference in the treatment outcomes of these two-drug combinations as the study was not designed or powered to answer this question. Although it is undoubted that availability of nanoxel gives a cheaper option for patients who are to be treated with non-cremophor paclitaxel, which may be an important factor in a resource-limited setting. The other interesting point was that the grade III/IV toxicity in this study was lower than that reported in another phase III study evaluating this combination, which as per authors could be due to the dose modification incorporated in the treatment protocol. This gives a useful insight into means of limiting toxicity without affecting the efficacy and treatment outcome by tailoring the dose of chemotherapy regimen for an individual patient in a palliative setting. Other means have also been used to reduce the toxicity of this regimen, for example, administering gemcitabine (1000 mg/m²) and nab-paclitaxel (125 mg/m²) every two weeks. This regimen has comparable efficacy and reduced toxicity as compared to standard dosing schedule of nab-paclitaxel-gemcitabine. Patients who are fit for combination chemotherapy but who continue to have bilirubin >1.5 mg/dl despite biliary stenting can be treated with FOLFOX regimen. Other combination regimens have also been evaluated, for example, gemcitabine-5-fluorouracil and gemcitabine-capecitabine which have a doubtful benefit over single-agent gemcitabine. In view of the dismal outcome of patients with advanced pancreatic cancer several novel approaches have been evaluated. Pancreatic cancer has been shown to express epidermal growth factor receptor (EGFR). The vascular endothelial growth factor (VEGF) is also deemed important in the pathogenesis and spread of pancreatic cancer. In keeping with these findings, a phase III study compared the gemcitabine (1000 mg/m² weekly) with or without erlotinib (100 mg daily). Patients in the combination arm (gemcitabine and erlotinib) had a significant though small OS benefit of two weeks (6.2 vs. 5.9 months). This clinically insignificant benefit comes at a greater financial cost and toxicity. The combination of gemcitabine with VEGF inhibitor, for example, bevacizumab, or multikinase inhibitors, for example, axitinib or sorafenib, on the other hand, failed to show any benefit over single-agent gemcitabine.

The patients who are not candidate for intensive chemotherapy and ECOG PS ≤2 can be offered single-agent gemcitabine (1000 mg/m² weekly 7 of 8 wk and then for 3 of 4 wk). The use of single-agent gemcitabine is associated with low ORR (11%) with clinical benefit observed in nearly 30 per cent of the patients. Clinical benefit was defined by decrease in pain, analgesic use and weight gain. This discordance between response assessed by response evaluation criteria in solid tumors (RECIST) criteria (recist.eortc.org) and clinical benefit may be due to the extensive desmoplastic reaction seen in pancreatic cancer which makes assessment of response at primary site difficult. Immunotherapy is another option for patients with metastatic pancreatic cancer with high microsatellite instability.

Palliative care forms the bedrock for an appropriate management of metastatic/locally advanced metastatic PDAC. Care must be taken for adequate analgesia, nutrition, management of obstructive jaundice, gastric outlet obstruction and psychosocial well-being of these patients. Patients with significant comorbidities and a poor performance status should be referred for palliative care.

Genetically, PDAC is a very heterogeneous disease; important mutations are KRAS (>90%), p53 (60-70%), CDKN2A, (>50%), SMAD4 (approximately 50%). About 4-5 per cent of patients have mutations in BRCA1/2; these cases have enhanced sensitivity to platinum-based chemotherapy as well as poly(ADP-ribose) polymerase (PARP) inhibition. This improved understanding has led to initiation of a number of clinical trials (https://www.cancer.gov/types/pancreatic). Several new drugs such as methyl ethyl ketone (MEK) inhibitors, pegvorhyaluronidase alpha (PEGH20) which degrades hyaluronic acid in tumour microenvironment (TME), nab-paclitaxel (BBI-608), which inhibits cancer stem cells, and AM0010, a PEGylated interleukin-10 are at various stages of drug development and are being evaluated in combination with various agents. Similarly, poly(ADP-ribose) polymerase (PARP) inhibitors are being evaluated as single-agent or as maintenance therapy in breast cancer (BRCA)-mutated patients with metastatic pancreatic cancer who have had a stable disease or objective response to chemotherapy.
In PDCA, the stroma is very dense, fibrotic and heterogeneous and is possibly responsible for resistance to therapy. Recent progress in the field of immunotherapy and checkpoint inhibitors have shown promise in a number of cancers particularly in melanoma, lung cancer, urinary bladder cancer, renal cancer and head and neck cancer. Results of single-agent checkpoint inhibitors have not met with success. Possibly lower levels of neoantigens, the unique immunosuppressive TME and low levels of intratumoral infiltrating T-lymphocytes are some of the barriers. Currently, a number of phase I/II trials are underway to evaluate the use of checkpoint inhibitors along with gemcitabine or targeted therapies for modulating and enhancing T-cell immunity. Results of these trials are awaited with hope.

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Universal Health Coverage (UHC) and Health Technology Assessment (HTA)

The government of India (GOI) is committed to provide universal health coverage to assure the availability of free and comprehensive primary healthcare services to its 1.2 billion population. The challenging task of extending the healthcare services to each and every patient can only be achieved with optimal utilization of the resources available. Therefore, it is required that decisions on resource allocation are policy relevant and evidence informed. The evidence-based decision-making involves clinical effectiveness, cost-effectiveness, budget impact, as well as ethical, social and political feasibility studies. Health technology assessment (HTA) is an internationally accepted tool to ensure that technology choice is participatory and is well guided by considerations of scientific evidence, safety, consideration on cost-effectiveness and social values.

Establishment of Health Technology Assessment in India (HTAIn)

The HTA in India (HTAIn) previously known as Medical Technology Assessment Board (MTAB) was set up under the Department of Health Research (DHR), by Ministry of Health and Family Welfare (MoHFW), to help GOI in evidence-informed decision-making in healthcare. HTAIn has been given the responsibility to conduct health technology assessment studies for the requests coming from central and State health ministry that includes systematic literature reviews, economic evaluations, primary costing as and when required, and measuring and valuing the health outcomes pertaining to that health technology. Along with clinical and cost effectiveness, the studies will also analyse evidences related to equity issues regarding the deployment of health technologies, that ensures efficient use of the limited health budget and provide people access to quality healthcare at minimum cost.

How HTAIn functions in India

HTAIn consists of three core bodies including HTAIn Secretariat, HTAIn Technical Appraisal Committee (TAC) and HTAIn Board. The HTAIn Secretariat works in collaboration with its identified technical partners (TPs) and regional resource hubs (RRHs) across India. The requests for HTA study are first processed by HTAIn Secretariat experts and then allocated to TPs. TPs develop the study proposal and present the study to TAC members. Once approved by TAC members, a stakeholder consultation meeting is convened to inform and apprise all concerned stakeholders regarding the HTA study. After completion of the HTA study, outcomes of the study are again presented to TAC members and after approval by TAC, a second stakeholder’s consultation meeting is convened to inform them about the study results and final recommendations. Finally, the study is put up to the HTAIn Board, and once approved by the Board, a policy brief along with the HTA outcome report is sent to the user department from where the request has originally come.

Need of National HTA database

HTAIn has started functioning congruously in January 2017 in DHR. Since its beginning, requests for HTA studies started to come from different policymakers with topics from different divisions of the Union MoHFW, State MoHFW, Rashtriya Swasthya Bima Yojana, National Pharmaceutical Pricing Authority, National Health Mission, National innovation portal, etc. As HTAIn is now more than 1½ year old and there are more than 25 HTA topics...
under process, it is crucial to record all the information properly and systematically. To manage the HTA study related information by storing it systematically and to make the information available in public domain transparently, a national HTA database is initiated under DHR. The national HTA database will be available on DHR website and will be periodically updated (Figure).

Data collection

A common ‘HTA information sheet’ (HIS) will be developed to collect the data. This HIS will be used to record the information on each ongoing and completed HTA study in an organized manner. Specific information regarding each HTA study including HTA topics, study investigators, interventions and comparators, user department, decision taken by TAC, stakeholder’s consultation will be collected. The scope for inclusion will be broad, encompassing any study designated as a HTA by the HTAIn Secretariat or by TPs. The national HTA database will be hosted by DHR and it will be made available on DHR website. The information on each HTA study will be periodically updated.

Proposed overview of DHR-HTA database

The DHR-HTA database will provide free access to bibliographic information about ongoing and completed HTAs studies commissioned and undertaken across the nation under HTAIn, DHR. All the studies will be funded by DHR, MoHFW, India. This database will provide specific information regarding each HTA study. There are practical and scientific benefits of a freely accessible transparent database. It is thus envisaged that providing free access to HTA information will reduce unnecessary duplication and will help in preventing wasted effort and precious research funds. It will encourage global collaboration between researchers, increase transparency and improve quality.

Other HTA databases worldwide

The National Institute for Health Research (NIHR) HTA database is the most comprehensive, international register and provides free access to bibliographic information about ongoing and published HTAs commissioned or undertaken by members of the International Network of Agencies for HTA (INAHTA) and other HTA organizations from around the world. It is funded by the UK NIHR and is currently produced by the Centre for Reviews and Dissemination (CRD). NIHR database established in 1998 combined the HTA records identified by CRD and INAHTA’s HTA reports.

A new Canadian search interface for the HTA database was launched in January 2015. The new interface was developed through collaboration between CRD and the Pan-Canadian HTA Collaborative Working Group that is now serving as a Canadian HTA Repository.

Another database is a recently developed the EUnetHTA Planned and Ongoing Projects (POP) database. This database is developed to help HTA agencies in sharing the information with each other on planned, ongoing or recently published projects conducted at the individual agency level. The tool is created and managed by LBI-HTA (Austria) and the database is developed and maintained by DIMDI (Germany). Access to the POP database is currently restricted to EUnetHTA Partners and Associates who contribute to the POP database.
Besides these, there are many national HTA databases such as in Australia\textsuperscript{16}, Thailand\textsuperscript{17} and Singapore\textsuperscript{18} that provides information on HTA studies in their respective countries.

India is a relatively younger member joining the worldwide HTA community, but being unique in its own diversity in terms of healthcare challenges and healthcare system will surely have a major impact. Although there have been many studies on cost-effectiveness analysis, economic evaluations, cost-utility studies, etc.\textsuperscript{19-23}, coming from different researchers in India, most of these studies have been conducted for academic purpose. HTAIn being a central government programme will bring such studies under a broader umbrella, where these studies will be more policy driven. The DHR-HTA database will be showcasing all these studies, and it will be informative not just for India but also for a wider HTA community spread worldwide.

\textbf{Conclusion}

The DHR-HTA database will become a valuable resource for locating literature and information, which will be freely available from a single source. This database will enable funders and researchers to identify work already in progress and will help reduce unintended duplication of effort. The database will also serve as a one-point source for policymakers where they can have a quick glance at the website to have an idea about studies under process, which can later become a basis for new public health programmes. The database will also be helpful to private manufacturers and clinicians as keeping them well informed about what all-new interventions are being considered under different HTA studies. Open access of such a database will bring the much-required transparency in the field of research and development.

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A fascinating story of the discovery & development of biologicals for use in clinical medicine

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A young physician starting a fresh career in medicine in this millennium would hardly stop to think about the genesis of a particular biological drug that he/she will be prescribing for a patient evaluated in the morning outpatient department. For him/her, this is now routine, and the question of ‘Who’, ‘How’ and ‘When’ about these biologicals would be the last thing on their mind. However, for those who came to the medical profession in the 1950s, 1960s and 1970s, these targeted drugs are nothing short of ‘miracles’. It would be a fascinating story for the young doctor to learn about the long journey that the dedicated biomedical scientists of yesteryears took to reach the final destination of producing such wonder drugs. The story is much like an interesting novel, full of twists and turns, heart-breaking failures and glorious successes. The biologicals acting as ‘targeted therapy’ have not only changed the natural history of a large number of incurable/uncontrollable diseases but have also transformed the whole approach towards drug development. From the classical empirical process, there is now a complete shift towards understanding the disease pathobiology focusing on the dysregulated molecule(s), targeting them with greater precision and aiming for better results. Seminal advances in understanding the disease mechanism, development of remarkably effective new technologies, greater knowledge of the human genome and genetic medicine have all made it possible to reach the stage where artificially developed ‘targeted’ drugs are now therapeutically used in routine clinical medicine.

Key words Biological drugs - drug discovery - history of medicine - infliximab - monoclonal antibody - tumour necrosis factor-α

Introduction

Most orally administered drugs are organic compounds with a relatively small molecular weight (usually <500 g/mol), except calcineurin inhibitors that have a slightly higher molecular weight (but still <1000 g/mol). These molecules are chemically synthesized in laboratories without using any biological systems. Indeed, orally taken small molecules are easily absorbed by diffusion through the intestinal lining. Although the gut can ingest large molecules through the process of endocytosis, use of this delivery system for orally administered drugs is yet to find its way in clinical medicine. Therefore, drugs with a large molecular weight perform need to be administered parenterally. Biologicals, a new category of drugs that are not synthesized chemically in the laboratory, need biological systems for their
production. These are ‘targeted’ drugs that ‘home’ on and bind to a specific key molecule involved in the pathogenesis of that disease. These drugs have been named ‘biological response modifiers’ or ‘biologics’ in short. Since their addition in the armamentarium of the clinicians around the end of the 20th century and the beginning of the millennium, these drugs have revolutionized therapy and significantly influenced the outcome of several so-called ‘untreatable diseases’ across all the medical specialities. These drugs are now widely used in routine day-to-day medical practise. Yet, only a few might actually know the underlying fascinating story of scientific discoveries of the past few decades that made it possible to develop these drugs. This review attempts to recreate the successes and travails of a large number of medical scientists whose astute observations and painstaking work over decades culminated in the production of a clinical-grade monoclonal antibody (mAb) that was highly specific against a key molecule in the pathogenesis of rheumatoid arthritis (RA), namely the tumour necrosis factor-α (TNF-α). The rest as they say is history.

**Biological drugs**

Definition of a biological drug as provided by the National Cancer Institute (NCI, USA) is ‘a substance that is made from a living organism or its products and is used in the prevention, diagnosis, or treatment of cancer and other diseases. Biological drugs include antibodies, interleukins (ILs), and vaccines; also called biologic agent and biological agent’ (https://www.cancer.gov/publications/dictionaries/cancer-terms/def/biological-drug). It may be mentioned that agents used in clinical therapeutics and derived from living beings are not a new phenomenon in medicine. ‘Serum therapy’ or ‘antitoxin therapy’ was first discovered by Shibasaburo Kitasato (1852-1931) and Emil von Behring (1854-1917) using serum from animals (usually sheep or horse) injected with appropriate antigens to produce the desired antibodies. This method of treatment was widely prevalent in the last two centuries. Indeed, diphtheria, tetanus and rabies antitoxins continue to be used even today. Not only was the method of producing such sera and their purification process tedious and time-consuming, but also the main disadvantage was the frequent appearance of a disease often called ‘serum sickness’, caused by the body’s immune response to foreign (animal) proteins. Based on pure nomenclature, even the clotting factors used in bleeding disorders and several growth factors (used in haematology and other disciplines) are biologically derived. Even the older insulin, used widely for decades, was a biologically derived molecule (from the pancreas of pigs).

**Monoclonal antibodies (mAbs)**

With rapid advances in biotechnology along with complementary DNA (cDNA) cloning of the relevant molecules, substances such as insulin (and several others including erythropoietin) can now be synthesized without involving the biological systems (e.g. cell cultures, genetically modified bacteria, and yeast cells). However, conceptually, if treatment requires highly specific antibodies (derived from a single clone of antibody-producing plasma cells against aberrant molecules in the body leading to pathology), an in-depth understanding of the biological processes involved is essential for producing monoclonal antibodies (mAbs) in large amounts. The mAbs are now widely used in the treatment of a large number of diseases encompassing almost all speciality/superspeciality boundaries in medicine. Nevertheless, modern-day young physicians may not be fully aware of the seminal contributions and painstaking efforts of a dedicated group of passionate clinicians, biologists, biochemists, molecular biologists and pharmacologists, who made it all possible. The story of the development of biologicals is that of a remarkable breed of researchers who, despite several heart-breaking failures, ultimately succeeded in discovering a family of new therapeutic molecules that have not only changed the treatment landscape of a large number of chronic ‘untreatable’ diseases but have also provided new leads for ‘targeted therapy’. These monumental discoveries have changed the way the medicine is practised today. This review highlights the salient historical events during the development of first such therapeutic mAb, an anti-TNF-α, that was given the name ‘infliximab’.

**Biotechnology made it possible**

The advent of several path-breaking technologies has made it possible to develop new biologicals. While most of these were developed for different objectives and variable research needs, the story of their discovery and the key researchers involved are both interesting and educative.

**Tissue culture and the study of culture supernatants**

Tissue culture has been central to the study of cell biology, particularly for understanding the cellular function in health and disease. Wilhelm Roux, a student of Rudolf Virchow (a German physician with
lifetime contributions in experimental embryology), was the first to successfully grow medullary plate of an embryonic chicken in the laboratory in 1885. This discovery was followed by the ‘hanging drop’ method of cell culture developed by Ross Harrison, an American biologist and anatomist credited to be the first to work successfully with artificial tissue culture in 1907. From 1911 to 1935, the tissue culture technique reached a level of sophistication that made it the pivot for the study of cell physiology and behaviour. During this period, several other dedicated biologists contributed significantly towards making the technique more user-friendly. One of them was Alexis Carrel, a French surgeon and biologist who won the Nobel Prize (1912) and pioneered the culture techniques to perfection. Carrel worked at the Rockefeller Institute from 1906 to 1927 and teamed up with Montrose Burrows (1911), a US surgeon and pathologist with special interests in cancer research. Together, they developed the long-term aseptic technique of in vitro cell culture. Burrows is credited with coining the phrase ‘tissue culture’ and was among the first to adapt the technology to the study of tissues from warm-blooded animals. From the standpoint of rheumatology research, Vaubel at the Rockefeller Institute Hospital was the first to study synovial cells in tissue culture way back in 1933. He is credited with the description of two distinct cell types in the synovial tissue, namely a fibroblast-like synoviocytes (FLS) and a macrophage-like synoviocytes (MLS).

**Intercellular cross-talk and the concept of cytokines**

While the technique for long-term cell cultures was being developed during the early 20th century, it was soon realized that the harvested culture supernatants provided a rich source of molecules secreted by cells. This observation led to a plethora of studies defining their biological properties. Most of these were found to be small protein molecules (~5-20 kDa). In the absence of a defined system of nomenclature, different investigators gave different names, often two different names for the same molecule. In 1974, Cohen an American (New Yorker) biochemist and 1986 Nobel Laureate along with his colleagues coined the term ‘cytokine’ to designate products of cell cultures. They argued that many non-leucocyte cells in the body could also produce cytokines. Strictly speaking and depending on the cell types involved, the ‘cytokine umbrella’ encompasses besides cytokines, the chemokines, interferons (INFs), ILs, lymphokines, TNF, and others. At that time, it was speculated that these molecules must be involved in cell signalling (‘cross-talk’ between cells) and could thus be considered ‘short-range’ hormones (autocrines, paracrines) that influence the functioning and behaviour of the neighbouring cells. However, to date, the terminology of these molecules remains in flux, and many different names have been popular including migration factors, lymphokines, monokines, ILs, chemokines, autocrines, and paracrines. These are also named after the tissue in which they are found, for example, neurokines, adipokines, and others. Conventionally, hormones and growth factors are not included under the head ‘cytokines’.

**Impact of the discovery of cDNA cloning - Obtaining molecules in the purest form**

For studying molecules in different tissues in health and disease, tracer antibodies raised in small laboratory animals have conventionally been used. However, there were inherent problems of specificity and batch-to-batch variation for the use of these ‘polyclonal’ antibodies. This problem was soon resolved with the discovery of DNA cloning technology, capable of generating pure molecules. Although it is beyond the scope of this review to discuss biotechnological cloning methods for the production of pure forms of molecules and proteins, suffice to say that without these sophisticated techniques, it would not have been possible to conduct high-class basic research, leading to the production of pharmaceutical-grade biologicals for clinical use. One such technique for the production of specific antibody molecules that uniformly (clonally) carry the same three-dimensional structure (e.g. the same heavy and light chains, as well as the variable and hypervariable regions along with the same glycosylation pattern reacting exactly with the same epitope of the given antigen) was the discovery of the Nobel Prize-winning technique called ‘Hybridoma Technology’. However, before we dwell on the story of hybridomas, there is a lot to discuss about cytokines.

**Discovery of IL-1, TNF-α/cachectin and their receptors - Cohen’s (Nobel Laureate) and Jean-Michel Dayer’s contributions**

The history of the discovery of cytokines goes back to the early 20th century when tuberculosis and tuberculin research was gaining strength. The work of Zinsser and Tamiya and Arnold Rich in 1926-1927 on ‘permeability factor’ released by tissues sensitized by tuberculin might have been the first clear demonstration of what we call today, the ‘cytokine effect’. From 1957
to 1974, several investigating groups reported on the functional aspects of molecules secreted by ‘stimulated’ (sensitized) cells using different biological systems. Isaacs and Lindenmann while working with live influenza virus grown in chicken embryo chorioallantoic membranes observed that cells pre-treated with heat-inactivated influenza virus inhibited the growth of live influenza virus. They conjectured that the phenomenon was mediated by a protein released by cells in the chorioallantoic membrane that was pre-treated with heat-inactivated influenza virus. They named it ‘interferon’ (interference with the growth of the virus). Byron Waksman and Margit Matolfsky (1958) showed that tuberculin sensitization of macrophages, instead of causing damage, led to their stimulation (macrophage activation)\cite{10}, a mechanism earlier utilized by Freund and McDermott\cite{12} as an adjuvant for boosting an immune response to weak antigens. In 1966, migration-inhibitory factor that inhibited the migratory property of normal macrophages was discovered\cite{13,14}. Soon in 1968, several groups almost simultaneously discovered a protein secreted by stimulated lymphocytes, which they named ‘lymphotoxin’\cite{15,16} that was later renamed as ‘TNF-β’\cite{17}. Dudley Dumonde (1969) is credited with the use of ‘lymphokine, monokine’ terminology\cite{10,18}. He defined them as non-antibody mediators of cellular immunity generated by lymphocyte activation possibly pointing to lymphocytes of the innate immune system. While working in the laboratory of Byron Waksman in 1972, Igal Gery, described a ‘lymphocyte-activating factor’ (LAF)\cite{19}. Soon after in 1974, Cohen introduced the term ‘cytokine’ as discussed above. Indeed, the saga of cytokines is replete with the names of Cohen and Byron Waksman; the former earned the Nobel Prize, while the latter went onto become a legendary physician-immunologist from New Haven, Connecticut, USA.

Besides the basic scientists including biochemists and molecular biologists, many of those who contributed significantly in this field were clinicians interested in autoimmune diseases. At this point in time, several new departments got established around the world with the designation ‘Rheumatology and Clinical Immunology’ or vice versa. Some of the prominent names include Division of Clinical Immunology and Rheumatology, University of Alabama, Birmingham; Department of Rheumatology and Clinical Immunology, Charité - University Medicine Berlin; UMC Utrecht Rheumatology and Clinical Immunology; Amsterdam Rheumatology and Immunology Canter; Rheumatology and Clinical Immunology, University of Groningen, The Netherlands, and many others. For those engaged in this field, RA was the main disease that needed to be better understood for developing effective treatment modalities. Incidentally, joints are relatively easily accessible for obtaining tissue (pathologic synovial membrane) for deciphering the pathogenesis of synovitis and pannus that erodes the cartilage and bones in joints. Obviously, synovial tissue was the earliest to be grown in tissue culture to understand its physiology and pathology in diseases such as RA. In this regard, the work of Vauber\cite{5} in successfully culturing the synovial tissue in the laboratory has already been mentioned.

Till the late 1960s, the pathogenesis of RA was being debated from gum infection (this theory has seen great revival in recent times with Porphyromonas gingivalis as the possible culprit) to abnormal extracellular matrix (ECM) caused by alterations in enzymes, proenzymes and possible auto-activation, exposing the altered antigens of ECM and thus causing autoimmunity. It needs to be reminded that in 1962, interstitial collagenase (now called metalloproteinase-1 or MMP-1) had already been discovered along with its property of altering tissue structure\cite{20}. The legacy of ‘abnormal extracellular matrix’ in those days had translated in the name of ‘collagen diseases’ that we call today systemic immunoinflammatory diseases (two categories: autoimmune inflammatory diseases and auto-inflammatory diseases). However, advances in enzymology (1970s-1980s) and their application in RA refuted any direct role of altered enzymes in ECM damage. One had to go back to the ‘cells in rheumatoid synovium’ especially the pannus that was central to the process of joint destruction. However, the burning question at the time was ‘How do cells in the pannus (immune cells, inflammatory cells) cause damage to the ECM?’

In 1974, Dayer et al\cite{21,22} from the Faculty of Medicine, University of Geneva, Switzerland, joined the famous Arthritis Unit at Massachusetts General Hospital headed by Krane. Here, Dayer and colleagues studied the synovial cultures from patients with RA and discovered that synovial fibroblasts were able to produce large amounts of collagenase and prostaglandin E2 (PGE2). They demonstrated that physical contact of synovial fibroblasts with mononuclear cells was essential for the accelerated production of collagenase and PGE2, a process that becomes autonomous (start to behave like tumour cell) with the passage of time. They succeeded in purifying a small 15 kDa molecule that acted as the sensitizera
stimulator for the synovial fibroblasts to produce large amounts of collagenase and PGE2. The molecule was termed ‘mononuclear cell factor’ or MCF\textsuperscript{21,22}. Based on the detailed chromatographic studies, MCF was re-christened as IL-1 at the Second International Lymphokine Workshop held in 1979 in Ermatingen, Switzerland\textsuperscript{23}. Again, while analyzing the respective function of mononuclear cell subpopulations, Nardella \textit{et al}\textsuperscript{24} demonstrated that T-lymphocyte-stimulated monocyte-macrophages (MΦ) produced MCF, thus establishing the sequential pathways from TL → MΦ → synovial fibroblast activation, where direct physical contact of the mononuclear cells was essential. This paradigm for the joint disease in RA still holds true even after >40 yr\textsuperscript{25,26}. The contribution of humoral immune response (B-cell involvement) was later added to the schema when self-associating IgG isotype rheumatoid factor was shown to directly stimulate MΦ that activate synovial fibroblasts without the requirement for T-cells\textsuperscript{24,27}. Seckinger \textit{et al}\textsuperscript{28} also discovered the first IL-1 receptor antagonist (IL-1Ra) and subsequently described how a natural cytokine antagonist blocked the binding of another cytokine from the same family. Thus, the principle of the competitive binding assay of the IL-1 inhibitor to IL-1 at the receptor level was developed. The observation was crucial for researchers at Synergen, a biotechnology company at Boulder, Colorado, USA, who purified and cloned IL-1Ra in 1990\textsuperscript{10}. Cloning of cDNA of IFN-α and IFN-β in 1980 and of cDNA of T lymphocytes (TNF-β) and TNF-α and IL-1 was achieved in 1984, followed by that of IL-1Ra in 1990. These were remarkable discoveries, and since then, the recombinant molecules and specific mAbs have revolutionized the field of signal transduction and the dissection of the hierarchy of different cytokines depending on the type of disease and therapeutic approaches. A key observation of Dayer \textit{et al} was related to the behaviour of synovial fibroblasts in RA\textsuperscript{21,22}. As already mentioned, he showed that normal synoviocytes exposed to MCF show striking morphological changes, become elongated with modified spatial structure and start producing large amounts of collagenase (MMP-1) and PEG2. Such modified cells are called synovial fibroblasts. More importantly, Croft and colleagues\textsuperscript{29} demonstrated that after a period of continuous exposure to MCF, this subpopulation of synovial fibroblasts switches to an autonomous pattern of cell growth with evidence for the expression of different genes and a variable pattern of response to different cytokines. One wonders that with such elegant work of Dayer, (Fig. 1) was it simply bad luck that later studies involving inhibition of MCF (later renamed IL-1) could not find a discernible effect in controlling rheumatoid disease? However, that is a different story (discussed below).

**Coining of the term TNF - work of Lloyd Old’s and Anthony Cerami’s teams including Jean-Michel Dayer and Bruce Beutler (Nobel Laureate)**

There has been some confusion among biologists regarding the discovery and coining of the term TNF. In hindsight, the reason for this confusion is easy to understand. Historically, it was Carswell \textit{et al}\textsuperscript{30} at the Memorial Sloan-Kettering Cancer Center, New York, who published their seminal work in the prestigious ‘Proceedings of the National Academy of Science USA’ in 1975. Using the model of sarcoma Meth A and other transplanted tumours caused by endotoxins, they demonstrated that mice primed with substances inducing non-specific hyperplasia of the reticuloendothelial system (e.g. BCG, Zymosan, Corynebacteria) release a substance that caused haemorrhagic necrosis mimicking the effect of endotoxin itself. They gave it a name, ‘tumour necrosis factor’ or simply TNF, not the best of names yet; this is the name being used until today. Being an experimental finding in mice, it failed to receive the desired attention of the researchers. Exactly 10 years later, Beutler \textit{et al}\textsuperscript{31} at the Rockefeller University, New York (just across the street from Lloyd Old’s laboratory at the Memorial Sloan-Kettering), published the first demonstration of the important role of TNF in the pathogenesis of disease. The significance of the work done by Beutler and his colleagues on TNF was elegantly emphasised by Jan Vilček in 2008.\textsuperscript{32} Having something to do with disease pathogenesis, the discovery of TNF caught the
imagination of basic researchers and clinicians alike around the world. There are two important points about the paper by Beutler et al\textsuperscript{31}: (i) the first author Bruce Beutler together with Jules A. Hoffmann went on to share the 2011 Nobel Prize in Physiology or Medicine for ‘their discoveries concerning the activation of innate immunity’, (ii) the paper appeared exactly 10 yr after the report from Lloyd Old’s Laboratory that had described and used the term ‘TNF’. In all fairness to Beutler and colleagues, they had simultaneously used the term cachectin and TNF in this 1985 paper. Cerami must be credited for building a team of brilliant biologists and immunologists that had besides Beutler, others including the famous Swiss scientist Jean-Michel Dayer (his work already described above). This group published a series of papers describing a lipoprotein lipase-suppressing hormone secreted by endotoxin-induced RAW 264.7 cells that they named ‘cachectin’. Nevertheless, they also mentioned the name TNF for the same molecule\textsuperscript{33-35} (Figs 2 and 3).

Most endeavours of great value require some inspiration, a lot of perspiration and a little bit of luck. Cerami’s main work was on ‘Neglected Tropical Diseases’ for which he frequently travelled to Africa making Kenya as his base station. There he became interested in the dramatic wasting in cattle suffering from African sleeping sickness caused by protozoa, \textit{Trypanosoma brucei} spread by Tsetse flies. He established that the blood of these cattle carried a molecule that caused wasting. Further work on this molecule led to the discovery of ‘cachectin’. It was then argued that if a neutralizing antibody to cachectin is administered, the cachexia (and the other ill effects of cachectin) could be reversed. However, most devastatingly, in his experiments in Kenya, the administration of antibodies against cachectin to cattle suffering from ‘sleeping sickness’ caused their death. The failed experiment was a great deal of disappointment for team-Cerami\textsuperscript{36}. The failure of cachectin/TNF antibody in the treatment of sepsis became one of the main stumbling blocks for several investigators in later years including Ravinder Nath Maini and Marc Feldmann when they wanted a small amount of mAb against TNF for human trials in another disease namely RA. Most Biotechnology Companies did not agree to initiate clinical trials in humans. In later studies on the use of anti-TNF in sepsis in mice, it became clear that the issue had to do with the timing of administration of antibodies; given before the onset of sepsis, it completely prevented the clinical features but not in an established infection\textsuperscript{31}. However, by then, the reputation of mAb as therapeutic agents had taken a hit.

\textbf{Purification and cDNA cloning of TNF: Meeting of 25 scientists at the Memorial Sloan-Kettering Cancer Center, New York, December 1984 - Lloyd Old and Jan Vilček to the fore}

Vilček\textsuperscript{37} had just completed medical graduation in 1958 in Bratislava (in the Old Czechoslovakia) when he attended talks by Alick Isaacs, the co-discoverer of INF, and Albert Sabin, best known for the development of live polio vaccine. He got highly motivated and started working in the field of INFs. While still in Bratislava, he published a brilliant paper on INF in the next three years\textsuperscript{38}. In 1964, he migrated from communist Czechoslovakia to the United States and joined the New

\textbf{Fig. 2.} Dr Anthony (Toni) Cerami (Photograph personally provided for use in this article).

\textbf{Fig. 3.} Dr Bruce Beutler (\textit{Courtesy}: Brian Coates for UT Southwestern medical Center).
York University (NYU) School of Medicine as Assistant Professor to fulfill his zest for advanced basic research in biological systems. In 1982, his team had already done advanced research in INFs describing the two types, IFN-α and IFN-β (now known as type-I INFs). During the production of these INFs by mononuclear cells by the prevailing laborious cell culture methods of that time, his team observed an additional molecule that they called ‘immune INF’ (or INF-γ now known as type-II INF). On further analysis, there were two different molecules; one produced by the lymphocytes and the other by monocytes. In 1984, his team named the former as ‘lymphokine’ (was also known as TNF-β) and the latter as ‘monocyte-derived cytotoxin’ that subsequently turned out to be the classical TNF now called TNF-α\(^39\). This was a unique effort involving nine scientists from three different laboratories across USA (Department of Microbiology, NYU School of Medicine; Developmental Hematopoiesis Laboratory, Sloan-Kettering Institute for Cancer Research, New York; and the department of Protein Biochemistry, Genentech, Inc., South San Francisco, California, USA). In December 1984, Lloyd Old and colleagues at the Memorial Sloan-Kettering Cancer Center organized a small workshop that was attended by scientists interested in TNF and lymphokines. It included several authors of the above-mentioned paper including Bharat Bhushan Aggarwal of the Protein Biochemistry Department of the Genentech Corp, California, USA. It was during this workshop that Bharat announced the seminal work on the first complete amino acid sequence of human TNF, the partial sequence of human lymphotoxin protein and cDNA sequences, the chromosomal location of the TNF and lymphotoxin structural genes by his colleague scientists at the Genentech\(^40,41\). Since that historical workshop, Vilček developed an active ongoing collaboration with Genentech, including one with Bharat Aggarwal and David Goeddel, who provided samples of the pure form of recombinant human TNF. This greatly facilitated studies on understanding the biological function and mechanism of action of TNF in Vilček’s Laboratory. Till then, the only thing known about TNF-α was their cytotoxic action on some tumours and cachexia in chronically infected animals\(^35\). For deciphering the biological actions of TNF, it was essential to study different cells to find out which ones had on their surface specific high-affinity binding receptors. Two easily available human cell lines in Vilček’s laboratory were (i) the HeLa cell, an immortal cell line from a patient with cervical cancer who had died in 1951 (HeLa cell line is one of the most common and the oldest human cell line available in tissue culture and is used widely in laboratories around the world), (ii) FS-4 strain of human diploid fibroblasts cell-line that was grown in Vilček’s Laboratory\(^42\). These cells showed high-affinity receptors for TNF. Further experiments in Vilček’s laboratory showed that INF-γ synergizes the cytotoxic action of TNF by increased expression of TNF receptors\(^43\). In the case of human diploid FS-4 fibroblasts, an exposure of even nano mol strength of TNF caused a stunning change in the cell morphology, which could be noticed even under a light microscope. The fibroblasts became elongated with a change in their spatial orientation. A student at Vilček’s Laboratory, Vito Palombella, demonstrated similar morphological changes even in normal human fibroblasts exposed to TNF\(^44\). Lin and Vilček also showed that like other growth factors, TNF led to an increased expression of c-Fos and c-Myc mRNA in human fibroblasts\(^45\). Their subsequent work showed that TNF induced a large number of genes in different human cells and was also a strong inducer of a molecule that later came to be known as IL-6, a strong inducer of inflammation including acute-phase reactants\(^46\) (Figs 4 and 5).

**Nature’s experiment: Multiple myeloma-The natural monoclonal antibodies (mAbs)**

The story of the discovery of biologicals for clinical use cannot be complete without mentioning the legendary clinical immunologist-physician Henry Kunkel (of ‘Kunkel-girls’ fame, ‘guru’ of a whole generation of American clinical immunologists and autoimmune experts in 1970s-1980s) of Rockefeller Institute, New York. In 1951, he had made a unique

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**Fig. 4.** Dr Jan Vilček (Photo credit: Peter Hurley, provided by Dr Jan Vilček personally for use in this article).
observation that malignant plasma cells harvested from the bone marrow of patients with multiple myeloma not only had their antibody-making machinery intact but that also all cells produced identical antibody molecules in every parameter tested. Serum electrophoresis of these patients showed a ‘monoclonal (M)-spike’. Thus, myeloma was recognized as one of the ‘monoclonal gammopathies’, and myeloma cell lines became the main source of studies on antibodies. This was made even simpler by a serendipitous discovery by Michael Potter (a molecular biologist at the NCI, Bethesda) who in 1962 reported that injecting mineral oil in the peritoneal cavity of a particular strain of mice (BALB/c) induced the growth of myeloma cells. His laboratory thus became a source of several lines of myeloma cells for researchers around the world. The propagation of these cells was made easier by establishing an in vitro culture technique by Kengo Horibata and AW Harris (under the supervision of Melvin Cohn at the famous Salk Institute, San Diego). These cells continuously produced antibodies in a clonal pattern using different heavy chains and light chains specific for each clone. However, the exact specificity of these antibodies could not be established. Indeed, determining their specificity out of a billion possible antigens and their epitopes would have been like finding a needle in a haystack. The principal question in the mind of most researchers of the time was, ‘Could one re-direct the clonal antibody producing machinery of these immortal myeloma cell lines to make antibody to a single specified epitope’? That way one could solve all the difficulties of purifying polyclonal antibodies produced in either horses or small laboratory animals and make them relatively specific for directed research on cells and molecules.

Path-breaking discoveries by Sinkovics, Askonas, Klinman and Niels Jerne on the generation of monoclonal antibodies (mAbs)

In 1970s, most investigators in the field of antibodies and antibody-producing cells (later called B cells) had recognized the prime need for producing large amounts of antibodies with known specificity to a particular antigen, unlike myeloma cell lines where specificity of the antibodies remained unknown. Some of the prominent names engaged in this research included Joseph Sinkovics, Brigitte Ita Askonas and Norman Klinman.

Sinkovics, a Hungarian immunologist based at the M.D. Anderson Hospital and Tumor Institute in Texas, reported in 1970 successful natural fusion of a splenic plasma cell with the mouse lymphoma cells. Although the secreted antibodies were highly specific and functional, their work failed to catch the imagination of others in the field of cell fusion. The credit for using the term ‘hybridoma’ goes to Sinkovics who generated a hybrid cell that was capable of producing mAb and had imbibed the quality of immortality of a malignant cell.

Brigitte Ita Askonas was an Austrian by birth. However, with the increasing Nazi influence and after a short stay in Paris, she migrated to Montreal, Canada, to work as a researcher at the McGill University and obtained her degree in Biochemistry. After a stint at the Basel Institute of Immunology, Switzerland, she moved to Cambridge University, UK, where she worked for her PhD. Finally, she moved to the National Institute of Medical Research, Mill Hill, London, in 1952 and worked in the chemistry division till 1989. She was interested in the study of antibody synthesis in vivo. While working with myeloma cells and through the background knowledge of Kunkel’s work in the 1950s and her own vast experience in biochemistry, she was the first to elucidate biochemical steps involved in the synthesis of immunoglobulin molecules within the antibody-producing plasma cells (progeny of B-cells). She was also the first to demonstrate that a single antibody-producing cell clonally produced a single (monoclonal) type of antibody, the work for which she was elected a Fellow of the Royal Society in 1973. While at Mill Hill, she made several other seminal discoveries related to antibody heterogeneity. Historically, she was the first scientist to have produced a mAb in the laboratory. For this work, she used the genetically identical irradiated mice to produce the mAbs. However, for reasons not obvious, her work failed to receive the due recognition. One possible
reason could have been that these antibody-producing clones were fragile, not surviving for an adequate length of time to be used for bulk production of mAbs (Fig. 6).

Norman Klinman, an American immunologist, based at the University of Pennsylvania with joint attachment to the Wistar Institute, published a technique in 1969, which he called ‘monofocal antibodies’. He used irradiated mice that were injected with fresh cells with antibody-producing capacity, some of which ‘homed’ in the spleen. This spleen was cut in small cubes and placed in tissue culture that contained a specific antigen. The hypothesis was that the spleen fragment that contained a specific antibody-producing cell would produce antibodies specific to that antigen and these could then be harvested from the culture supernatants and used for experiments.

Although Niels Jerne (First Director of the Basel Institute of Immunology) was not directly involved in the development of therapeutic mAbs (biological disease-modifying drugs or bDMARDs), his ‘Natural Selection Theory’ of antibody specificity had put him among the best minds in the field of immunology. He shared the 1984 Nobel Prize with Köhler and Milstein for their work on the important contribution of theory and practice in shaping our understanding of the body’s immune system (Figs 7 and 8). Jerne’s haemolytic plaque technique (in soft agar plates) was an elegant method of studying single mAb-producing cells and their biology. The technique was widely used to study the monoclonality and specificity of the produced antibodies [The first author (ANM) while working at the New England Medical Centre, Boston, in 1968, used the Jerne-plaque technique extensively for his work on ‘Lifecycle of antibody-producing cells’].

César Milstein and Georges J. F. Köhler: Hybridoma technology and production of monoclonal antibodies (mAbs) - The 1984 Nobel Prize

Much of the story described in this section has been comprehensively narrated by Lara Marks, a summary of which is recounted here. César Milstein, an Argentinian biochemist (1927-2002), had graduated from the University of Buenos Aires and obtained his PhD in 1956. Funded by the British Council, he moved to the United Kingdom in 1958, became a naturalized British citizen and joined the Biochemistry Department of Darwin College, University of Cambridge. He also had a short-term appointment with the Medical Research Council (MRC). From the late 1960s, his
research revolved around the fascinating subject of antibody diversity and the mechanism of its generation. The ‘germline’ versus ‘somatic-mutation’ theory was indeed the moot question under great debate by the immunologists of the day. Those were also the days when the first author of this paper (ANM), undertook a ‘primer’ course in immunology in Boston and was thus privy to hot debates on ‘germline versus somatic-mutation’, the two theories being the major basis of antibody diversity\textsuperscript{57}. Based on simple common sense, the famed American molecular biologist, Joshua Lederberg in 1959 argued in favour of somatic mutations as the basis of antibody diversity because otherwise Nature cannot be wasting innumerable genes only to produce a diverse range of antibodies. Soon after, Brenner (Harvard) and Milstein (Cambridge) published the now-famous ‘Brenner-Milstein model’ for the generation of antibody diversity\textsuperscript{58}. For further details, one might refer to the landmark paper on the life history of César Milstein published by Neuberger\textsuperscript{59}.

In the early 1970s, Dick Cotton a postdoctoral fellow investigator from Australia with interest in immunogenetics joined Milstein’s Laboratory, and together, they perfected the technique of cell fusion using a variety of myeloma cell lines. The technique involved the use of inactivated Sendai virus that he obtained from Abraham Karpas working in the laboratory next door, who used it to promote cellular fusion. In 1973, Milstein presented their work on myeloma cell fusion at the Basel Institute of Immunology (Niels Jerne was the Director of the Institute). In the audience was a young German biologist, Georges Köhler, who was completing his PhD from the University of Freiburg. Excited by Milstein’s work, Köhler joined his research team in Cambridge in April 1974 as a postdoctoral scientist to carry forwards the research on antibody diversity. For proving the hyper-mutation theory, they needed immortalized antibody-producing cell lines. By this time, Milstein had already obtained Potter’s mouse myeloma cell line (MOPC21) that Horibata and Harris had used to establish long-term tissue culture during the early 1970s\textsuperscript{60}. Working with Brownlee, Milstein was able to establish the myeloma cell line, but the problem was that these were making antibodies against unknown antigens as discussed earlier.

By the time Köhler joined Milstein’s Laboratory, the hybridoma technology had already been established in several laboratories around the world. However, these two were struggling to discover a hybrid cell that must have three basic properties: (i) induction of immortality for synthesis of an antibody molecule, (ii) it must have the properties of producing a single antibody molecule, i.e. a specific mAb, and (iii) it should be able to grow in tissue culture for indefinite periods. Using the techniques of isolating single antibody-forming cells from sheep red cell immunized mice spleen fragments (Klinman technique described above) and the already established cell-fusion technique with Sendai virus perfected in their laboratory by Dick Cotton and Abraham Karpas (with skilled technical assistance provided by Shirley Howe), Köhler and Milstein succeeded in producing ‘hybridomas’ that could make mAbs against certain antigens of sheep red cells. With such positive results in their hand (proven by using the Jerne haemolytic plaque technique), these two biologists suddenly realized that they had successfully discovered a tool that everybody else had been striving to make for many years. In critical scientific terms, they had developed an immortal antibody-producing cell line that was capable of producing an endless supply of identical antibodies with known specificity. The method would soon become popular under the name ‘hybridoma technology’. The antibodies thus produced were named ‘mAbs’ signifying that they were derived from a single hybrid cell. Köhler and Milstein\textsuperscript{61} published their epoch-making discovery in 1975 for which they were awarded the Nobel Prize in 1984, which they shared with Niels Jerne who had laid down the theoretical framework years before their discovery. Although these hybridomas could be propagated indefinitely in large amounts, there was a small problem due to the dwindling supply of Sendai virus in Milstein’s Laboratory. Further, the Sendai virus inactivation and its use for cell fusion were tedious. As luck would have it, Giovanni Galfré, a postdoctoral student who had recently joined Milstein, resolved the issue by using polyethylene glycol (PEG), a chemical that had been used successfully in other cell-cell fusion experiments. Galfré’s introduction of PEG significantly enhanced the hybridoma technology for generating immortal mAbs on an unprecedented scale. The discovery of mAbs heralded a major advance over the conventional polyclonal antibodies.

\textbf{Saga of monoclonal antibody (mAb) patent - A jarring note in a flawless melody}

In 1975, Milstein presented his work on hybridoma technology at an internal meeting of the MRC, UK, the laboratory where this discovery was made. Tony Vickers, a scientist by training, was working as an official in the
Administration Department of MRC\textsuperscript{47}. Realizing the immense commercial value of the discovery, he alerted the National Research Development Corporation (NRDC), the body responsible for patenting MRC inventions. To the great disappointment and surprise of the MRC administration, the reply from NRDC (dated 7th October 1976 later tracked down, the details of which can be accessed\textsuperscript{47}, the subject heading of the NRDC reply letter was ‘Continuous Culture of Fused Cells’) was rather disheartening. It read and we quote\textsuperscript{47} ‘It is certainly difficult for us to identify any immediate practical application which could be pursued as a commercial venture,.....and it is not immediately obvious what patentable features are at present in the Nature paper’. In hindsight, this misjudgement about hybridoma technology might have caused a loss of billions of dollars to British economy!

In those early days, investigators took ethics in science extremely seriously and any thoughts about the commercial exploitation of a discovery would have been considered ‘sinful’. Indeed, there was free exchange of thoughts/ideas and research methods among scientists across the world with little worry about intellectual property or methodological copyrights. In such a scientific atmosphere of free exchange of ideas and material, Milstein in September 1976 received a request from Hilary Koprowski, a scientist working at the Wistar Institute, Philadelphia (founded in 1892 and now a NCI-designated Cancer Centre affiliated to NYU since 1972), for parting with some of his hybridoma cell lines. In the spirit of ‘free exchange in science’, Milstein immediately dispatched a hybridoma cell line to Koprowski; and the rest, as they say, is history\textsuperscript{47}. Koprowski (1913-2013) was a Polish scientist who pioneered the development of oral polio and modern rabies vaccines. In the history of the discovery of mAbs, it is mentioned that Hilary Koprowski, Carlo Croce and Walter Gerhard were granted two patents (October 1979 and April 1980) for making mAbs against tumours and influenza virus. This was the first time ever that the scientists were granted any patents for producing mAbs. Almost simultaneously, Koprowski also co-founded Centocor, one of the first biotechnology companies for commercial production of mAbs for diagnostics and therapeutics [‘Remicade’ (infliximab), the first biological used in the treatment of RA is a Centocor product). It was a pity that Köhler and Milstein’s contributions were not acknowledged. It may be mentioned that 1970s were the days of political and economic anxiety for Britain. The failure to patent such an important discovery raised a political storm in the country that ultimately reached the then Prime Minister Margaret Thatcher’s (a chemist by training) desk. To say the least, she was furious; a great opportunity was missed by Britain\textsuperscript{47}.

**Monoclonal antibody (mAb) against TNF-\(\alpha\), ‘Infliximab’**

Vilček’s contributions to the development of mAbs against TNF-\(\alpha\), infliximab - the first mAb ever to be therapeutically used in human have been immense\textsuperscript{37}. Much of this and the story of Centocor Laboratories in Malvern, Pennsylvania, and the role of Kapowski at Centocor, has been described above. Michael Wall, the founder of Centocor and its future Director, was well known to Vilček through an earlier collaboration with a small life science company working on IFN-\(\beta\) (old name lymphotoxin). These gentlemen were seeking intellectual support from the academia for developmental plans of their new company. That was the year 1983. Discussions led to the idea of generating different mAbs through scientific collaboration to be exploited by the company commercially (possibly in developing enzyme-linked immunoassay utilizing mAbs). It is of note that Vilček had also mentioned their possible therapeutic use in human\textsuperscript{37}. Thus, came about a convenient financial arrangement where Vilček’s Laboratory would produce the monoclonals and Centocor would exploit them commercially and in return, give a grant to Vilček’s Laboratory for a postdoctoral position and also pay royalties to NYU. As luck would have it, Junming (Jimmy) Lee an expert in mAb technology joined on a Centocor grant. He produced the first mAb against human IFN-\(\gamma\) in Vilček’s Laboratory. By the late 1980s, Centocor wanted to diversify into therapeutic use of mAbs. With the background work of Cerami and colleagues from mid-1980s on the role of TNF in bacterial sepsis, and the combined efforts of Vilček’s laboratory and several scientists from Centocor led to the production of a human-mouse chimeric mAb (called cA2) against human TNF in early 1990s. However, it failed to produce any clinically positive effects in patients with septicemia and, therefore, did not get FDA approval. The work had to be diverted to other mAb development that would be therapeutically useful. In 1993 two scientists from the Kennedy Institute of Rheumatology, London, (RN Maini & M Feldmann) approached Centocor to part with some of their cA2 mAb against human TNF. The rest was again history. Centocor became a billion-dollar company and the work of Maini and Feldmann opened a whole new era
of targeted drugs\cite{37}. Ever since the success of anti-TNF biological therapy, developing drugs without known mechanism (found empirically to be effective, e.g. low-dose methotrexate for the treatment of RA) of action has been abandoned. Targeted therapies are being developed for only those diseases where the cellular-molecular mechanisms of pathogenesis are known.

**First use of ‘infliximab’ in clinical medicine - Maini and Feldmann show the way**

In the late 1960s and early 1970s, Maini et al\cite{61} and Feldmann and Basten\cite{62} working at the Kennedy Institute of Rheumatology, Imperial College, London, had noted remarkable biological activities in the supernatant of immune cells grown in tissue culture. The technologies of the time were not advanced enough for an in-depth analysis of molecules from these supernatants. By the mid-1970, the technology of cDNA cloning reflecting the mRNA for any naturally produced molecule became available and this simplified the study of cytokines in tissues in health and disease. Maini being a rheumatologist and a keen student of immunology teamed up with Feldmann for an in-depth study of the synovial tissue and of cytokines in RA. Since joint replacement surgeries, occasional biopsies and frequent joint aspirations are routine in a department of Rheumatology; this made their task easier for they could obtain samples of pathological synovial tissues easily. By this time, the synovial tissue culture techniques had already been established since the seminal work of Vabuel\cite{5}. With some local modifications, it became possible for Buchan et al\cite{63,64} and Feldmann et al\cite{65} to study the role of cytokines generated by cells of the synovium from RA patients by mRNA expression. They observed a spectrum of inflammatory cytokines including TNF, IFN-γ, IL-1, lymphotoxin, IL-6, and granulocyte-macrophage colony-stimulating (GM-CSF) factor in the culture supernatants. Interestingly, the synovial cells had the ability of continuous production of cytokines, which is much in contrast to the transient appearance of most cytokines in a normal physiological process. This phenomenon was termed ‘dysregulation of cytokine production’ (transient physiological vs. continuous pathological production). Unlike previous studies on synovial tissue cytokines (Dayer, 1976 - mentioned in details earlier\cite{10}), released by FLS, Maini and Feldmann used enzymatically treated synovial cells which could survive for up to a week in culture. This novel technique permitted them to study cytokine dysregulation and overproduction in more details. By this time (late 1980s and early 1990s), several laboratories had generated mAbs against most of the known cytokines. For example, Michael Shepard generated a mAb against TNF at Genentech Laboratories USA, which he generously provided to Maini and Feldmann for their research. Using this neutralizing antibody against TNF, the latter group of investigators observed a most unusual phenomenon, i.e. blocking of TNF inhibited the synthesis of several other important proinflammatory cytokines in cell culture. This led to the pivotal concept that TNF was at the ‘apex’ of a cascade of inflammatory cytokines in the pathogenesis of RA. These findings were published in a seminal paper in 1989 whose first author was Fionnuala Brennan\cite{66}, a postdoctoral fellow working in the team. The implications of this finding were stunning; targeting a single cytokine leads to complete suppression of the inflammatory cascade in the synovial tissue from RA. Using the hamster anti-mouse-TNF (provided by Bob Schreiber), Richard Williams, a PhD student provided the proof of concept in the mouse that administration of anti-mouse TNF antibody ameliorated collagen-induced arthritis\cite{67}. The next obvious step was to initiate clinical trials using anti-human TNF antibodies in RA patients (Fig. 9).

Despite scientific evidence of protection with anti-TNF antibodies against endotoxin shock in mice\cite{31}, Cerami’s\cite{36} unsuccessful experiments with cattle sleeping sickness in Africa had made most biotech companies wary to provide their anti-human TNF monoclonals for clinical trial in humans. That was the main stumbling block faced by Maini and Feldmann in their quest for testing the efficacy of anti-TNF mAb in RA. Fortunately, Centocor Company in Philadelphia

**Fig. 9.** Sir Ravinder Nath Maini and Sir Marc Feldmann (Courtesy: Sir Marc Feldmann and reproduced with permission from The Lasker Foundation).
in collaboration with Vilček’s group at NYU got interested in producing mAbs for ELISA test kits and for treating human disease. They hired James N. Woody, an ex-student of Maini and Feldmann as the Chief Scientific Officer at the Centocor and this facilitated the availability of anti-human anti-TNF mAbs for a small clinical trial on 10 patients with RA. The trial conducted in May 1992 onwards produced dramatic results. Patients showed marked clinical improvement as well as decrease in inflammation, i.e. the acute phase reactants, which normalized rapidly. This grand success of Centocor’s anti-TNF mAb, given the name ‘infliximab’ was publicly announced by Marc Feldmann in September 1992 in a small group meeting organized by David Naor in Arad, Israel, and the results were published.68. Several clinical trials followed in quick succession proving high efficacy of infliximab in the treatment of RA.69-72. A remarkable aspect of the clinical benefits was that the drug stopped the appearance of new erosions and some of the existing erosions started to heal. Such an effect had never been seen with any previous drug used for treating RA.

Conclusions

While in the past, most scientific discoveries of note were serendipitous, quite often with a ‘Eureka moment’ in the life of a scientist, there has been a major paradigm shift in recent years. For example, a window of the laboratory of Alexander Fleming’s in St Mary’s Hospital Medical School, Imperial College London, was left open overnight. Next morning, Fleming saw a small clean halo on a bacterial culture plate. At the centre of the halo was Penicillium notatus, a fungus producing penicillin. That is how the antibiotics were discovered, and Fleming was awarded Nobel Prize in 1945 for the discovery (shared with Chain and Walter). Since those early days, there has occurred a major paradigm shift from ‘empirical’ to ‘mechanistic’ approach where the disease mechanism is studied for abnormalities/dysregulations, zeroing in on the most rational target to be hit for normalizing the dysregulation. Over time, step-by-step, one-after-the-other, newer technologies, newer molecular paths in physiology, newer regulatory circuits, newer possibilities of modulating cellular and molecular functions, were discovered. Using this background knowledge and steps involved in the pathological (disease) process, the treatment targets are identified, and drugs targeting those molecules developed. In such a scenario, it becomes difficult to pinpoint a single individual’s effort for the development of a drug with profound effects, never seen before. With sound reasoning and proven scientific basis, clinician(s) who use the drug for the first time are often remembered as the discoverer(s) of that drug. The story of the development of infliximab is a typical example of such a drug discovery. Decades of work in different areas of biology, biochemistry and biotechnology carried out by scores of dedicated scientists made it possible to develop a medical-grade mAb against TNF-α. The rheumatologist-immunologist team at the Imperial College, Kennedy Institute, London, led by Mani and Feldmann, would always be remembered, not only for their path-breaking discovery that TNF-α was at the centre of a cascade of inflammatory cytokines in the pathogenesis of RA, but also to be the first to actually use it clinically and demonstrate its high efficacy never seen earlier with any treatment.

The therapeutic success of infliximab opened the door for clinical use of mAbs in medicine and provided a major boost to the Pharma companies for developing additional targeted therapies for a variety of diseases. The public announcement of the high efficacy of infliximab in patients with RA caught the imagination of a large number of biotechnology companies. They could now divert their attention from the disastrous pursuit of infection and septic shock towards diseases where dysregulated cytokine network had been demonstrated such as RA, spondyloarthritis, and other similar systemic immunoinflammatory diseases including inflammatory bowel disease, psoriatic arthritis and juvenile idiopathic arthritis. Without that public announcement, this epoch-making discovery could have gone unnoticed, hidden in the pages of some of the academic journals.

Among the 10 best-selling drugs of 2015, at least six were mAbs.73 Most of the present generation of physicians may not be aware that the discoverer of mAb for therapeutic use was a team of rheumatologist-immunologist-clinicians at the Imperial College, Kennedy Institute of Rheumatology, London, headed by Sir Ravinder Nath Maini and his long-time colleague, Sir Marc Feldmann. For his outstanding contribution to science and medicine, Maini has been honoured with several high awards that include the Crafoord Prize (2000), Albert Lasker Award for Clinical Medical Research (2003), Dr Paul Janssen Award for Biomedical Research (2008), Gairdner Foundation International Award (2014), while Prof. Marc Feldmann has been honoured with Crafoord Prize (2000), Albert Lasker Award for Clinical Medical Research (2003),...
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References


Introduction

The Indian Council of Medical Research (ICMR) has been at the forefront in setting up the ethical guidance for the conduct of biomedical and health research in India. The latest version of National Guidelines for Biomedical and Health Research Involving Human Participants, 2017 was planned in order to provide a more detailed guidance to the existing topics in view of emerging ethical concerns and to add a number of newer areas in which guidance was lacking. The scope of the guidelines has been expanded to include socio-behavioural research related to health and research involving biological material and datasets. The guidelines have 12 sections which cover a wide range of topics and areas of research. The first six sections are more generic, applying to all types of biomedical and health research, while the next six sections are more subject specific. The guidelines have been revised in consultation with a large number of experts and stakeholders and went through an exhaustive process stretching over a period of two years in its drafting, review, consultation and finalisation. This commentary seeks to explain the process and key components of the Guidelines.

National ethical guidelines for biomedical & health research involving human participants, 2017: A commentary

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The Core Advisory Group consisted of Vasantha Muthuswamy (Chairperson), S.D. Seth (Co-Chairperson) and members Nandini K. Kumar, N.K. Arora, Urmila Thatte, Vijay Kumar and Roli Mathur (Member Secretary)

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socio-behavioural research, conduct of research during emergency situations, use of stored biological material and data and others. In October 2015, the first meeting of the Core Advisory Group* set up by ICMR (Annex. 2.B)\(^4\) decided on the topics to be included in the latest revision and the approach to involve a variety of stakeholders in the process of revision. In order to ensure the widest possible participation, the core group appointed a sub-committee for each of the 12 identified topics, comprising of 48 members (Annex. 2.B)\(^4\). They were drawn from various research organisations and included trained bioethicists and ethics committee members, clinician and researchers, sponsors and the public. Following a series of meetings, an initial draft capturing the latest national requirements and global standards was circulated for comments and was posted on the ICMR website for a period of eight weeks to obtain comments from the public.

Efforts were made to consult with stakeholders from across the country to ensure responsiveness to health needs while accommodating our varied socio-cultural ethos. The WHO-Country Office India partnered with the ICMR Bioethics Unit and supported two consultation programmes at the regional and national levels\(^5\). The regional consultation programme was organised on October 4, 2016, at Bangalore and was attended by the relevant stakeholders from across various regions of the country, who provided valuable suggestions. At the National Consultation meeting held on December 14, 2016, in New Delhi, representatives of various public and private institutions, the relevant government departments and agencies, members of the Central Ethics Committee on Human Research (CECHR), international agencies, and others provided relevant feedback. Comments received from all these sources, through public consultation, both regional and national, were extensively discussed for updating of the draft document. In addition, a number of separate expert group meetings were held to get the relevant advice in specific areas such as ethical review procedures, public health research, socio-behavioural research, human genetic testing and research, clinical trials, new technologies, etc.

The National Ethical Guidelines for Biomedical and Health Research Involving Human Participants, 2017, were released on October 12, 2017 by the Hon’ble Union Minister of Health and Family Welfare at ICMR\(^4\). It is a detailed document covering a wide range of topics in which the existing chapters have been updated and new sections of current importance have been added (Table). The general principles in the present document have been simplified for easy understanding (Sec 1)\(^4\). The principles of social responsibility and environmental protection have been added in order to stress the need for protecting social and cultural harmony and conserving our limited resources in the conduct of biomedical and health research. In the General Ethical Issues section (Sec 2)\(^4\), the addition of the topic of risk categorisation will help ethics committees (EC) conduct a more objective benefit-risk assessment. The earlier version of the Ethical Guidelines had separate chapters on transplantation and assisted reproductive technologies, which were dropped in this version because they are more applicable to medical practice rather than to research.

Some topics dealt with in brief in the earlier version have been expanded into complete sections, such as those on informed consent, vulnerability, biological materials and biobanking. Newer sections were created to cover areas like the responsible conduct of research (including publication ethics), public health research, socio-behavioural research, and research during humanitarian disasters and emergencies. Another important inclusion in the revised guidelines is the introduction of research using datasets which has now been added to the section on Biological materials and Biobanking since the basic ethical requirements for both are common. The chapters on ethical review procedures, clinical trials, and genetics research have also been also elaborated considerably, and will be enhanced in future versions.

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RCR, Responsible Conduct of Research
helpful for researchers as well as for ethics committees (EC) in their day to day functioning.

Guidance was needed for researchers in the country regarding the responsible conduct of research (RCR) since there is a lack of formal education/training on this. The newly created section on RCR will help the scientist understand the measures required for data acquisition, management and sharing, collaboration (both national and international), responsible authorship and publication ethics (Sec 3). In the section on Ethical review procedures (Sec 4), each EC member’s affiliation, qualifications, role and responsibilities have been described to remove the existing confusion about their appointment, composition of the committee, and quorum. It is hoped that the document will help, especially the non-medical members, to have greater clarity about their roles and responsibilities and make their participation at EC meetings more meaningful and effective beyond just fulfilling quorum requirements. In addition, efforts have been made to harmonise and explain the differences between regulatory and non-regulatory/academic clinical trials. Clear guidance has been given regarding the setting up of independent ethics committees with special reference to when and how the services of other ECs can be utilised. Review of multicentre research has been a challenge in view of the varied requirements put forth by the different participating ECs. For the first time in India, the guidelines have proposed that a common EC may be identified from the participating sites to act as the main designated EC (Sec 4.2). This can have representatives from ECs of other participating sites and a common review can be carried out. It is hoped that this would greatly reduce the time and effort required for reviewing a common proposal at multiple sites and would help to initiate a dialogue among the concerned ECs and build an EC network with communication channels. In the long run, this would help to streamline and strengthen ethical review systems in the country.

The Guidelines advise ECs to undertake regular monitoring of research and explain conditions when site monitoring may be essential. Institutions are now requested to make adequate provision (manpower, infrastructure, funds) to run the ethics committee office smoothly. EC work should no longer be regarded as a part-time voluntary activity but as an essential function requiring protected time of the member secretary for efforts to improve EC efficiency. The Guidelines have explained the need for building quality EC systems, laying down conflict of interest policies, and stressed the need for registration of an EC as well as its participation in national or international recognition or accreditation programmes (Sec 4).

These Guidelines have not only highlighted the need for payment of compensation in case of research-related injury, but also suggested mechanisms for putting a system in place to make such payments. At present, only sponsored clinical trials may have the provision for paying compensation for research-related injury, since that is required by law. However, there is a complete lack of clarity regarding payment of compensation in academic, or investigator-initiated, or non-funded research. The institutions where research is conducted will now be required to create a corpus fund, or to seek insurance cover, or grants to cover compensation, if required to be paid to research participants.

There is an entire section on the informed consent process, detailing the information required to be effectively communicated for understanding and seeking voluntary consent of the participant (Sec 5). High risk research may require even a test of understanding. There is a description regarding use of electronic methods for seeking consent, waiver of consent, re-consent/fresh consent, consent under special situations involving gatekeepers, community and vulnerable groups obtaining assent for children and processes involved after obtaining consent etc.

The Guidelines describe the additional protections needed for conducting research involving vulnerable people (Sec 6). Besides women and children, others such as sexual minorities, sex workers, tribal populations, persons who are cognitively affected/impaired, those with reduced autonomy, terminally ill patients or those who are economically and socially disadvantaged may be vulnerable and this must be determined. The underlying principle is that since they are unable to protect themselves adequately they are prone to exploitation and need protection. The Guidelines also discuss the need to be inclusive so that no group is deprived of the probable benefits that are likely to emerge from research.

The clinical trials section has been expanded considerably and guidance has been included regarding investigator-initiated trials, academic research, student research, multicentre trials or those involving...
communities, or traditional systems of medicine or using new technologies etc (Sec 7)\(^4\). The importance of a priori arrangements for post-trial access and benefit sharing after completion of research has been highlighted and this is to ensure that the outcomes are translated into benefits and meaningful outcomes for participants or communities and do not remain limited to publication alone. It has been clarified that the clinical trials protocols for marketing approval of products need to follow the Drugs and Cosmetics Act 1940, and Rules, 1945, and the relevant amendments, from time to time\(^6\). The need for registration of all such trials under the Clinical Trial Registry of India has also been highlighted (Sec 7.1.10)\(^6\).

The epidemiology chapter of the earlier Guidelines has been replaced with the Public Health Research section (Sec 8)\(^4\). On this subject, there is an overlap between service and research and therefore ethical aspects are often not clearly understood. This section has provided specific guidance for the conduct and review of surveys, implementation research, demonstration projects, community trials, surveillance studies, program evaluation studies etc. Relevance of informed consent and EC review depending on type of research has been elaborated.

A new section on ethical aspects of social and behavioural research related to health has been included for the first time (Sec 9)\(^4\). In this area, there was lack of clarity about the requirements such as review by EC, informed consent and others. In addition, sometimes socio behavioural research involves research on sensitive topics or involves risk, which requires more guidance. The Guidelines discuss the need for community engagement whenever possible and to understand the requirements and health needs of the participants. They suggest the need for reaching out to leaders, community advisory boards, or community representatives or having them participate in EC discussions so that the research is more responsive to and customised for the health needs of the community.

There is a very narrow gap between routine genetic testing and research raising several ethical, legal and social issues warranting monitoring and responding to emerging ethical concerns (Sec 10)\(^4\). The importance of genetic counselling as well as having appropriate safeguards to maintain privacy and confidentiality is explained in order to prevent stigma or discrimination. Ethical issues specific to different types of screening programmes such as prenatal or new-born screening are explained. Newer technologies, especially the recent CRISPR technology and the ethical dilemmas that it poses are discussed and there is a hope that this would show a way forward for research despite the unclear challenges to human health and safety.

The section on biomaterials, biobanking and datasets makes it clear that the donor / research participant owns the biological sample. For data that is collected, institutions are the custodians or trustees through their ECs and, researchers have no claim for either ownership or custodianship (Sec 11)\(^4\). The different options for consent, maintenance of confidentiality, use of left over clinical samples, transfer of biospecimens, long term storage, return of results and benefit sharing are explained and should be considered by researchers, biobanks, forensic laboratories and ECs.

The last section on research on humanitarian emergencies and disasters, has been prepared on account of recent domestic and international events like the tsunami, the Chennai floods, and the Ebola and Zika virus infection which necessitated emergency research (Sec 12)\(^4\). The requirements for emergency review by the EC, prior preparedness, consent documentation, sensitivity involved in dealing with the affected community and planning as well as protection from invasion of privacy are described, while balancing these with the need for conducting research.

In order to increase the awareness of and use of ICMR’s ethical guidelines by researchers, EC members and others, a series of dissemination programmes are being organised across the country. The first such event took place on November 16, 2017, at the All India Institute of Medical Sciences, New Delhi, on December 14, 2017, at the Postgraduate Institute of Medical Education and Research, Chandigarh, followed by dissemination programmes on February 7, 2018, at Chennai and on February 17, 2018, at Bhubaneswar. These were attended by researchers, EC members, clinicians, students from medical colleges, dental colleges, pharmacy, nursing colleges, research institutions, NGOs, patient representatives, sponsors, government agencies and other stakeholders. ICMR Bioethics Unit, National Centre for Disease Informatics and Research (NCDIR), and Clinical Development Services Agency (CDSA) under the Translational Health Sciences and Technology Institute (THSTI) have further collaborated in organising four such events.
Clinical trials for marketing approval are regulated under The Drugs and Cosmetics Act and Rules and biomedical and health research must follow the ICMR National Ethical Guidelines. There is therefore a need to harmonise and make sure that research participants whether participating in clinical trials, or basic or applied biomedical, health or socio behavioural research, are protected.

In our country, ethics is, unfortunately, still not part of the existing teaching curriculums in both the medical and non-medical streams. This influences both the quality of output in biomedical and health research and the protection of human participants for which the ethical conduct of research is essential. The ICMR National Ethical Guidelines document sets the standards for the ethical requirements to be followed in biomedical research in India. It is expected that all biomedical and health research in the country should follow this guidance which will go a long way towards improving the quality and outcomes of research.

Acknowledgment: The revision of ICMR ethical guidelines was led by the *Core Advisory Group of the ICMR Bioethics Unit whose contribution is gratefully acknowledged. Special thanks to the 48 members of the 14 subcommittees who drafted the initial draft, the experts who reviewed it and provided inputs, stakeholders from all parts of the country as well as from outside the country who provided their valuable comments. The support of the WHO-Country Office for India, in jointly hosting the consultation meetings in Bengaluru and New Delhi, is deeply appreciated and acknowledged.

Conflicts of Interest: None.

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Experience with non-cremophor-based paclitaxel-gemcitabine regimen in advanced pancreatic cancer: Results from a single tertiary cancer centre

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Background & objectives: Gemcitabine combined with non-cremophor-based paclitaxel is one of the standards of care in advanced inoperable pancreatic cancer. This study was undertaken to retrospectively evaluate real world non-trial outcomes with this combination.

Methods: Patients with histologically proven advanced inoperable pancreatic adenocarcinoma (PDAC), treated with non-cremophor-based paclitaxel-gemcitabine combination (PG) (gemcitabine-nanoxel or gemcitabine-abraxane) between January 2012 and June 2015, were retrospectively analyzed. Response assessment was done every 8-12 wk with computed tomography scan and responses were measured as per the Response Evaluation Criteria in Solid Tumours 1.1 criteria where feasible. Toxicity was recorded as per the Common Terminology Criteria for Adverse Events (CTCAE) v4 criteria. Progression-free survival (PFS) and overall survival (OS) were calculated using the Kaplan-Meier method.

Results: A total of 78 patients with PDAC were treated with the combination. Of these, 83.3 per cent of patients had metastatic disease. The median number of chemotherapy cycles administered was three. The objective response rate for the whole group was 30.8 per cent. Grade III/IV toxicities were seen in 35.9 per cent of patients. Median PFS was 5.6 months and median OS was 11.6 months.

Interpretation & conclusions: Non-cremophor-based paclitaxel in combination with gemcitabine appeared efficacious for advanced pancreatic cancers in routine clinical practice. Within the confines of a single-centre retrospective analysis, gemcitabine-nanoxel and gemcitabine-abraxane appeared to have similar efficacy and toxicity in advanced pancreatic cancers.

Key words Advanced pancreatic cancer - non-cremophor-based paclitaxel-gemcitabine - PDAC - unresectable cancer

Advanced pancreatic cancer remains a major clinical problem with a very high mortality to incidence ratio and accounts for roughly seven per cent of all cancer-related deaths worldwide, although it does not figure amongst the top ten most common cancers in India as per population-based registries1–4. Although research in the area has improved our understanding of pancreatic cancers, this has not yet translated into improvement
in outcomes\textsuperscript{5}. Conventionally, locally advanced and metastatic pancreatic cancers have been associated with poor prognosis with median survival of about 8-14 and 4-8 months, respectively\textsuperscript{5,8}. Various chemotherapeutic agents and regimens have been evaluated, and a few have been shown to improve survival. Gemcitabine-based combination chemotherapy regimens and FOLFIRINOX (5 Fluorouracil-Irinotecan-Oxaliplatin-Leucovorin) appear to be the most active regimens\textsuperscript{9-11}. Results of the phase III Metastatic Pancreatic Adenocarcinoma Clinical Trial (MPACT) study have established gemcitabine in combination with albumin-bound paclitaxel as one of the standard first-line regimens for metastatic pancreatic cancer based on an overall survival benefit compared to gemcitabine monotherapy\textsuperscript{12}.

Cremophor free paclitaxel has the advantage of lesser pre-medications and fewer allergic infusion reactions compared to conventional paclitaxel\textsuperscript{13}. Abraxane is also proposed to have some unique molecular and biological characteristics that contribute to its anti-tumour mechanisms\textsuperscript{14-16}. Nanoxel is a polymer and surfactant bound paclitaxel that also avoids the infusion reactions associated with paclitaxel\textsuperscript{17}. A cost-efficacy analysis by Ranade \textit{et al}\textsuperscript{18} showed a three-week cycle with nanoxel cost-effective when compared with cremophor-based paclitaxel using a complex economic model that took all costs associated with administration and adverse events with the two drugs into consideration\textsuperscript{19}. Other nanotechnology-based non-cremophor paclitaxel formulations have been used in advanced pancreatic cancers with reasonable efficacy\textsuperscript{19,20}. The primary objective of this study was to evaluate the survival of patients with unresectable/metastatic pancreatic cancer treated with non-cremophor-based paclitaxel-doublets (abraxane-gemcitabine and nanoxel-gemcitabine) in the routine clinical practice while the secondary objective was to assess adverse events and toxicity profile.

\textbf{Material & Methods}

All patients with histologically proven locally advanced or metastatic pancreatic cancers diagnosed at the department of Medical Oncology at Tata Memorial Hospital, Mumbai, India, between January 2012 and June 2015 and treated with either gemcitabine-abraxane (GA) or gemcitabine-nanoxel (GN) were included in this retrospective analysis. The study was approved by the Institutional Ethics Committee (IEC No IEC/0216/1644/001). Baseline clinical and demographic variables were recorded. The decision to use either abraxane or nanoxel-based therapy was taken by the primary treating physician based on the in-house availability of the drug, discussion of the cost-benefit ratio of the regimens and patient preference. From January 2014 onwards the patients were offered nanoxel as an alternative non-cremophor paclitaxel. Patients not able to afford abraxane were also offered nanoxel.

\textit{Treatment details:} Patients received a 30 min intravenous infusion of abraxane or nanoxel at a dose of 125 mg/m\textsuperscript{2} followed by an infusion of gemcitabine at a dose of 1000 mg/m\textsuperscript{2}, on days 1, 8 and 15 every four weeks. The dose was reduced to 75 per cent in cycle 1 in patients with serum albumin <3.0 g/dl and in subsequent cycles in case of a grade III/IV toxicity in the previous cycle and restarted once the toxicity had settled to grade I or completely recovered as per our institutional practice. Therapy was withheld in the event of any life-threatening toxicity, deterioration in patient’s performance status or disease progression. Response assessment was done every 8-12 wk or as and when felt clinically relevant with a contrast-enhanced computed tomography (CECT) scan of the thorax, abdomen and pelvis. Response was assessed using Response Evaluation Criteria in Solid Tumours (RECIST) v1.1 by treating clinician or with the help of radiologists associated with gastrointestinal disease management group\textsuperscript{21}. In case of non-measurable lesions, the response was not quantified. Complete response, partial response, stable disease and progressive disease were defined as per RECIST 1.1. Clinical benefit (CB) was defined as lack of disease progression at two consecutive response assessments (16-24 wk after starting treatment). Toxicity was documented using the Common Terminology Criteria for Adverse Events (CTCAE) criteria v4.03\textsuperscript{22}. Subsequent therapy on progression was based on patient’s performance status and was at the treating physician’s discretion.

\textit{Statistical analysis:} The data were retrieved from a prospectively maintained database. Overall survival (OS) was calculated from the date of starting chemotherapy to date of death from any cause or date of the last follow up. In patients with locally advanced pancreatic cancer, once the decision for inoperability was taken, then the date of start of chemotherapy was retrospectively used for calculation of OS. Progression-free survival (PFS) was calculated from the date of start of GA or GN until the date of
documented radiologic or clinical progression, death or loss to follow up. Survival functions were calculated using the Kaplan-Meier method. The median PFS and OS of the two treatment groups, GA and GN, were compared using the log-rank test.

**Results**

Seventy eight patients of locally advanced or metastatic pancreatic cancer were treated with GA or GN between January 2012 and June 2015. Median age at diagnosis of metastatic disease or inoperable disease was 60 yr (range: 24-84 yr) and 62.8 per cent of patients were males. Majority of the patients had metastatic disease at presentation (83.3%) and were treatment naïve (68%). The baseline characteristics are summarized in Table I.

GA combination chemotherapy was used in 57 (73.1%) patients, whereas 21 patients (26.9%) received the GN combination. The median number of cycles of chemotherapy received for the whole group was three. Eighteen patients (23.1%) received cycle 1 at a reduced dose of 75 per cent because of low albumin (<3.0 g/dl). Dose reduction in subsequent cycles was done in 14 patients (17.9%) due to grade III/IV toxicity. Delay in chemotherapy due to toxicity was seen in 18 patients (23.1%). The CB rate was 48.7 per cent [95% confidence interval (CI) –37.2-60.3] and overall response rate was 30.7 per cent (95% CI –21.8-42.3).

**Toxicity profile:** Grade III/IV toxicity was seen in 28 patients (35.9%). There was no significant difference in any grade III/IV toxicity between the GA and GN regimens. The rate of grade III/IV neutropenia and thrombocytopenia was higher in the GN group, but this did not reach statistical significance. Chemotherapy was withheld in 11 patients (14.1%) of whom nine had received GA chemotherapy. One patient died due to chemotherapy-related neutropenic sepsis. The toxicity details are shown in Table II.

**Treatment outcomes:** At a median follow up for all patients of 11.4 months (range: 2-23 months), the median overall survival was 11.6 months (95% CI –8.8-14.3) and two-year estimated OS was 12 per cent. There was no significant difference in the median overall survival between the chemotherapeutic regimens [median OS - GA vs. GN –9.3 months (95% CI –5.7-13) vs. 14 months (95% CI –6.0-22); \( P=0.255 \)] (Fig. 1).

<table>
<thead>
<tr>
<th>Table I. Baseline demographic and clinical characteristics of patients (n=78)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Characteristic</td>
</tr>
<tr>
<td>Age (yr), median (range)</td>
</tr>
<tr>
<td>Gender</td>
</tr>
<tr>
<td>Male</td>
</tr>
<tr>
<td>Female</td>
</tr>
<tr>
<td>Disease status</td>
</tr>
<tr>
<td>Locally advanced</td>
</tr>
<tr>
<td>Metastatic</td>
</tr>
<tr>
<td>Site of metastatic disease</td>
</tr>
<tr>
<td>Liver</td>
</tr>
<tr>
<td>Peritoneal</td>
</tr>
<tr>
<td>Lungs</td>
</tr>
<tr>
<td>No metastatic disease</td>
</tr>
<tr>
<td>Site of primary</td>
</tr>
<tr>
<td>Head</td>
</tr>
<tr>
<td>Body and tail</td>
</tr>
<tr>
<td>Obstructive jaundice</td>
</tr>
<tr>
<td>Prior intervention</td>
</tr>
<tr>
<td>Surgery</td>
</tr>
<tr>
<td>Chemotherapy</td>
</tr>
<tr>
<td>Radiotherapy</td>
</tr>
<tr>
<td>No prior intervention</td>
</tr>
<tr>
<td>ECOG PS</td>
</tr>
<tr>
<td>0</td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>≥2</td>
</tr>
<tr>
<td>Serum albumin (g %)</td>
</tr>
<tr>
<td>Median</td>
</tr>
<tr>
<td>Mean</td>
</tr>
<tr>
<td>Protocol received</td>
</tr>
<tr>
<td>Abraxane - Gemcitabine</td>
</tr>
<tr>
<td>Nanoxel - Gemcitabine</td>
</tr>
<tr>
<td>Median number of chemotherapy cycles</td>
</tr>
<tr>
<td>Dose reduction</td>
</tr>
<tr>
<td>Cycle 1</td>
</tr>
<tr>
<td>Subsequent cycles</td>
</tr>
<tr>
<td>Delay in chemotherapy (wk)</td>
</tr>
<tr>
<td>&lt;1</td>
</tr>
<tr>
<td>&gt;1</td>
</tr>
<tr>
<td>Reason for delay in chemotherapy (n=23)</td>
</tr>
<tr>
<td>Toxicity</td>
</tr>
</tbody>
</table>

Contd...
<table>
<thead>
<tr>
<th>Characteristic</th>
<th>n (% where applicable)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Logistic</td>
<td>5 (21.8)</td>
</tr>
<tr>
<td>Stopped chemotherapy due</td>
<td></td>
</tr>
<tr>
<td>to toxicity (%)</td>
<td>11 (14.1)</td>
</tr>
<tr>
<td>Response rate</td>
<td></td>
</tr>
<tr>
<td>CR</td>
<td>2 (2.6)</td>
</tr>
<tr>
<td>PR</td>
<td>22 (28.2)</td>
</tr>
<tr>
<td>SD</td>
<td>14 (17.9)</td>
</tr>
<tr>
<td>PD</td>
<td>22 (28.2)</td>
</tr>
<tr>
<td>Not evaluable</td>
<td>18 (23.1)</td>
</tr>
<tr>
<td>Median PFS (months)</td>
<td>5.6 (95% CI: 3.7-7.4)</td>
</tr>
<tr>
<td>Abraxane - Gemcitabine</td>
<td>5.7 (95% CI: 2.7-7.6)</td>
</tr>
<tr>
<td>Nanoxel - Gemcitabine</td>
<td>5.2 (95% CI: 2.5-8.8)</td>
</tr>
<tr>
<td>Median OS (months)</td>
<td>11.6 (95% CI: 8.8-14.3)</td>
</tr>
<tr>
<td>Abraxane - Gemcitabine</td>
<td>9.3 (95% CI: 5.7-13)</td>
</tr>
<tr>
<td>Nanoxel - Gemcitabine</td>
<td>14 (95% CI: 6-22)</td>
</tr>
</tbody>
</table>

ECOG, Eastern Cooperative Oncology Group; CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; PFS, progression free survival; OS, overall survival; CI, confidence interval; PS, performance status

Table II. Toxicity with gemcitabine - abraxane and gemcitabine - nanoxel (Grade 3 and Grade 4 events)

<table>
<thead>
<tr>
<th>Toxicity</th>
<th>Abraxane-Gemcitabine</th>
<th>Nanoxel-Gemcitabine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n=57)</td>
<td>(n=21)</td>
</tr>
<tr>
<td>Mucositis</td>
<td>1 (1.8)</td>
<td>1 (4.8)</td>
</tr>
<tr>
<td>Diarrhoea</td>
<td>1 (1.8)</td>
<td>0</td>
</tr>
<tr>
<td>Neutropenia</td>
<td>6 (10.5)</td>
<td>6 (28.6)</td>
</tr>
<tr>
<td>Thrombocytopenia</td>
<td>6 (10.5)</td>
<td>5 (23.8)</td>
</tr>
<tr>
<td>Febrile neutropenia</td>
<td>5 (8.8)</td>
<td>1 (4.8)</td>
</tr>
<tr>
<td>Peripheral neuropathy</td>
<td>4 (7)</td>
<td>1 (4.8)</td>
</tr>
<tr>
<td>Fatigue (Grade 3 only)</td>
<td>4 (7)</td>
<td>0</td>
</tr>
<tr>
<td>Vomiting</td>
<td>2 (3.5)</td>
<td>0</td>
</tr>
<tr>
<td>Liver dysfunction</td>
<td>1 (1.8)</td>
<td>0</td>
</tr>
</tbody>
</table>

The median PFS for the whole group was 5.6 months (95% CI –3.7-7.4) with a one year PFS of 11.2 per cent. There was no significant difference in PFS between the two regimens [median PFS - GA vs. GN –5.7 months (95% CI –2.7-7.6) vs. 5.2 months (95% CI –2.5-8.8); P=0.84] (Fig. 2).

Discussion

Limited efficacies of chemotherapeutic agents and dismal outcomes have plagued the treatment of advanced pancreatic cancers over the years. With current evidence supporting the use of gemcitabine-based combination with albumin-bound paclitaxel and FOLFIRINOX, it is important to choose an appropriate regimen for patients, taking into account age, comorbidity status, Eastern Cooperative Oncology Group Performance Status (ECOG PS) amongst
other variables. The reduced infusional toxicity with non-cremophor-based paclitaxel leading to the lesser need of premedication with dexamethasone along with the preclinical evidence of improved efficacy with a more linear dose-response curve compared to traditional paclitaxel (Taxol) and improved survival have made these agents an important component in the therapeutic armamentarium against advanced pancreatic cancer. Our study looked at two non-cremophor-based paclitaxel preparations which were used in combination with gemcitabine for locally advanced and metastatic pancreatic cancers. The primary aim of the study was to examine the usage and performance of these agents in routine clinical practice. A retrospective analysis has suggested that a modified two weekly abraxane/gemcitabine schedule appears to retain its survival benefit along with lesser toxicity and is more cost effective.

In our study, 73.1 per cent of the patients opted for abraxane-based combination therapy. This was expected as the efficacy and overall survival advantage of abraxane-gemcitabine combination has been proven in phase III randomized controlled trial. Nanoxel has not been compared in a clinical trial with abraxane. Although a preclinical study conducted in athymic nude mice comparing abraxane and nanoxel along with a third cremophor free paclitaxel formulation found superior anti-tumour activity with abraxane, at equitoxic doses; the interpretability of this study was limited by the small numbers in each group (n=10 per group). Other non-cremophor paclitaxel formulations have shown efficacy in phase II studies in advanced pancreatic cancers further supporting the role of nanoxel as a potential option in advanced pancreatic cancers.

The overall survival of our patients compares well with published data from other studies using gemcitabine-non cremophor paclitaxel in advanced pancreatic cancers (Table III). The survival of the patients on abraxane and nanoxel was not significantly different in our study. This suggested that nanoxel might have comparable activity to abraxane, but the small numbers undermined the strength of this finding. This study was not powered to statistically compare the abraxane and nanoxel group and should only be considered as preliminary data to suggest the feasibility of using gemcitabine-nanoxel in pancreatic cancers. With regard to toxicity, both drugs were comparable with no significant difference in rates of grade III/IV adverse events. Compared with the toxicity data from the MPACT trial, the rates of grade III/IV neutropenia were much lower in our study. This could be due to the difference in the dose modification protocols used in our study. However, the febrile neutropenia rate was higher in our study. Grade III/IV peripheral neuropathy also appeared to be lower in our patients even though the median number of chemotherapy cycles were similar to that delivered in the MPACT trial. Amongst the other chemotherapy regimens used in advanced pancreatic cancers, FOLFIRINOX has shown improvement in survival and quality of life, but its applicability is limited to only the fit patients of the lot. This was reflected in a previous report from our centre where only 6.9 per cent of treatment-naïve metastatic pancreatic cancers received FOLFIRINOX as their first-line treatment. Barring the minuscule but significant survival benefit observed with gemcitabine/erlotinib combination in a single phase III randomized study, other combination chemotherapies like gemcitabine-capecitabine have failed to show improvement in survival over gemcitabine alone.
Thus, gemcitabine in combination with abraxane remains an important and a standard first-line regimen for metastatic pancreatic cancer.

Limitations of this study included the small numbers and its retrospective nature. The study was not powered to show the actual efficacy of a nanoxel-based chemotherapeutic regimen; it only suggested feasibility. However, it provided some evidence of the two regimens being relatively comparable in their toxicity and efficacy.

In conclusion, non-cremophor paclitaxel in combination with gemcitabine appeared to have modest efficacy in unresectable/metastatic pancreatic cancer, and the outcomes in this study were similar to previously published data. Within the confines of a single-centre retrospective analysis, gemcitabine-nanoxel and gemcitabine-abraxane appeared to have similar efficacy and toxicity in advanced pancreatic cancers. Prospective studies looking at cost-effective nanoparticle-based paclitaxel formulations represent an important area for future research.

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Conflicts of Interest: None.

References:


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e-mail: anantr13@gmail.com
Prevalence, clinical & biochemical correlates of non-alcoholic fatty liver disease in overweight adolescents

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Background & objectives: Non-alcoholic fatty liver disease (NAFLD) characterized by excessive accumulation of fat in the liver, which can progress to inflammation, and cirrhosis, has emerged as an important complication of obesity in adults as well as children. This study was undertaken to assess the prevalence of NAFLD and its correlation with clinical and biochemical parameters in overweight Indian adolescents.

Methods: In this cross-sectional study, 218 overweight adolescents aged 10 to 16 yr and their parents were included. Measurements included anthropometry, ultrasonography to diagnose NAFLD, fasting glucose, aspartate aminotransferase (AST), alanine aminotransferase (ALT) and lipids for adolescents and parents, and additional parameters of blood pressure, body fat percentage (BF%), fasting insulin, apolipoprotein C3, tumour necrosis factor-a and adiponectin for adolescents. The variables were compared between adolescents with and without NAFLD, and logistic regression analysis was performed.

Results: Mean age and body mass index (BMI)SD score (SDS) were 11.9±1.6 yr and 2.3±1.1, respectively. NAFLD was seen in 62.5 per cent of the adolescents. The prevalence of NAFLD in the parents was similar among the adolescents with and without NAFLD, while BMI and waist circumference SDS, BF per cent, blood pressure (BP), ALT, AST, insulin and homeostatic model assessment of insulin resistance (HOMA-IR) were significantly higher in the adolescents with NAFLD. On multiple logistic regression, abdominal obesity, HOMA-IR and body fat percentage were independently associated with NAFLD with odds ratios (95% confidence interval) of 2.77 (1.40-5.47), 2.21 (1.16-4.21) and 2.17 (1.12-4.22), respectively.

Interpretation & conclusions: NAFLD was noted among nearly two-thirds of the overweight adolescents. An independent association was observed between abdominal obesity, HOMA-IR and body fat percentage and NAFLD in overweight adolescents.

Key words Abdominal obesity - alanine aminotransferase - body fat percentage - childhood obesity - insulin resistance - steatohepatitis
The developing world while still struggling to devise ways and means of reducing the burden of childhood undernutrition has been thrown unprepared into the midst of the epidemic of childhood obesity. Up to 18 per cent of urban children and adolescents in the Indian subcontinent are overweight or obese\(^1\). Previous studies in overweight and obese Indian adolescents have documented a high prevalence of insulin resistance, hypertension and metabolic syndrome\(^2,3\).

Non-alcoholic fatty liver disease (NAFLD), characterized by the accumulation of fat in the liver without a history of alcoholism or known liver pathology, has emerged as an important health problem in India, with an overall prevalence of 9-32 per cent among adults, being higher in those who are overweight and/or diabetic\(^4\). The spectrum of NAFLD ranges from simple steatosis through steatohepatitis to advanced fibrosis and cirrhosis\(^5\). With an estimated mean prevalence in the general paediatric population of 7.6 per cent and in obese children of 34.2 per cent\(^6\), NAFLD is considered the most common cause of liver disease in paediatric population in the developed world\(^7\). Children with NAFLD have a high prevalence of concomitant metabolic syndrome, increased risk for developing type 2 diabetes, and progression to end-stage liver disease\(^8\). There is, however, a scarcity of data regarding the prevalence and determinants of susceptibility to NAFLD in paediatric age group in India. Recently, a study in 100 overweight/obese adolescents from a Mumbai school reported NAFLD in 62 per cent\(^6\).

We undertook the present study to assess the prevalence of NAFLD and gain an insight into the clinical and biochemical parameters, such as age, gender, pubertal status, body mass index (BMI), central adiposity, body fat per cent, serum insulin, transaminases, lipid profile, adiponectin and tumour necrosis factor (TNF)-α; and the prevalence of obesity and fatty liver in parents, that may be associated with a higher risk of NAFLD in overweight/obese adolescents.

**Material & Methods**

This cross-sectional study was undertaken at the department of Paediatrics, All India Institute of Medical Sciences, New Delhi, India, between November 2012 and October 2015. The study was approved by the Institutional Ethics Committee. There were no data on the prevalence of NAFLD in Indian overweight adolescents when we commenced the study. In overweight adolescents from other countries, the prevalence ranged from 30-50 per cent\(^7-9\). A sample size of 196 was estimated taking prevalence as 50 per cent, with 95 per cent level of confidence and absolute precision of seven per cent. Adolescents aged 10-16 yr with BMI >85\(^{th}\) percentile\(^10\) who presented to the paediatric OPD during the first two years of the study were approached. Those with obesity caused by endocrine or genetic disorders, and those with previously diagnosed diabetes mellitus or chronic liver disease were excluded. Both parents of all the enrolled adolescents were also invited to participate. Written informed consent from parents and assent from the adolescents were taken.

**General information, anthropometry and body composition:** Socio-economic status (assessed by modified Kuppuswamy scale) and demographic information, dietary intake, physical activity and family history of obesity, diabetes or liver disease were recorded. Blood pressure (BP) was measured using an automated BP instrument (Omron HEM-7203, Kyoto, Japan). Weight and height were measured to the nearest 0.1 kg and 0.5 cm, respectively and BMI calculated. Waist (WC) and hip circumferences (HC) were measured using non-stretchable tape according to WHO guidelines\(^11\), and waist to HC ratio (WHR) was calculated. Skinfold thickness was measured at biceps, triceps, mid-thigh, subscapular and supra-iliac regions using Holtain’s skinfold calipers (Holtain Ltd, Crymych, UK). The anthropometric measurements were converted to standard deviation scores (SDS) based on Indian reference data\(^10,12\). Tanner staging was done, and the adolescents were grouped into prepubertal, early pubertal (stage 2 and 3) and late pubertal (stage 4 and 5). Acanthosis nigricans over the neck was classified as absent, mild (if visible on close visual inspection and limited to the base of skull), and moderate-to-severe (if extending to lateral margins of the neck or further anteriorly)\(^13\). Body fat percentage was measured using bioelectrical impedance analysis (Maltron BF-907, Essex, UK).

**Biochemistry:** Fasting venous sample (5 ml) was obtained after a 12 h overnight fast for estimation of alanine aminotransferase (ALT), aspartate aminotransferase (AST), glucose, insulin, triglycerides (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL), apolipoprotein C3, TNF-α and adiponectin levels. Modified oral glucose tolerance test was done by administering oral glucose at 1.75 g/kg (maximum 75 g) and taking a repeat blood sample after...
two hours. ALT, AST and glucose were estimated by Roche automated clinical analyzer; TG, TC and HDL by Randox kits (Randox Ltd, Antrim, United Kingdom), and Insulin by electrochemiluminescence (Roche Cobas e411, Roche Diagnostics Germany GmbH). Commercial enzyme-linked immunosorbent assay kits were used to measure TNF-α (Diaclone, Besancon Cedex, France), apolipoprotein C3 (Assaypro St. Charles, USA), and adiponectin (R&D, Minneapolis, USA). Homeostatic model assessment of insulin resistance (HOMA-IR) was calculated as fasting insulin (µU/ml) × fasting glucose (mmol/l)/22.5. Low-density lipoprotein cholesterol (LDL) was calculated using Friedewald’s equation 4.

**Diagnosis of non-alcoholic fatty liver disease (NAFLD):** The diagnosis of NAFLD was based on ultrasonography (USG), performed using curvilinear probe (2-5 Hz) of Acuson S2000 (Siemens, Germany). Fatty liver was diagnosed and graded as mild, moderate and severe based on echogenicity, visualization of vasculature, parenchyma and diaphragm15. In adolescents with fatty liver, hepatitis B surface antigen (HBsAg) and anti-hepatitis C virus (HCV) antibodies were assayed using electrochemiluminescence (Roche Cobas e411, Roche Diagnostics Germany GmbH).

**Parents’ data:** Parents’ age was recorded, and height and weight measured. Fasting venous samples were collected for ALT, AST, lipid profile and glucose; USG was performed to diagnose and grade fatty liver.

**Definitions:** WC was considered high if >90th percentile for Indian adolescents12, or more than the adult cutoffs of 90 and 80 cm for Asian males and females, respectively16. WHR was considered to be high if >0.95 for boys and >0.85 for girls17. Hypertension was diagnosed at systolic BP ≥130 mmHg and/or diastolic BP ≥85 mmHg18. Elevated ALT was defined as >26 U/l for boys and >23 U/l for girls19. Parents were considered overweight and obese at BMI ≥23 and ≥25 kg/m², respectively20. Fasting and two hours blood glucose were considered elevated if ≥100 and 140 mg/dl, respectively21, and HOMA-IR was considered high if >2.521. TG was considered high if ≥150 mg/dl; HDL was considered low if <40 mg/dl in adolescents of either gender, and fathers, and <50 mg/dl in mothers6,18. TC and LDL cholesterol were considered elevated if ≥200 and 130 mg/dl, respectively23. Metabolic syndrome in adolescents was diagnosed using the International Diabetes Federation criteria18.

**Statistical analysis:** Data were analyzed by Stata 11.2 (Stata Corp 4905 College Station, Texas 47845 USA) and presented as mean±SD, median (range) or frequency (percentage). Parameters of the adolescents with and without NAFLD were compared using Chi-square test or Fisher’s exact test for categorical variables, one-way ANOVA or independent t test for continuous variables following normal distribution, and Kruskal-Wallis or Wilcoxon rank sum test for parameters following non-normal distribution. Receiver operating characteristic (ROC) curves were generated to identify the cut-offs for various parameters for association with NAFLD. Univariate and step-wise multivariate logistic regression was used to calculate unadjusted and adjusted odds ratios after assessing multicollinearity and mediators among the variables.

**Results**

**Clinical, demographic and anthropometric parameters of the adolescents:** A total of 218 adolescents and their parents were included in the study. Mean age of the adolescents was 11.9±1.6 yr. Mean BMI SDS was 2.3±1.1; 52 (23.9%) were overweight, and 166 (76.2%) were obese. Acanthosis nigricans was present in 88 per cent, abdominal obesity in 66 per cent and hypertension in 22.9 per cent (Table I).

**Radiological and biochemical profile of the adolescents:** NAFLD was present in 130 (62.5%) of the adolescents [95% confidence interval (CI) 56.2-69.4%], and serum ALT was elevated in 78 per cent. Among the other metabolic derangements, elevated HOMA-IR and low HDL were the commonest, noted in 65.6 and 58.8 per cent, respectively. Metabolic syndrome was present in 26.4 per cent (Table II). None of the adolescents with fatty liver or elevated ALT had positive HBsAg or anti-HCV antibody test.

**Clinical, radiological and biochemical parameters in the parents:** Overweight/obesity was present in 90.6 per cent of the fathers and 90.5 per cent of the mothers. Diabetes was present in 8.5 and 6.4 per cent of fathers and mothers, respectively. Fatty liver was observed in 72.7 and 60.6 per cent of the fathers and mothers, respectively (Table III). Moderate and severe fatty liver was commoner among fathers compared to mothers. The most common dyslipidemia was low HDL.

**Comparison among adolescents with and without NAFLD:** The comparison of the clinical and biochemical
## Table I. Clinical, demographic and anthropometric parameters of the adolescent study participants (n=218)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Mean±SD or n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr)</td>
<td>11.9±1.6</td>
</tr>
<tr>
<td>Gender, male/female</td>
<td>151 (69)/67 (31)</td>
</tr>
<tr>
<td>State</td>
<td></td>
</tr>
<tr>
<td>Delhi</td>
<td>114 (52.3)</td>
</tr>
<tr>
<td>Uttar Pradesh</td>
<td>55 (25.2)</td>
</tr>
<tr>
<td>Haryana</td>
<td>27 (12.4)</td>
</tr>
<tr>
<td>Others</td>
<td>22 (10.1)</td>
</tr>
<tr>
<td>Urban/rural</td>
<td>181 (83)/37 (17)</td>
</tr>
<tr>
<td>Socio-economic status</td>
<td></td>
</tr>
<tr>
<td>Upper</td>
<td>33 (15.1)</td>
</tr>
<tr>
<td>Upper middle</td>
<td>92 (42.2)</td>
</tr>
<tr>
<td>Lower middle</td>
<td>74 (34.0)</td>
</tr>
<tr>
<td>Upper lower/lower</td>
<td>19 (8.7)</td>
</tr>
<tr>
<td>Height SDS</td>
<td>-0.4±1.1</td>
</tr>
<tr>
<td>Pre-pubertal/early pubertal/late pubertal†</td>
<td>52 (25)/115 (54)/45 (21)</td>
</tr>
<tr>
<td>Acanthosis nigricans over neck</td>
<td></td>
</tr>
<tr>
<td>Absent/mild/moderate or severe†</td>
<td>24 (12)/60 (30)/116 (58)</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>27.3±4.3</td>
</tr>
<tr>
<td>BMI SDS</td>
<td>2.3±1.1</td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>87.7±11.0</td>
</tr>
<tr>
<td>Abdominal adiposity</td>
<td>140 (66.0)</td>
</tr>
<tr>
<td>Waist circumference SDS</td>
<td>1.6±1.3</td>
</tr>
<tr>
<td>Waist hip ratio</td>
<td>0.94±0.6</td>
</tr>
<tr>
<td>Boys with WHR &gt;0.95</td>
<td>84 (55.6)</td>
</tr>
<tr>
<td>Girls with WHR &gt;0.85</td>
<td>56 (83.6)</td>
</tr>
<tr>
<td>Systolic BP (mm Hg)</td>
<td>120±10</td>
</tr>
<tr>
<td>Systolic BP ≥130 mm Hg</td>
<td>32 (14.7)</td>
</tr>
<tr>
<td>Diastolic BP (mm Hg)</td>
<td>75±9</td>
</tr>
<tr>
<td>Diastolic BP ≥85 mmHg</td>
<td>33 (15.1)</td>
</tr>
<tr>
<td>Hypertension</td>
<td>50 (22.9)</td>
</tr>
<tr>
<td>Body fat percentage</td>
<td>38.0±5.2</td>
</tr>
<tr>
<td>Biceps SDS</td>
<td>4.5±1.3</td>
</tr>
<tr>
<td>Triceps SDS</td>
<td>3.1±1.0</td>
</tr>
<tr>
<td>Subscapular SDS</td>
<td>2.6±1.1</td>
</tr>
<tr>
<td>Sum of skinfolds (biceps, triceps, subscapular, suprailliac, thigh) (mm)</td>
<td>135±23</td>
</tr>
<tr>
<td>Ratio of central (subscapular + suprailliac) to peripheral (biceps + thigh) skinfolds</td>
<td>0.9±0.1</td>
</tr>
</tbody>
</table>

†WC >90th percentile for age and gender, or >90 cm in boys or >80 cm in girls; †data from few participants were not available.

SD, standard deviation; SDS, standard deviation score; BMI, body mass index; BP, blood pressure

## Table II. Radiological and biochemical parameters of the adolescents

<table>
<thead>
<tr>
<th>Parameter</th>
<th>n</th>
<th>Mean±SD or n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fatty liver on USG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absent</td>
<td>208</td>
<td>78 (37.5)</td>
</tr>
<tr>
<td>Mild</td>
<td>84</td>
<td>(40.4)</td>
</tr>
<tr>
<td>Moderate</td>
<td>39</td>
<td>(18.8)</td>
</tr>
<tr>
<td>Severe</td>
<td>7</td>
<td>(3.3)</td>
</tr>
<tr>
<td>ALT (U/l)</td>
<td>218</td>
<td>43.7±29.8</td>
</tr>
<tr>
<td>Elevated ALT (&gt;26 U/l in boys, &gt;23 U/l in girls)</td>
<td>170 (78)</td>
<td></td>
</tr>
<tr>
<td>AST (U/l)</td>
<td>218</td>
<td>37.2±17.6</td>
</tr>
<tr>
<td>AST: ALT ratio</td>
<td>218</td>
<td>1.0±0.3</td>
</tr>
<tr>
<td>Fasting glucose (mg/dl)</td>
<td>216</td>
<td>84.3±11.2</td>
</tr>
<tr>
<td>Fasting glucose ≥100 and &lt;126 mg/dl</td>
<td>20 (9.3)</td>
<td></td>
</tr>
<tr>
<td>2 h glucose (mg/dl)</td>
<td>211</td>
<td>107.6±16.9</td>
</tr>
<tr>
<td>2 h glucose ≥140 mg/dl</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Fasting insulin (µU/ml)</td>
<td>214</td>
<td>18.0±10.4</td>
</tr>
<tr>
<td>Fasting insulin ≥20 µU/ml</td>
<td>69</td>
<td>(31.6)</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>212</td>
<td>3.7±2.1</td>
</tr>
<tr>
<td>HOMA-IR &gt;2.5</td>
<td>139</td>
<td>(65.6)</td>
</tr>
<tr>
<td>Triglyceride (mg/dl)</td>
<td>212</td>
<td>124.6±59.2</td>
</tr>
<tr>
<td>Triglyceride &gt;150 mg/dl</td>
<td>52</td>
<td>(24.5)</td>
</tr>
<tr>
<td>Total cholesterol (mg/dl)</td>
<td>213</td>
<td>162.7±33.6</td>
</tr>
<tr>
<td>Total cholesterol ≥200 mg/dl</td>
<td>25</td>
<td>(11.7)</td>
</tr>
<tr>
<td>HDL cholesterol (mg/dl)</td>
<td>209</td>
<td>38.3±11.3</td>
</tr>
<tr>
<td>HDL cholesterol &lt;40 mg/dl</td>
<td>123</td>
<td>(58.8)</td>
</tr>
<tr>
<td>LDL cholesterol (mg/dl)</td>
<td>211</td>
<td>100.1±33.6</td>
</tr>
<tr>
<td>LDL cholesterol &gt;130 mg/dl</td>
<td>35</td>
<td>(16.6)</td>
</tr>
<tr>
<td>TNF-α (pg/ml)</td>
<td>205</td>
<td>15.9 (7.6-37.4)</td>
</tr>
<tr>
<td>Apolipoprotein C3 (mg/dl)</td>
<td>212</td>
<td>7.6 (4.1-10.9)</td>
</tr>
<tr>
<td>Adiponectin (µg/ml)</td>
<td>209</td>
<td>4.7 (3.2-7.1)</td>
</tr>
<tr>
<td>Metabolic syndrome by IDF criteria</td>
<td>208</td>
<td>55 (26.4)</td>
</tr>
</tbody>
</table>

†Median (IQR). HOMA-IR, homeostatic model assessment of insulin resistance; TNF-α, tumour necrosis factor α; HDL, high-density lipoprotein; LDL, low-density lipoprotein; IQR, interquartile range; USG, ultrasonography; ALT, alanine aminotransferase; AST, aspartate aminotransferase; IDF, International Diabetes Federation

Parameters in the adolescents with and without fatty liver is presented in Table IV. Age, distribution of gender and pubertal status and socio-demographic profile were similar in the two groups. BMI SDS, WC SDS, WHR, BP, FM per cent, ALT, AST, fasting insulin, HOMA-IR and prevalence of acanthosis nigricans were higher in the adolescents with NAFLD compared to those without NAFLD. Parents’ mean BMI as well as the proportion
Table III. Clinical, radiological and biochemical parameters of the parents

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Fathers</th>
<th>Mothers</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>Mean±SD or n (%)</td>
</tr>
<tr>
<td>Age (yr)</td>
<td>212</td>
<td>41.0±4.6</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>201</td>
<td>28.7±5.21</td>
</tr>
</tbody>
</table>

Fatty liver on USG

<table>
<thead>
<tr>
<th></th>
<th>Fathers</th>
<th>Mothers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absent</td>
<td>183</td>
<td>197</td>
</tr>
<tr>
<td>Mild</td>
<td>43 (23.5)</td>
<td>78 (39.6)</td>
</tr>
<tr>
<td>Moderate</td>
<td>69 (37.7)</td>
<td>31 (15.7)</td>
</tr>
<tr>
<td>Severe</td>
<td>21 (11.5)</td>
<td>10 (5.1)</td>
</tr>
<tr>
<td>ALT (U/l)</td>
<td>171</td>
<td>180</td>
</tr>
<tr>
<td>AST (U/l)</td>
<td>171</td>
<td>180</td>
</tr>
<tr>
<td>Fasting glucose (mg/dl)</td>
<td>129</td>
<td>140</td>
</tr>
<tr>
<td>≥100 and &lt;126 mg/dl</td>
<td>28 (21.7)</td>
<td>16 (11.4)</td>
</tr>
<tr>
<td>≥126 mg/dl</td>
<td>11 (8.5)</td>
<td>9 (6.4)</td>
</tr>
<tr>
<td>Triglyceride (mg/dl)</td>
<td>167</td>
<td>181</td>
</tr>
<tr>
<td>&gt;150 mg/dl</td>
<td>142.0±69.2</td>
<td>126.8±74.6</td>
</tr>
<tr>
<td>≥150 mg/dl</td>
<td>65 (38.9)</td>
<td>52 (29.1)</td>
</tr>
<tr>
<td>Total cholesterol (mg/dl)</td>
<td>167</td>
<td>179</td>
</tr>
<tr>
<td>≥200 mg/dl</td>
<td>176.0±42.4</td>
<td>172.3±39.3</td>
</tr>
<tr>
<td>≥200 mg/dl</td>
<td>49 (29.3)</td>
<td>46 (25.4)</td>
</tr>
<tr>
<td>HDL cholesterol (mg/dl)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;40 mg/dl in men</td>
<td>166</td>
<td>182</td>
</tr>
<tr>
<td>&lt;50 mg/dl in women</td>
<td>113 (68.1)</td>
<td>143 (78.5)</td>
</tr>
<tr>
<td>LDL cholesterol (mg/dl)</td>
<td>167</td>
<td>178</td>
</tr>
<tr>
<td>&gt;130 mg/dl</td>
<td>112.7±40.6</td>
<td>107.5±35.8</td>
</tr>
<tr>
<td>&gt;130 mg/dl</td>
<td>50 (29.9)</td>
<td>45 (25.3)</td>
</tr>
</tbody>
</table>

BMI, body mass index; USG, ultrasonography; ALT, alanine aminotransferase; AST, aspartate aminotransferase; TG, triglyceride; TC, total cholesterol; HDL, high-density lipoprotein; LDL, low-density lipoprotein

of parents who had fatty liver was similar in both the groups.

The anthropometric and biochemical parameters were further compared between the adolescents without NAFLD (n=130), with mild NAFLD (n=84) and with moderate or severe NAFLD (n=46). The parameters that were significantly different among the adolescents in these three groups are presented in Table V. A clear gradation of BMI, WC, acanthosis nigricans, ALT, AST, fasting insulin and HOMA-IR was observed across the three groups, elucidating further the association between severity of obesity, insulin resistance and NAFLD. Subscapular skinfold thickness, a measure of central adiposity was higher and HDL significantly lower among the adolescents with moderate or severe NAFLD as compared to those with mild NAFLD. Mean serum adiponectin in adolescents with moderate or severe NAFLD (4.9±3.8 μg/ml) was lower compared to those without NAFLD (5.5±3.1 μg/ml, P=0.08).

Receiver operating characteristic (ROC) and regression analysis: ROC analysis was performed to obtain the cut-offs of anthropometric and biochemical parameters for predicting a higher risk for NAFLD. WC SDS had the highest discriminating ability, with area under the curve (AUC) of 0.73 (95% CI 0.66-0.80), and the cut-off being 1.4 sensitivity 70.1% and specificity 70.6%). This was followed by ALT ≥33 U/l (AUC ROC 0.70; 95% CI 0.63-0.77, sensitivity 60.8% and specificity 66.2%); BMI SDS ≥2.2 (AUC ROC 0.70; 95% CI 0.62-0.76, sensitivity 69% and specificity 63.6%); insulin ≥15 μU/ml (AUC ROC 0.68; 95% CI 0.61-0.76, sensitivity 67.4%, specificity 64.9%); HOMA-IR ≥3.2 (AUC ROC 0.67; 95% CI 0.59-0.74, sensitivity 64% and specificity 64.5%) (Figure) and body fat per cent ≥37.7 (AUC ROC of 0.63; 95% CI 0.55-0.71, sensitivity 63.6% and specificity 64.0%).

Association of various parameters with NAFLD was checked by univariate followed by multiple logistic regression analysis. Abdominal obesity, HOMA-IR and body fat percentage (BF%) emerged as
Table IV. Comparison of clinical and biochemical parameters in the adolescents with and without non-alcoholic fatty liver disease (NAFLD)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Adolescents with NAFLD (n=130)</th>
<th>Adolescents without NAFLD (n=78)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr)</td>
<td>12.0±1.6</td>
<td>11.6±1.4</td>
<td>0.06</td>
</tr>
<tr>
<td>Gender male/female</td>
<td>91 (70)/39 (30)</td>
<td>51 (68.4)/27 (34.6)</td>
<td>0.29</td>
</tr>
<tr>
<td>Prepubertal/early pubertal/late pubertal†</td>
<td>30 (23.6)/73 (57.5)/24 (18.9)</td>
<td>21 (27.6)/35 (46.0)/20 (26.4)</td>
<td>0.59</td>
</tr>
<tr>
<td>Acanthosis nigricans</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absent/mild/moderate-to-severe†</td>
<td>10 (8.0)/27 (21.8)/87 (70.2)</td>
<td>14 (18.2)/33 (44.2)/29 (37.7)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>28.6±4.5</td>
<td>25.7±3.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>BMI SDS</td>
<td>2.6±1.1</td>
<td>1.8±0.8</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>91.1±10.7</td>
<td>82.9±9.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Waist circumference SDS</td>
<td>1.9±1.3</td>
<td>1.0±0.9</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Waist-hip ratio</td>
<td>0.94±0.05</td>
<td>0.91±0.05</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Sum of skinfolds (mm)</td>
<td>135.4±24.9</td>
<td>133.7±18.9</td>
<td>0.43</td>
</tr>
<tr>
<td>Ratio of central to peripheral skinfolds</td>
<td>0.91±0.14</td>
<td>0.93±0.14</td>
<td>0.58</td>
</tr>
<tr>
<td>Systolic BP (mm Hg)</td>
<td>120.9±10.3</td>
<td>117.9±9.7</td>
<td>0.05</td>
</tr>
<tr>
<td>Diastolic BP (mm Hg)</td>
<td>76.4±9.2</td>
<td>74.1±8.1</td>
<td>0.07</td>
</tr>
<tr>
<td>Body fat %</td>
<td>39.0±4.8</td>
<td>37.0±5.3</td>
<td>0.006</td>
</tr>
<tr>
<td>ALT (U/l)</td>
<td>49.8±32.1</td>
<td>32.9±16.9</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>AST (U/l)</td>
<td>40.6±20.5</td>
<td>31.7±10.0</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>AST: ALT ratio</td>
<td>0.9±0.3</td>
<td>1.0±0.3</td>
<td>0.002</td>
</tr>
<tr>
<td>Fasting glucose (mg/dl)</td>
<td>83.7±12.4</td>
<td>84.7±9.5</td>
<td>0.54</td>
</tr>
<tr>
<td>2 h glucose (mg/dl)</td>
<td>108.7±18.9</td>
<td>105.6±12.8</td>
<td>0.20</td>
</tr>
<tr>
<td>Fasting insulin (µU/ml)</td>
<td>20.6±11.2</td>
<td>14.2±7.8</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>4.2±2.3</td>
<td>3.0±1.7</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Triglyceride (mg/dl)</td>
<td>126.5±56.0</td>
<td>124.2±65.5</td>
<td>0.79</td>
</tr>
<tr>
<td>LDL cholesterol (mg/dl)</td>
<td>99.7±31.4</td>
<td>98.9±36.9</td>
<td>0.87</td>
</tr>
<tr>
<td>Total cholesterol (mg/dl)</td>
<td>162.2±32.3</td>
<td>161.7±36.2</td>
<td>0.92</td>
</tr>
<tr>
<td>HDL cholesterol (mg/dl)</td>
<td>37.6±11.4</td>
<td>39.0±11.2</td>
<td>0.41</td>
</tr>
<tr>
<td>Apolipoprotein C3 (mg/dl)</td>
<td>7.2 (4.6-10.8)</td>
<td>7.8 (3.2-11.1)</td>
<td>0.68</td>
</tr>
<tr>
<td>TNF-α (pg/ml)</td>
<td>16.2 (9.5-36.6)</td>
<td>15.8 (6.8-38.0)</td>
<td>0.69</td>
</tr>
<tr>
<td>Adiponectin (µg/ml)</td>
<td>4.5 (2.9-6.9)</td>
<td>4.9 (3.5-7.1)</td>
<td>0.27</td>
</tr>
<tr>
<td>Metabolic syndrome</td>
<td>38 (29.2)</td>
<td>17 (21.7)</td>
<td>0.23</td>
</tr>
<tr>
<td>Fathers’ BMI (kg/m²)</td>
<td>29.3±5.7</td>
<td>28.0±4.3</td>
<td>0.11</td>
</tr>
<tr>
<td>Mothers’ BMI (kg/m²)</td>
<td>29.9±5.8</td>
<td>29.6±4.9</td>
<td>0.68</td>
</tr>
<tr>
<td>Fatty liver in fathers</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absent/present</td>
<td>28 (25.4)/82 (74.5)</td>
<td>22 (30.1)/51 (69.9)</td>
<td>0.49</td>
</tr>
<tr>
<td>Fatty liver in mothers</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absent/present</td>
<td>47 (39.5)/72 (60.5)</td>
<td>31 (39.7)/47 (60.3)</td>
<td>0.97</td>
</tr>
</tbody>
</table>

*Median (IQR); †data was not available for few participants. Statistical tests used: Independent t test for comparison of means, Chi-square test for comparison of proportions. IQR, interquartile range; SDS, standard deviation score; BMI, body mass index; BP, blood pressure; ALT, alanine aminotransferase; AST, aspartate aminotransferase; LDL, low-density lipoprotein; HOMA-IR, homeostatic model assessment of insulin resistance; TNF-α, tumour necrosis factor α; HDL, high-density lipoprotein
significant independent variables that were associated with NAFLD (Table VI).

**Discussion**

A high prevalence of NAFLD (62.5%) was observed in the overweight/obese adolescents in our study. BMI, WC, BF per cent, insulin resistance and levels of ALT and AST were positively associated with a higher risk of NAFLD. The presence of obesity and/or fatty liver in parents was not associated with a higher prevalence of NAFLD in the adolescents. WC SDS ≥1.4, BMI SDS ≥2.2, ALT ≥33 U/l, insulin ≥15 μU/ml and HOMA-IR ≥3.2 were identified as cutoffs predictive of higher risk for NAFLD.

In previous studies in overweight children and adolescents, the prevalence of NAFLD has ranged from about 30 per cent in studies from Europe7 to about 45-50 per cent in studies from China9 and Turkey8. In another study in Indian overweight adolescents, a similarly high prevalence has been reported6. Earlier studies have indicated an ethnic difference in the prevalence of NAFLD, with Hispanic adolescents being at the highest risk and Africans the least24. Our observations suggest a higher predilection for NAFLD in Asian Indian adolescents.

Asian Indians have a high prevalence of central adiposity and insulin resistance beginning at a young age, with clustering of cardiometabolic risk markers including high body fat percentage dyslipidaemia and fasting glucose2,25. The present study places NAFLD as a prominent member of the cluster of complications associated with central adiposity and insulin resistance in Indian adolescents. Considering that India is home to 243 million adolescents, more than 20 per cent of the world’s adolescent population26, up to 25 million of whom are overweight, this high prevalence of NAFLD has a huge potential implication for our health infrastructure and resources.

Among the fathers and mothers, majority of whom were overweight/obese, the prevalence of fatty liver was 72.3 and 60.4 per cent, respectively. This was similar to the prevalence of 72.3 per cent reported among overweight/obese adults from Chennai27. We did not find any difference in the prevalence of NAFLD in our study participants based on their age, pubertal

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Adolescents without NAFLDa (n=78) Mean±SD or n (%)</th>
<th>Adolescents with mild NAFLDb (n=84) Mean±SD or n (%)</th>
<th>Adolescents with moderate or severe NAFLDb (n=46) Mean±SD or n (%)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMI (kg/m²)</td>
<td>25.7±3.2</td>
<td>28.6±4.5</td>
<td>30.1±4.3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>BMI SDS</td>
<td>1.9±0.8</td>
<td>2.4±1.1</td>
<td>2.9±1.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>WC (cm)</td>
<td>82.9±9.2</td>
<td>88.7±9.9</td>
<td>95.7±9.9</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>WC SDS</td>
<td>1.1±0.9</td>
<td>1.8±1.4</td>
<td>2.3±1.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Acanthosis nigricans</td>
<td></td>
<td></td>
<td></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Absent</td>
<td>14 (18.2)</td>
<td>7 (8.6)</td>
<td>3 (7.0)</td>
<td></td>
</tr>
<tr>
<td>Mild</td>
<td>33 (44.2)</td>
<td>22 (27.2)</td>
<td>5 (11.6)</td>
<td></td>
</tr>
<tr>
<td>Moderate-severea</td>
<td>29 (37.7)</td>
<td>52 (64.2)</td>
<td>35 (81.4)</td>
<td></td>
</tr>
<tr>
<td>Subscapf (mm)</td>
<td>23.6±4.5</td>
<td>23.5±5.8</td>
<td>26.2±5.1</td>
<td>0.02</td>
</tr>
<tr>
<td>ALT (U/l)</td>
<td>32.9±16.9</td>
<td>41.4±24.8</td>
<td>65.0±38.2</td>
<td>0.001</td>
</tr>
<tr>
<td>AST (U/l)</td>
<td>31.7±10.0</td>
<td>35.2±12.3</td>
<td>50.3±27.9</td>
<td>0.001</td>
</tr>
<tr>
<td>AST: ALT ratio</td>
<td>1.0±0.2</td>
<td>1.0±0.3</td>
<td>0.8±0.3</td>
<td>0.001</td>
</tr>
<tr>
<td>HDL (mg/dl)</td>
<td>38.9±11.3</td>
<td>39.4±11.2</td>
<td>34.2±11.3</td>
<td>0.04</td>
</tr>
<tr>
<td>Insulin (µU/ml)a</td>
<td>14.2±7.8</td>
<td>19.4±10.4</td>
<td>22.7±12.3</td>
<td>0.001</td>
</tr>
<tr>
<td>HOMA-IRa</td>
<td>3.0±1.7</td>
<td>4.1±2.4</td>
<td>4.4±2.1</td>
<td>0.001</td>
</tr>
</tbody>
</table>

^ Statistical tests used: Fisher’s exact test for categorical variables, one-way ANOVA with post hoc Bonferroni correction for continuous variables following normal distribution; Kruskal-Wallis followed by multiple comparison using Dunn’s test for variables not following normal distribution. SDS, standard deviation score; BMI, body mass index; ALT, alanine aminotransferase; AST, aspartate aminotransferase; WC, waist circumference; Subscapf, subscapular skinfold thickness; HOMA-IR, homeostatic model assessment of insulin resistance; HDL, high-density lipoprotein cholesterol
stage or gender. Among parents, the prevalence, as well as the severity of NAFLD, was higher among fathers as compared to mothers. This is in agreement with previous literature suggesting that male gender is associated with a higher prevalence of NAFLD, while estradiol affords some protection in females.\textsuperscript{28}
Our results indicated that in adolescents, both genders were at similar risk of NAFLD. Higher BMI and WC SDS were significantly associated with NAFLD in the present study, as reported in several earlier studies. Presence and severity of acanthosis nigricans were also observed to be significantly higher in the adolescents with NAFLD compared to those without, thus indicating its usefulness as a clinical risk marker for complications of obesity.

In the present study, fasting and two hours glucose, triglyceride, total and LDL cholesterol and apolipoprotein C3 were similar in the adolescents with and without NAFLD. HDL cholesterol was lower in the adolescents with moderate or severe NAFLD compared to those with mild NAFLD. In a previous study on paediatric population, higher levels of LDL-C, TG, TC and apolipoprotein C3 have been found to be independent predictors of NAFLD. Cytokines and inflammatory mediators are considered important in the pathogenesis and progression of NAFLD. Adiponectin has insulin-sensitizing effects and has been reported to be lower in obese children and adults with NAFLD compared to those without NAFLD. In the present study, adiponectin was lower among the children with moderate or severe NAFLD as compared to those with mild or no NAFLD, but no such difference was noted for TNF-α.
The strength of the present study was that a reasonably large number of adolescents and both their parents were evaluated clinically, biochemically and by USG. The limitations were that the prevalence of NAFLD was not assessed in lean adolescent controls, and the diagnosis of NAFLD was not confirmed by more robust methods such as magnetic resonance imaging (MRI) or histology.

In conclusion, our results showed NAFLD in nearly two-thirds of the overweight adolescents. The clinical and biochemical parameters associated with higher risk for NAFLD were higher BMI and WC, the presence of acanthosis nigricans, and elevated ALT and HOMA-IR. Screening for NAFLD should be incorporated in the evaluation of all overweight adolescents, especially if one or more of the risk markers are present.

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Conflicts of Interest: None.

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Addition of power Doppler to grey scale transvaginal ultrasonography for improving the prediction of endometrial pathology in perimenopausal women with abnormal uterine bleeding

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Background & objectives: Transvaginal ultrasonography (TVS) is a non-invasive procedure and can be used as a screening tool among women with abnormal uterine bleeding (AUB). Power Doppler is useful in depicting the vascular architecture better than the conventional Doppler. Hence, this study was conducted to evaluate whether addition of power Doppler to grey scale TVS can replace invasive hysteroscopy for the prediction of endometrial pathology in perimenopausal women with AUB.

Methods: One hundred women (>45 yr) with perimenopausal AUB underwent evaluation with TVS, power Doppler and hysteroscopy-guided biopsy after a detailed history and examination. Histopathology was considered as gold standard and other tools such as grey scale TVS with power Doppler and hysteroscopy were compared with it.

Results: Fifty six per cent women had no vascularity on power Doppler. Among those who had vascularity, the vascular patterns noted were single-vessel in 18 per cent, scattered-vessel in 15 per cent and multiple-vessel in 11 per cent. The sensitivity, specificity, positive predictive value and negative predictive value of TVS-endometrial thickness with power Doppler in detecting hyperplasia were 50, 86.5, 13.3 and 97.6 per cent, respectively, whereas the same for hysteroscopy were 100, 97.6, 88.1 and 100 per cent, respectively.

Interpretation & conclusions: Addition of power Doppler to grey scale TVS improved the specificity and negative predictive value almost comparable to hysteroscopy for evaluation of AUB, but sensitivity and positive predictive value remained poor.

Key words Abnormal uterine bleeding - endometrial carcinoma - endometrial polyp - hysteroscopy - power Doppler - transvaginal ultrasonography

Abnormal uterine bleeding (AUB) at any age in a woman's life is disruptive and worrisome and is more common in perimenopausal age group. Although anovulation is the most common endometrial cause, endometrial adenomatous polyps, endometrial hyperplasia and carcinoma are the other important causes. Hence, pre- and postmenopausal women with AUB require careful evaluation to rule out endometrial malignancy.
Although postmenopausal bleeding (PMB) is a common complaint accounting for up to 69 per cent of postmenopausal women referred to the gynaecological outpatient department, only 10-15 per cent of them will actually have endometrial carcinoma. Hence, subjecting the rest of the women whose examination reveals no abnormality on invasive endometrial biopsy becomes questionable. Thus, there is a need for an efficient non-invasive technique that can predict endometrial pathologies.

Transvaginal ultrasonography (TVS) is a non-invasive procedure and can be used as a screening tool. TVS allows visualizing the endomyometrial interface and the entire cavity. TVS measurement of endometrial thickness (ET) has high negative predictive value and can avoid unnecessary biopsies. According to a study by Dijkhuizen et al., TVS can prevent around 40 per cent of endometrial biopsies. ET when combined with some morphologic parameters and Doppler velocimetry studies can improve the diagnostic accuracy of TVS in patients with PMB.

Power Doppler is a new technology, based on the amplitude of Doppler signal and not on Doppler frequency shift that makes it more sensitive to low-velocity blood flow. Hence, it is useful in depicting the vascular architecture better than the conventional Doppler. There are a few studies which suggested that power Doppler vascular pattern can be used to predict endometrial pathology in postmenopausal women with PMB.

The present study was aimed to evaluate the role of transvaginal ultrasound with power Doppler studies to predict endometrial pathologies as against gold standard hysteroscopy-guided biopsy in the pre- and postmenopausal women with AUB.

**Material & Methods**

This prospective study was conducted between December 2013 and May 2015 in Jawaharlal Institute of Postgraduate Medical Education and Research (JIPMER), Puducherry, a tertiary care centre in south India. One hundred women with premenopausal AUB (women aged above 45 yr with excessive bleeding or any deviation from normal frequency or cyclicity) or PMB were included in the study after taking informed written consent. The study protocol was approved by the Institute’s ethics committee. The study by El-Morsi et al. noted that the positive predictive value of power Doppler in predicting polyp and hyperplasia was 68 per cent. The sample size was calculated based on this positive predictive value of 68 per cent, expected precision of 10 per cent, alpha error of 0.05 and 10 per cent attrition.

Women with other apparent causes of AUB which were unlikely to be due to endometrial pathology (women on hormone replacement therapy/anticoagulants, known cases of thyroid dysfunction, chronic renal diseases, chronic liver diseases and haematological diseases and women with clinical evidence of cervical cancer, cervical polyp, fibroid and adenomyosis) were excluded.

A detailed history was taken and a systematic examination was performed. The different clinical parameters noted were age, parity, presenting complaints which included severity and duration, menstrual history that included age at menarche, regularity, duration and severity of flow, age at menopause, presence of inter-menstrual bleeding, dysmenorrhoea, comorbid illness such as diabetes, hypertension, history of surgery or significant illness and family history of malignancy. Examination parameters such as body mass index (BMI), any mass per abdomen, findings of per speculum and vaginal examination (the size of uterus and the presence of adnexal mass) were also noted.

On the same day or within two days of the first visit, TVS was performed using a 7.5 MHz probe of Voluson Pro 730 from GE (General Electric, USA) healthcare, India. Initially, grey scale examination was done to note the uterine length, width, ET, endomyometrial interphase, presence of intra-cavitary fluid and presence of adnexal lesion. Then, power Doppler was activated. The following settings were ensured: pulse repetition frequency between 0.3 and 0.9 KHz (usually 0.6) and wall filter 30-50 Hz. Gain was adjusted such that artefacts were minimal or absent. The vessel pattern was noted.

Within one week of TVS, hysteroscopy-guided biopsy was scheduled as a minor operation theatre procedure. A 5 mm Karl Storz rigid hysteroscope (Karl Storz, Germany) was used. Normal saline (0.9%) was used as the distension medium. The endocervical canal, endometrial cavity, anterior, posterior, lateral walls and fundus of the uterus were also examined systematically. Special attention was paid to the areas where increased vascularity was noted on the TVS. If there was a suspicious growth, biopsy was taken using biopsy forceps which was introduced through the biopsy port. If there was no evidence of focal lesion, a thorough curettage of the endometrial cavity was
done. Hysteroscopy was reported as atrophic, normal, focal abnormality such as polyp, myoma and growth or diffuse hyperplasia. Endometrial hyperplasia was classified as hyperplasia without atypia and hyperplasia with atypia according to the WHO classification not according to the original Kurman’s classification. The histopathology report was collected.

Statistical analysis: Statistical analysis was done using SPSS software version 13 (IBM Corporation, USA). Continuous data such as age, age at menarche and age at menopause are expressed as mean±standard deviation. Categorical data such as parity, comorbidities and BMI are expressed as median with range. The mean uterine length, width and ET of malignant and premalignant lesions were compared with other benign lesions using independent Student’s \( t \) test. The predictive power of TVS was determined by calculating the sensitivity, specificity, positive predictive value and negative predictive values using the cross-tabulations. The significance \( (P) \) of these data was calculated using Fisher’s exact test.

Results

Of the 100 women included, 46 were postmenopausal and the average age at menopause was 52.6±6 yr, the remaining 54 were premenopausal. The mean age of the women included in the study was 50.4±5.48 yr. The median parity of women was 2.5 (range 0-7). Majority of the women were multiparous (94%). The mean BMI was 26.29±4.14 kg/m\(^2\). More than half of the women (55%) were obese and around one-fourth (28%) were in the overweight category.

In the postmenopausal group, the median duration of menopause before the presenting symptom was four years (range: 1-25 yr). Most of the women (67.39%) had PMB within five years of menopause (n=31). Around 20 per cent of women had bleeding after five years but before 10 yr of menopause (n=9); only two women had PMB after 15 yr of menopause. Majority of postmenopausal women (74%) presented within three months of symptoms (n=34) as against 24 per cent in the premenopausal group (n=13). It was observed that more than half (51.8%) of premenopausal women approached higher institute after six months of the complaint (n=28).

These women were subjected to TVS with power Doppler, hysteroscopy and guided biopsy/thorough endometrial curettage. The histopathological report was considered as the gold standard and the predictive power of various diagnostic tools was evaluated. The diagnostic tools assessed in the study were grey scale transvaginal ultrasound, power Doppler pattern and hysteroscopy. Table I depicts the distribution of various histopathological diagnoses of AUB in post- and premenopausal groups.

Transvaginal ultrasound: The most important parameter in grey scale ultrasound is ET. ET is commonly expressed in millimetre (mm). ET of \( >4 \) mm in the postmenopausal women and \( >8 \) mm in the premenopausal women was considered as thick endometrium. Twenty six per cent of women in premenopausal group (n=14) and 61 per cent in postmenopausal group (n=28) had thick endometrium. Apart from ET, other parameters measured were

<table>
<thead>
<tr>
<th>Diagnosis by histopathology</th>
<th>Postmenopausal women (n=46), n (%)</th>
<th>Premenopausal women (n=54), n (%)</th>
<th>Total frequency (n=100)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atrophic endometritis</td>
<td>16 (34.8)</td>
<td>0</td>
<td>16</td>
</tr>
<tr>
<td>Chronic endometritis</td>
<td>3 (6.5)</td>
<td>5 (9.2)</td>
<td>8</td>
</tr>
<tr>
<td>Endometrial polyp</td>
<td>12 (26)</td>
<td>5 (9.2)</td>
<td>17</td>
</tr>
<tr>
<td>Proliferative endometrium</td>
<td>4 (8.6)</td>
<td>13 (24)</td>
<td>17</td>
</tr>
<tr>
<td>Disordered proliferative endometrium</td>
<td>2 (4.3)</td>
<td>14 (25.9)</td>
<td>16</td>
</tr>
<tr>
<td>Secretory endometrium</td>
<td>2 (4.3)</td>
<td>14 (25.9)</td>
<td>14</td>
</tr>
<tr>
<td>Endometrial hyperplasia*</td>
<td>3 (6.5)</td>
<td>5 (9.2)</td>
<td>8</td>
</tr>
<tr>
<td>Endometrial carcinoma</td>
<td>2 (4.3)</td>
<td>2 (3.7)</td>
<td>4</td>
</tr>
<tr>
<td>Uterine sarcoma</td>
<td>1 (2.1)</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Pill endometrium</td>
<td>0</td>
<td>1 (3.7)</td>
<td>1</td>
</tr>
<tr>
<td>Inadequate specimen</td>
<td>1 (2.1)</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

*One had simple hyperplasia without atypia, one had simple hyperplasia with atypia and two had complex hyperplasia with atypia.
uterine height and width; other findings such as the intra-uterine fluid collection, myoma and endometrial polyps were also noted. The mean uterine length, width and ET were higher in women with hyperplasia and carcinoma when compared to others. The difference was significant only for uterine width (5.1±0.95 cm vs. 4.5±0.73 cm, P=0.01).

Endometrial polyps were observed in three per cent, intrauterine fluid collection in three per cent myoma in five per cent. Among three patients who had intrauterine fluid collection, the final diagnosis was uterine sarcoma, endometrial carcinoma and atrophic endometrium. The distribution of ET in various histopathological reports is depicted in Fig.1.

Twenty six per cent of women in the premenopausal and 61 per cent in the postmenopausal group had thickened endometrium. Except one case, all other women with endometrial carcinoma had ET ≥4 mm. All cases of endometrial hyperplasia had abnormal ET. Considering the cut-off as ≥4 mm, the predictive power of TVS was calculated (Table II). Among premenopausal women with AUB, there is no consensus on the ET cut-off. Hence, different cut-offs were used and predictive power of ET was calculated. The cut-offs used were ≥8 and ≥12 mm (Table II). There were only two cases of hyperplasia in the premenopausal group. It was evident from Table II that if the cut-off was increased to ≥12 mm, it had a higher specificity without altering the sensitivity.

**Power Doppler:** The vascularity of endometrium was assessed using power Doppler. More than half of the women (56%) had no vascularity. Among women who had vascularity, 18 per cent had single-vessel, 15 per cent had scattered-vessel and 11 per cent had multiple-vessel pattern. Of the 18 women who had single-vessel pattern (Fig. 2), nine had polyp. The other diagnoses were atrophy (1), proliferative endometrium (2), disordered proliferative endometrium (3), secretory endometrium (1) and endometrial carcinoma (2). In 15 women who had scattered-vessel pattern, the final diagnoses were disordered proliferative endometrium (4), proliferative endometrium (3), secretory endometrium (3), uterine sarcoma (1), chronic endometritis (2) and endometrial hyperplasia (2). In those who had multiple-vessel pattern (Fig. 3), the final histopathological diagnoses were proliferative endometrium (4), disordered proliferative endometrium (2), hyperplasia (1), carcinoma (3) and chronic endometritis (1).

![Fig. 1. Distribution of endometrial thickness in various histopathological diagnoses.](image)

![Fig. 2. Single-vessel pattern.](image)

| Table II. Predictive power of transvaginal ultrasonography-endometrial thickness (ET) of diagnosing premalignancy and malignancy |
|-----------------|-----------------|-----------------|
| Parameter       | Postmenopausal women (ET ≥4 mm) (%) | Premenopausal women (ET ≥8 mm) (%) | Premenopausal women (ET ≥12 mm) (%) |
| Sensitivity     | 85.7            | 100             | 100             |
| Specificity     | 33.3            | 38.5            | 78.8            |
| Positive predictive value | 18.1            | 6               | 15.4            |
| Negative predictive value | 92.9            | 100             | 100             |
| P               | 0.4             | 0.5             | 0.05            |
Taking hysteroscopic-guided biopsy as gold standard, the efficacy of power Doppler patterns was compared. Single-vessel, scattered-vessel and multiple-vessel patterns could predict polyp, hyperplasia and carcinoma, respectively (Table III). The negative predictive values were more than 90 per cent, which implied that the presence of pathology in the absence of vascularity was less likely. The positive predictive values of detecting hyperplasia and carcinoma based on the vascularity were low because of the lesser number of cases. There were only four cases of hyperplasia and five cases of carcinoma. If abnormal ET and power Doppler pattern were combined, the specificity of detecting hyperplasia increased to 89.6 per cent and the specificity of detecting carcinoma increased to 92.6 per cent.

**Hysteroscopy:** Hysteroscopic observations were recorded as atrophy, normal, focal or diffuse abnormality. The classic description of chronic endometritis is micro polyps, stromal oedema and increased vascularity. This finding was noted in three of eight women who had chronic endometritis on histopathological examination. Predictive values for hysteroscopy are mentioned in Table IV.

Irrespective of the diagnosis, hysteroscopy had high negative predictive value. The sensitivity was 100 per cent in diagnosing atrophy, polyp (Fig. 4) and hyperplasia (Fig. 5). The sensitivity of detecting carcinoma was 60 per cent because there was one case (out of four) who had malignancy in the polyp. The positive predictive value for hyperplasia was less (26.7%). Of the 15 patients who had hyperplasia on hysteroscopy, only four had hyperplasia eventually on histopathology. Five patients had proliferative endometrium, three had disordered proliferative, two had secretory endometrium and one had endometrial carcinoma.

**Discussion**

AUB is an important symptom which requires careful evaluation, more so among perimenopausal

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**Table III.** Predictive values of various power Doppler patterns

<table>
<thead>
<tr>
<th>Test characteristics</th>
<th>Power Doppler pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Single-vessel (for polyp) (%)</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>52.9</td>
</tr>
<tr>
<td>Specificity</td>
<td>89.2</td>
</tr>
<tr>
<td>Positive predictive value</td>
<td>50</td>
</tr>
<tr>
<td>Negative predictive value</td>
<td>90.2</td>
</tr>
<tr>
<td>( P )</td>
<td>0.001</td>
</tr>
</tbody>
</table>

**Table IV.** Per cent predictive values for hysteroscopy

<table>
<thead>
<tr>
<th>Test characteristics</th>
<th>Atrophy</th>
<th>Endometrial polyp</th>
<th>Hyperplasia</th>
<th>Carcinoma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivity</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>60</td>
</tr>
<tr>
<td>Specificity</td>
<td>97.6</td>
<td>97.6</td>
<td>88.5</td>
<td>100</td>
</tr>
<tr>
<td>Positive predictive value</td>
<td>88.1</td>
<td>89.5</td>
<td>26.7</td>
<td>100</td>
</tr>
<tr>
<td>Negative predictive value</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>97.6</td>
</tr>
<tr>
<td>( P )</td>
<td>0.001</td>
<td>0.001</td>
<td>0.001</td>
<td>0.001</td>
</tr>
</tbody>
</table>
women. Although various diagnostic tools are available, there is a need for an effective non-invasive technique which can predict the endometrial pathology. Commonly used non-invasive tool to predict endometrial pathology is grey scale TVS, mainly ET. In the present study, the sensitivity and specificity of TVS in detecting endometrial hyperplasia/carcinoma were 85.7 and 33.3 per cent, respectively. Schramm et al11 evaluated the use of TVS in diagnosing endometrial carcinoma alone, and noted a sensitivity of 62 per cent and specificity of 50 per cent. In the present study, the negative predictive value of ET in predicting premalignant and malignant condition was 92.9 per cent. Similar observations were made by others12,13 who showed a negative predictive value of 100 per cent. A multicentre study by Karlsson et al14 included 1168 postmenopausal women and concluded that if a cut-off of ≤4 mm was used, the negative predictive value of TVS-ET was 100 per cent. Among premenopausal women with AUB, there is no consensus on the ET cut-off. Some researchers have used a cut-off of 8 mm, while others have used a cut-off of 12 mm15-17.

To overcome the shortcomings of TVS-ET alone, vessel pattern on power Doppler is studied. The vessel pattern can predict the pathology6-8,18. Single-vessel pattern predicts endometrial polyp, scattered-vessel pattern predicts hyperplasia and multiple-vessel pattern can predict endometrial carcinoma.

In the present study, the negative predictive value of various power Doppler patterns was more than 90 per cent, implying that the presence of pathology in the absence of vascularity was less likely. The positive predictive value for detecting hyperplasia and carcinoma based on the vascularity was low because of small sample.

There were seven cases where ET was less than 4 mm but had increased vascularity. Of these seven patients, one had endometrial carcinoma and one had uterine sarcoma. If biopsy was not performed in these seven patients considering the ET as normal, two cases of malignancy would have been missed. Hence, irrespective of ET, the presence of vascularity is a significant predictive factor. If abnormal ET and power Doppler pattern were combined, the specificity of detecting hyperplasia increased to 89.6 per cent and specificity of detecting carcinoma increased to 92.6 per cent.

In the present study, the specificity, positive predictive value and negative predictive value of hysteroscopy in detecting endometrial carcinoma were 100, 100 and 97.6 per cent, respectively. These results were in accordance with various other studies. In a study by El-Morsi et al7, the specificity was 95.89 per cent, positive predictive value was 62.5 per cent and negative predictive value of 97.2 per cent. The specificity of detecting endometrial carcinoma was 87.5 per cent in a study by Tandulwadkar et al19 and 100 per cent in the study by Loverro et al20.

There was one patient whose TVS, power Doppler and hysteroscopy suggested a polyp which turned out to be an endometrial adenocarcinoma on histopathology. This is likely to alter the results as there were only five patients with endometrial carcinoma including this patient.

The strengths of our study were use of all the available methods for the assessment of endometrial pathology which led to meaningful comparisons and
women with other apparent causes of AUB which were unlikely to be due to endometrial pathology were excluded. The limitations of the study were small number of women with endometrial carcinoma which led to poor sensitivity and positive predictive value of the evaluation methods. Transvaginal ultrasound, power Doppler and hysteroscopy for all the patients were not done by the same observer due to logistic reasons which could have led to inter-observer variations.

It was noticed that postmenopausal women approached the health facility earlier when compared to premenopausal women with AUB. Our results showed that for evaluation of AUB, addition of power Doppler to grey scale TVS improved the specificity and negative predictive value almost comparable to hysteroscopy. However, the sensitivity and positive predictive value did not improve probably owing to small number of endometrial hyperplasia/carcinoma (9 of 100) in the study.

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Conflicts of Interest: None.

References


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Labour & delivery monitoring patterns in facility births across five districts of India: A cross-sectional observational study

Shalini Singh¹, Jyotika A. Kashyap³, Nomita Chandhiok¹, Vipin Kumar⁴, Vishwajeet Singh² & Richa Goel⁵ for an ICMR-UNFPA Task Force study on reducing maternal mortality and morbidity through promotion of evidence based intrapartum and early postpartum care*

¹Division of Reproductive Biology, Maternal & Child Health, Indian Council of Medical Research, ²Department of Biostatistics, All India Institute of Medical Sciences, New Delhi, ³Department of Obstetrics & Gynaecology, Sir Sayajirao General Hospital, Medical College, Vadodara & ⁴Division of Clinical Oncology, ICMR-National Institute of Cancer Prevention & Research, Noida, India & ⁵Independant Consultant

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Background & objectives: India has recorded a marked increase in facility births due to government’s conditional cash benefit scheme initiated in 2005. However, concerns have been raised regarding the need for improvement in the quality of care at facilities. Here we report the monitoring patterns during labour and delivery documented by direct observation in reference to the government’s evidence-based guidelines on skilled birth attendance in five districts of India.

Methods: A cross-sectional study design with multistage sampling was used for observation of labour and delivery processes of low-risk women with singleton pregnancy in five districts of the country. Trained research staff recorded the findings on pre-tested case record sheets.

Results: A total of 1479 women were observed during active first stage of labour and delivery in 55 facilities. The overall frequency of monitoring of temperature, pulse and blood pressure was low at all facilities. The frequency of monitoring uterine contractions and foetal heart sounds was less than the expected norm, while the frequency of vaginal examinations was high at all levels of facilities. Partograph plotting was done in only 15.8 per cent deliveries, and labour was augmented in about half of the cases.

Interpretation & conclusions: The findings of our study point towards a need for improvement in monitoring of maternal and foetal parameters during labour and delivery in facility births and to improve adherence to government guidelines for skilled birth attendance.

Key words Guidelines - labour monitoring - partograph - quality of care - skilled birth attendance

The following nodal centres (Site Investigators) participated in the study* listed in alphabetical order:
Motilal Nehru Medical College, Allahabad (Ragini Mehrotra & Meena Dayal); Government Kilpauk Medical College, Chennai (A.M. Famida); National Institute of Applied Human Research and Development, Cuttack (Saraswati Swain); Sawai Man Singh Medical College, Jaipur (Vimla Jain); Sir Sayajirao General Hospital and Medical College, Vadodara (S.L. Pagi).
The presence of skilled care during delivery and soon after childbirth is considered one of the most cost-effective interventions to attain the Sustainable Development Goal 3 of reducing maternal mortality ratio to <70 per 100,000 live births\(^3\). The Government of India (GOI) in 2005 initiated incentivizing facility births through the Janani Suraksha Yojana to increase the coverage of skilled attendance at birth\(^4\) and published Guidelines for Skilled Birth Attendance to assist the health personnel to effectively provide good quality maternity care\(^4\). The overall institutional delivery rate has doubled from 43 per cent in 2008 to 84 per cent in 2013\(^5\), however, India lags behind in achieving the goal for reduction in maternal death\(^6-8\). The need of improving training and skills of healthcare providers as well as service delivery have been raised in the reports of the Joint Review Mission and the Common Review Mission which were commissioned for monitoring and evaluating the programme\(^2,8\). There is a paucity of data on implementation of the guidelines on skilled birth attendance at the facility level. Therefore, this cross-sectional direct observational study was conducted to evaluate the provider practices and processes during labour and early post-partum period vis-à-vis the government guidelines on skilled birth attendance in five districts of the country.

**Material & Methods**

**Study area and design:** The study was conceptualized and coordinated by the division of Reproductive Biology, Maternal & Child Health, Indian Council of Medical Research (ICMR), New Delhi, India. This direct observational study was carried out using a cross-sectional study design during June 2010-April 2011. Multistage sampling was followed; in the first stage, five States were selected, namely Rajasthan and Gujarat in the West, Uttar Pradesh in the North, Odisha from the East and Tamil Nadu from the Southern region of India. In the next stage, one district from each State was selected based on the convenience of the core research unit and the suggestions received from the Ministry of Health & Family Welfare, GOI. These were Jaipur (Rajasthan), Allahabad (Uttar Pradesh) and Cuttack (Odisha) from Empowered Action Group States with poor performance in maternal health, Vadodara (Gujarat) with medium performance and Thiruvallur (Tamil Nadu) with good performance in maternal health. In the third stage, all public health facilities in the selected districts were enumerated for random selection. In the urban areas, 11 public health facilities were selected which included five medical colleges (MCs), five district hospitals (DHs) and one sub-DH. In the rural areas, first referral units (FRUs)/community health centres (CHCs) with more than 500 deliveries and primary health centres (PHCs) with more than 200 deliveries in the past year and accessible by road from the MC within 2-3 hours were selected. These included 20 FRUs/CHCs and 24 PHCs.

**Sample size calculation:** Assuming the prevalence of the use of antepartum oxytocin to be around 50 per cent in the deliveries observed, the sample size was calculated at 95 per cent confidence level, with margin of error of 10 per cent and a design effect of 1.5; a sample of 144 women each, in urban and rural areas of the chosen study districts was required.

**Site preparation:** One nodal officer from the State Health Department coordinated the study implementation in the chosen district. The research staff underwent centralized training at ICMR headquarters, New Delhi on study protocol, implementing strategies, data collection and data entry procedures and regular monitoring was done by site investigators and central coordinating unit from ICMR.

**Study population:** Women with singleton pregnancy of more than 34 wk gestation, in active first stage of labour (cervical dilation >4 cm), with no obvious obstetric or medical complications and willing to participate in the study were enrolled. Women were observed up to four hours after delivery or till discharge, whichever was earlier. Women who were shifted out of labour room due to referral to a higher centre or for caesarean section or for any other reason and whose observation could not be completed were excluded from the study. Institutional ethical clearance certificates were obtained from all the five district nodal centres and written informed consents were obtained from all the participants.

**Variable measurement:** A structured pre-tested case record form based on the GOI guidelines for skilled birth attendance was used to record the study data. The observers recorded the number of times the provider(s) carried out different examinations/procedures on each participant from the time of enrolment till delivery. The action of keeping the stethoscope or doppler on the woman’s abdomen was considered as examination of foetal heart sound (FHS), keeping hand over uterus during contractions and during interval between two
consecutive labour pains was considered as examination of uterine contractions (UCs), applying blood pressure cuff over the upper arm and using stethoscope was considered as measurement of blood pressure.

**Statistical analysis:** Data entry was done on case record forms and entered in excel sheets at site offices with appropriate coding and de-identification. Range checks and logical checks were run to minimize the errors. For descriptive statistics, quantitative variables were organized as mean (standard deviation) and continuous variables as median (mode), respectively, in analysis.

For each case, the total duration of observation from enrolment to delivery was calculated in minutes, and number of examinations of UCs, FHS and vaginal examination were recorded. Using one examination of UC and FHS in every 30 min and one vaginal examination in every four hours as the standard norm, the number of observations per unit time for each case was calculated.

Kruskal-Wallis test followed by Dunn’s test was used for non-parametric pair-wise multiple comparisons in different independent facilities (MCs, DHs, FRUs/CHCs and PHCs) to compare median observation per unit time of UC, FHS examination and vaginal examination. Data were analyzed using STATA (version 13, StataCorp LP, College Station, TX, USA).

**Results**

A total of 1922 women were screened of whom 332 women were excluded as they did not meet the inclusion criteria. Of the remaining 1590 women, 111 whose observations could not be completed due to transfer for Caesarean section, referral and other reasons were further excluded. Thus, a total of 1479 women from 55 facilities completed the study (Fig. 1).

The sociodemographic characteristics of participants are described in Table I. The mean age of the participants was 24.3±0.09 yr. The average number of antenatal visits was 4.7 for all women enrolled. Nearly 30.7 per cent women in the PHCs were uneducated which was three times higher than those enrolled in the MCs.

**General & obstetric examination during observation period:** The overall frequency of examinations carried out during the observation period is shown in Table II. Uterine contractions was the least measured parameter in obstetric examination at enrolment, and vaginal examinations were done the maximum number of times both at enrolment and during the 1st stage of labour. Plotting of partograph was very low (15.8%), the highest being 21.7 per cent in FRUs/CHCs and the lowest in district/sub DH (1.6%).

Fig. 2 depicts the proportion of participants in whom four parameters, namely blood pressure, UC, FHSs and vaginal examination were not performed even once during the entire observation period. Monitoring of UCs was poorest in DHs followed by PHCs and FRUs/CHCs. Monitoring of blood pressure and FHS of participants was poor in FRUs/CHCs and PHCs.

**Monitoring during labour:** Table III depicts the facility-wise distribution of labour monitoring pattern and its comparison to the expected norm. The median value of the observation time in MCs was significantly higher than all other facilities. The difference in the median observation time between FRUs and PHCs was not significant. The median number of UC examinations per hour was low in all the facilities when compared to the standard norm (2/h). MCs had significantly higher median values for UCs and FHS examination as compared to all other facilities. The difference in the median number of UC examination between FRUs and PHCs was not significant. The difference in the median number of FHS examination between FRUs and PHCs and DHs and PHCs was also not significant. In contrast to the examination pattern of UCs and FHS, the mean number of vaginal examination per woman was nearly three to four times higher than the standard norm of one examination per four hours across different levels of facilities. Median number of vaginal examinations per hour was higher in MCs as compared to other health facilities. There was no

![Fig. 1. Flow diagram showing enrolment of study participants.](image-url)
significant difference in the frequency of vaginal examination between FRUs/CHCs and PHCs.

**Drugs used during labour**: Labour was augmented in more than half (48.8%) of the total cases observed (Table IV). Nearly 44.7 per cent women observed in PHCs were given drugs to augment labour. In nearly 4.0 per cent women, intramuscular oxytocin was administered during labour before delivery. Intravenous oxytocin was administered in the first stage of labour to 76.6 per cent of women at MCs and only 22.5 per cent of women at DHs. Providers other than doctors (SNs/LHVs/ANMs) were also seen administering oxytocic drugs before delivery (Data not shown).

**Outcome of delivery**: A total of 1480 newborns were delivered including a set of twins (Table V). There were one fresh stillbirth and one early neonatal death during the observation period. Overall, meconium was present in 7.6 per cent deliveries and 5.5 per cent babies required resuscitation.

**Discussion**

In general, between 70 and 80 per cent of all pregnant women may be considered as low-risk at the start of labour. While most women go through labour without any need for intervention, some develop complications rapidly. Therefore, regular monitoring of woman and the foetus are crucial to identify any
departure from safe delivery and to manage these complications early. Several countries, including Malaysia and Sri Lanka, have demonstrated reduction in maternal and perinatal mortality using the services of skilled birth attendants in facilities supported by adequate equipment, supplies and transport\textsuperscript{11}. Detailed clinical guidelines for intrapartum care published by National Institute for Health and Care Excellence (NICE) provide a framework to the caregivers for monitoring of mother and the foetus\textsuperscript{12}. The Indian guideline on skilled birth attendance incorporates evidence-based best practices as norms for optimal care during normal labour\textsuperscript{4}. Accordingly, after initial assessment of the woman, it is important to monitor maternal pulse, FHS and UCs every 30 min during active first stage of labour and vaginal examination, and blood pressure is recommended at four hourly intervals\textsuperscript{4}.

This study indicates a deficiency of monitoring the general physical well-being of the woman during labour at all levels of healthcare facilities; and the deficiency was higher in the primary and secondary healthcare facilities. Parameters, such as temperature, blood pressure and pulse, were never examined in 19.3 per cent of women enrolled in the study. A study carried out in a teaching hospital in Egypt indicated the omission of assessing temperature (88%), pulse (60%) and blood

<table>
<thead>
<tr>
<th>Parameters examined during each stage of study</th>
<th>MCs (n=368) (%)</th>
<th>DHs (n=378) (%)</th>
<th>FRUs/CHCs (n=489) (%)</th>
<th>PHCs (n=244) (%)</th>
<th>Total (n=1479) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>At enrolment</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Temperature</td>
<td>189 (51.4)</td>
<td>28 (7.4)</td>
<td>89 (18.2)</td>
<td>53 (21.7)</td>
<td>359 (24.3)</td>
</tr>
<tr>
<td>Pulse</td>
<td>355 (96.5)</td>
<td>283 (74.9)</td>
<td>191 (39.1)</td>
<td>91 (37.3)</td>
<td>920 (62.2)</td>
</tr>
<tr>
<td>BP</td>
<td>355 (96.5)</td>
<td>369 (97.6)</td>
<td>216 (44.2)</td>
<td>104 (42.6)</td>
<td>1044 (70.6)</td>
</tr>
<tr>
<td>Chest/CVS examination</td>
<td>241 (65.5)</td>
<td>134 (35.5)</td>
<td>133 (27.2)</td>
<td>68 (27.9)</td>
<td>576 (39.0)</td>
</tr>
<tr>
<td>FHS</td>
<td>352 (95.7)</td>
<td>371 (98.2)</td>
<td>232 (47.4)</td>
<td>130 (53.3)</td>
<td>1085 (73.4)</td>
</tr>
<tr>
<td>UCs</td>
<td>155 (42.1)</td>
<td>31 (8.2)</td>
<td>10 (2)</td>
<td>20 (8.2)</td>
<td>216 (14.6)</td>
</tr>
<tr>
<td>PV examination</td>
<td>351 (95.4)</td>
<td>370 (97.9)</td>
<td>270 (55.2)</td>
<td>129 (52.9)</td>
<td>1120 (75.8)</td>
</tr>
<tr>
<td><strong>During 1\textsuperscript{st} stage</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Temperature</td>
<td>124 (33.7)</td>
<td>12 (3.7)</td>
<td>113 (23.1)</td>
<td>39 (15.9)</td>
<td>288 (19.5)</td>
</tr>
<tr>
<td>Pulse</td>
<td>314 (85.3)</td>
<td>258 (68.3)</td>
<td>225 (46)</td>
<td>113 (46.3)</td>
<td>910 (61.5)</td>
</tr>
<tr>
<td>BP</td>
<td>319 (86.7)</td>
<td>289 (76.5)</td>
<td>242 (49.5)</td>
<td>130 (53.3)</td>
<td>980 (66.3)</td>
</tr>
<tr>
<td>FHS</td>
<td>351 (95.4)</td>
<td>289 (76.5)</td>
<td>306 (62.6)</td>
<td>160 (65.6)</td>
<td>1106 (74.8)</td>
</tr>
<tr>
<td>UCs</td>
<td>308 (83.7)</td>
<td>176 (46.6)</td>
<td>236 (48.3)</td>
<td>144 (59)</td>
<td>864 (58.4)</td>
</tr>
<tr>
<td>PV examination</td>
<td>357 (97)</td>
<td>341 (90.2)</td>
<td>397 (81.2)</td>
<td>201 (82.4)</td>
<td>1296 (87.6)</td>
</tr>
<tr>
<td>Partograph plotting</td>
<td>71 (19.3)</td>
<td>6 (1.6)</td>
<td>106 (21.7)</td>
<td>51 (10.9)</td>
<td>234 (15.8)</td>
</tr>
<tr>
<td><strong>During 2\textsuperscript{nd} stage</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Temperature</td>
<td>74 (20.1)</td>
<td>4 (1.1)</td>
<td>36 (7.4)</td>
<td>19 (7.8)</td>
<td>133 (8.9)</td>
</tr>
<tr>
<td>Pulse</td>
<td>184 (50)</td>
<td>81 (21.4)</td>
<td>122 (24.9)</td>
<td>56 (22.9)</td>
<td>443 (29.9)</td>
</tr>
<tr>
<td>BP</td>
<td>164 (44.6)</td>
<td>91 (24.1)</td>
<td>106 (21.7)</td>
<td>67 (27.5)</td>
<td>428 (28.9)</td>
</tr>
<tr>
<td>FHS</td>
<td>187 (50.8)</td>
<td>108 (28.6)</td>
<td>162 (33.1)</td>
<td>71 (29.1)</td>
<td>528 (35.7)</td>
</tr>
<tr>
<td>UCs</td>
<td>245 (66.6)</td>
<td>115 (30.4)</td>
<td>182 (37.2)</td>
<td>87 (35.7)</td>
<td>629 (42.5)</td>
</tr>
<tr>
<td><strong>After delivery</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pulse</td>
<td>270 (73.4)</td>
<td>104 (27.5)</td>
<td>162 (33.1)</td>
<td>67 (27.5)</td>
<td>603 (40.8)</td>
</tr>
<tr>
<td>BP</td>
<td>234 (63.6)</td>
<td>106 (28.4)</td>
<td>161 (32.9)</td>
<td>74 (30.3)</td>
<td>575 (38.9)</td>
</tr>
<tr>
<td>Abdomen checked</td>
<td>247 (67.1)</td>
<td>138 (36.5)</td>
<td>177 (36.2)</td>
<td>70 (28.7)</td>
<td>632 (42.7)</td>
</tr>
<tr>
<td>Perineum checked</td>
<td>231 (62.8)</td>
<td>225 (59.5)</td>
<td>266 (54.4)</td>
<td>100 (41)</td>
<td>822 (55.6)</td>
</tr>
</tbody>
</table>

FRUs/CHCs, first referral units/community health centres; PHCs, primary health centres; CVS, cardiovascular; BP, blood pressure; UCs, uterine contractions; FHS, foetal heart sounds; MCs, medical colleges; DHs, district hospitals; PV, per vaginal
pressure (19%) among women, which was similar to observations in our study. Indicators, such as measuring temperature, pulse and blood pressure, are well established, easy to measure and do not require expensive equipment. It is also known that adverse changes in these indicators can affect labour management and pregnancy outcome. A qualitative study featuring observations of women in labour in rural Rajasthan revealed inadequate monitoring of the maternal and foetal condition (by way of blood pressure and FHSs). Nearly one-third of the maternal deaths in India occur during intrapartum period and the absence of or infrequent monitoring of these parameters during labour deprives the women of a chance for early diagnosis and management or timely referral to a higher facility when the need arises. The omission of monitoring temperature, pulse and blood pressure may delay the diagnosis of sepsis and preeclampsia which contribute to 11 and 5 per cent of maternal mortality, respectively in India.

The plotting of partograph is an effective tool for monitoring progress of labour, prevents prolonged and obstructed labour and helps to undertake decision for augmentation, Caesarean section and transfer of women to higher facility for comprehensive emergency obstetric care. These benefits may be more in low-resource settings where the use of standard labour management protocol is inconsistent. Low use of partograph by providers in peripheral centres of Nigeria was similar to the findings of this study. In the MCs, the UCs and FHS examinations were close to the standard norm, but vaginal examinations were nearly six times higher. Preference of monitoring labour progress by frequent vaginal examinations seen in this study has also been reported from other studies in India.

### Table III. Labour monitoring pattern of the women enrolled in the study

<table>
<thead>
<tr>
<th>Parameters examined during labour</th>
<th>MCs (n=368)</th>
<th>DHs (n=378)</th>
<th>FRUs/CHCs (n=489)</th>
<th>PHCs (n=244)</th>
<th>P (Kruskal-Wallis test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total observation time from enrolment to delivery (h)</td>
<td>1292.0</td>
<td>1099.1</td>
<td>1210.3</td>
<td>621.6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Mean±SD observation period from enrolment to delivery (h)</td>
<td>3.5±2.5</td>
<td>2.9±2.1</td>
<td>2.5±2.0</td>
<td>2.6±2.0</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Median (range) observation period from enrolment to delivery (h)</td>
<td>3.0 (0.1-17.5)</td>
<td>2.4 (0.1-14.7)</td>
<td>1.8 (0.1-16.9)</td>
<td>2.0 (0.1-15.7)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

**UCs**

| Number of cases ever examined (%) | 308 (83.7) | 176 (46.6) | 236 (48.3) | 144 (59.0) | <0.001 |
| Mean±SD number of UC examinations per hour | 1.09±2.1 | 0.80±1.5 | 0.68±1.5 | 0.55±0.8 | <0.001 |
| Median (range) number of UC examinations per hour | 0.7 (0-20) | 0 (0-9.7) | 0 (0-20) | 0.2 (0-5.8) | <0.001 |

**FHS**

| Number of cases ever examined (%) | 353 (95.9) | 289 (76.5) | 306 (62.6) | 160 (65.6) | <0.001 |
| Mean±SD number of FHS examinations per case per hour | 1.47±2.7 | 0.98±1.7 | 0.90±1.8 | 0.69±1.1 | <0.001 |
| Median (range) number of FHS examinations per hour | 0.9 (0-30) | 0.4 (0-12.9) | 0.2 (0-30) | 0.2 (0-6) | <0.001 |

**Vaginal examination (PV)**

| Number of cases ever examined (%) | 357 (97.0) | 341 (90.2) | 400 (81.8) | 206 (84.4) | <0.001 |
| Mean±SD number of PV examinations per hour | 1.4±2.2 | 1.4±1.9 | 1.3±1.9 | 1.2±1.6 | <0.001 |
| Median (range) number of PV examinations per case per hour | 0.9 (0-20) | 0.8 (0-15) | 0.7 (0-20) | 0.7 (0-12) | <0.001 |

*Standard norm for UCs, FHS is two per hour and one per four hours for PV examination. Abbreviations are as given in Table II*
reported from a teaching hospital of Egypt\textsuperscript{21}. This study also indicated the inappropriate use of oxytocics such as use of unapproved vaginal misoprostol for labour augmentation, administration of intramuscular oxytocin before delivery, giving unlabelled intravenous oxytocin infusion without proper regulation of dose, administering oxytocin prior to delivery in clinical settings without facilities for Caesarean section such as PHCs and without plotting partograph for monitoring labour. Misuse of intrapartum oxytocin has been reported on the pretext of providing warmth for labour pains and was found to be culturally acceptable and demanded by women and her relatives who want to shorten the process of labour\textsuperscript{14}.

Nearly two-thirds of maternal deaths arise from complications during labour and early postpartum period\textsuperscript{22}. Routine care and early identification of complications play an important role in preventing maternal death and morbidity\textsuperscript{20}.

The strength of the study included reporting on directly observed patterns of labour monitoring covering different geographical regions of the country including from the good, medium and poor performing States with respect to maternal health indicators. The practices in the teaching hospitals were compared with district health system. Limitations of the study included the presence of external observers to document provider practices which might have resulted in modified or better practices (Hawthorne effect). Our study was not designed to evaluate the effect of provider practices on maternal and foetal outcome.

In conclusion, our results indicated an urgent need for strong reforms in the healthcare system, in-service training and monitoring of service delivery at all levels including teaching hospitals and district health system. With an increase in the proportion of institutional deliveries in India, the outcomes for mothers and newborns can be improved by optimizing the use of interventions during birth and improving standards of care through adhering to practice guidelines.

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**Conflicts of Interest:** None.

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9. StataCorp. **Stata statistical software: Release 13.** College Station, TX: StataCorp LP; 2013.


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Association of reduced count of interleukin-13-producing cells in breast milk with atopic dermatitis in infancy

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Background & objectives: Atopic dermatitis (AD) is one of the most common pathologic conditions of skin in children. The effect of breastfeeding on the risk of AD remains controversial. The aim of this study was to determine the counts of cytokine-producing cells in the mothers’ breast milk of infants with and without AD to assess association, if any.

Methods: Breast milk samples (10 ml) were obtained from mothers of 25 infants with AD and of 26 healthy infants as a control group. The number of cytokine-producing cells including interferon-gamma (IFN-γ), tumour necrosis factor-alpha (TNF-α), interleukin-13 (IL-13) and IL-4 in the milk samples was determined using an enzyme-linked immunospot assay technique.

Results: The mean of IL-13-producing cells in milk was significantly lower in mothers of AD-affected infants in comparison with mothers of normal infants (324.91±255.45 vs. 538.93±465.39, P<0.05). There were no significant differences between mothers of infants with and without AD regarding milk count of IFN-γ, TNF-α and IL-4-producing cells.

Interpretation & conclusions: Our results showed lower number of IL-13-producing cells in milk of mothers of infants with AD. Therefore, lower count of IL-13-producing cells in mothers’ milk may confer a susceptibility to AD. Further studies with a large number of samples need to be done to confirm our findings.

Key words: Atopic dermatitis - breastfeeding - cytokine-producing cells - infants - interleukin-13 - milk

Atopic dermatitis (AD) is a common dermatological disorder in infants. The age of onset for nearly 45 per cent of all cases is the first six months of life1. The prevalence of AD varies from two per cent in Iran to 20 per cent in Australia, the UK and Scandinavian countries2. AD is identified to be a complex of genetic predisposition, environmental factors, inflammatory skin mechanisms and some immunologic reactions3.
Two mechanisms contribute to the development of AD: immunoglobulin (Ig)E-mediated sensitization and/or dysfunction of the skin barrier. Clinically, AD may present as acute, sub-acute or in chronic phase. Acute type applies to a rapidly developing red rash which may be blistered and swollen. Chronic form refers to a longstanding irritable area. It is often darker than the encircling skin, thickened (lichenified) and much scraped. In acute phase, helper T-type 2 (Th2) cells initiate and maintain local inflammation through secretion of interleukin-4 (IL-4) and IL-13 which in turn stimulate IgE production and through IL-5 which mediates eosinophil differentiation. The cytokine IL-4 induces IgE and IgG1 secretion from B-cells. IL-13 also plays an important role in cutaneous Th2-related immune responses and can directly induce IL-5 expression and eosinophil infiltration. However, a shift from Th2 to Th1/Th0 cell-related immune responses was determined in acute-chronic transition. Interferon gamma (IFN-γ) induces Th1 cells and inhibits Th2 cell-related cytokine production. Tumour necrosis factor-alpha (TNF-α) is a mediator of both Th1 and Th2 responses, which induces the production of chemotactic agents in skin. Human milk is the preferable way for infant nutrition. The breast milk is important for immune development, tolerance and regulation of inflammation in infants. However, its contribution to the prevention of allergic disorders is controversial. While some reported its protective effect and increasing the risk of disease, others showed no association.

There is no information regarding the relation of the cytokine-producing cells in the breast milk and AD in infancy. Therefore, this study was undertaken to determine the number of cytokine-producing cells [including Th1 cell-related cytokines (IFN-γ and TNF-α) and Th2-related cytokines (IL-13 and IL-4)] in the mother’s breast milk of infants with and without AD to assess the association.

Material & Methods

This study was conducted in the department of Immunology, Kerman University of Medical Sciences, Kerman, Iran, during March to September 2013. Breast milk samples were obtained from mothers of 25 infants (age 10.02±5.82 months) with AD during seven months from March to September 2013 in Afzalipour Hospital of Kerman (a city located in the southeast of Iran). An expert dermatologist and an allergy specialist confirmed the presence of AD, according to the well-known Hanifin and Rajka diagnostic criteria. The women were chosen based on convenience criteria. A questionnaire including age of breastfed infants and their mothers, type of allergy, drug(s) taken, gender, parents’ consanguineous relationship and current diet was filled. The infants who received any immunomodulating and immunosuppressive agents such as antihistamine and local and systemic steroid for two weeks were excluded from the study. Perseverance of mothers for exclusive breastfeeding and abstinence of them from prevalent allergenic foods was considered as inclusion criteria. In all, mothers of 60 infants with AD visited the hospital during the study, of whom 35 women were excluded based on the exclusion criteria. This study was approved by the Ethical Committee of Kerman University of Medical Sciences, Kerman, Iran, and written informed consent was obtained from mothers if they agreed for breast milk sampling.

The control group consisted of 26 mothers of age-matched (9.84±5.50 months) healthy infants, with no history of AD disease and other dermatologic diseases. Age-matching was on the basis of group matching (not individual matching). The mothers of healthy infants visited the health centres for vaccination and monitoring of height and weight of the breastfed infants. All mothers were healthy, with no acute or chronic illnesses. The mothers with a history of recurrent infections, asthma, allergy and atopic diseases, any suspected immunological disorders, cigarette smoking and use of any drugs were excluded from the study. Other exclusion criteria were malignancy, surgery and major trauma in previous months. None of the mothers received any immunomodulating treatment within six months before milk collection.

A breast milk sample (10 ml) was obtained from all mothers included in the study, and the water layer was separated and stored at 4°C until the time of analysis. The number of cells producing cytokines including IFN-γ, TNF-α, IL-13 and IL-4 in milk samples was determined using the commercial ELISPOT kits (Mabtech, Stockholm, Sweden) according to the manufacturer’s guidelines.

Briefly, fresh milk samples were centrifuged at 2000 g for 10 min. After elimination of the fat layer, the pellet was washed with Hank’s balanced salt solution and the viable-floating cells (epithelial, mononuclear cells) were counted by a standard method using a haemocytometer slid and trypan blue staining (Merck, Germany).
ELISpot 96-well flat-bottomed plates were coated overnight at 4-8°C with 100 µl/well of specific capture antibody including anti-TNF-α and anti-IL-13 [15 and 10 µg/ml in phosphate-buffered saline (PBS), 100 µl/well, respectively]. The coated plates of IFN-γ and IL-4 were prepared. Then, the wells were washed with PBS and subsequently blocked with a complete culture medium (Gibco, Invitrogen Ltd, Paisley, UK) containing 10 per cent heat-inactivated foetal calf serum (FCS) (Gibco). The milk cells (2.5×10^6/well) were plated to a final volume of 200 µl/well of complete culture medium and incubated for 24 h at 37°C with 5 per cent CO_2. After washing, the cytokine-producing cells were visualized using detection antibodies; 100 µl/well [(anti-TNF-α, 0.5 µg/ml), (anti-IL-13, 1 µg/ml), (IFN-γ, 7-B6-ALP diluted 1:200) and (IL-4, IL4-Π-ALP diluted 1:300)]. Streptavidin-conjugated alkaline phosphatase diluted 1:1000 (v/v) in PBS/0.5 per cent FCS (for TNF-α and IL-13) was added and kept for one hour at room temperature (the washing were repeated) and 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt and nitroblue tetrazolium chloride substrate incubated for 10-30 min at room temperature in the dark successively (was washed with distilled water). The wells were observed using a stereomicroscope (Macro-Eye, Gordak Instruments, China). The number of specific cytokine-producing cells [spot-forming cells (SFCs)] was calculated by subtracting the number of spots in negative control wells from experimental ones.

Statistical analysis: Comparison between demographic characteristics of breastfed infants in the two groups was made by Chi-square test. Differences in variables were analyzed using unpaired t test as appropriate. The unadjusted and adjusted odds ratios (ORs) and 95 per cent confidence intervals (CIs) of the risk factors for AD were assayed by binary logistic regression model. The data were analyzed by SPSS statistical software (version 18, Chicago, IL, USA).

Results

Demographic characteristics of mothers and their infants are summarized in Table I. In the present study, the age range of infants was 1-21 months [9.93±5.60 (mean±SD)] (n=51). The age of the mothers of infants with AD was significantly higher than those mothers with infants without AD. A significant difference was also observed between infants with and without AD in case of their sex ratio (M/F) (18/7 in case and 10/16 in control, respectively) (P<0.05) and also the parents’ consanguineous relationship (P<0.05).

The ORs and 95 per cent CIs of the risk factors of AD are given in Table II. Both unadjusted and adjusted ORs were also calculated. In the adjusted model, between demographic variables, parents’ consanguineous relationship and the gender had no significant association with AD, when these variables were matched between the two groups, whereas mother’s age has significant association with AD (P=0.03). Moreover, in AD-breastfed infants, one unit increase in age of mother was associated with 32 per cent increase in odds of getting the disease in comparison with healthy breastfed infants.

The quantity of Th1 cell-related cytokine-producing cells in milk is summarized in Table III. The number of IFN-γ-producing cells was higher in the milk of mothers with AD infants compared to mothers with non-AD infants, but the difference was not statistically significant (P=0.08). No significant difference was also observed between mothers with and without AD infants with respect to the TNF-α-producing cells; however, this parameter was found to be augmented in mothers with AD infants.

The mean count of IL-13-producing cells was significantly lower in mothers of infants with AD in
Discussion

In this study cytokine-producing cells were measured in the breast milk of mothers feeding their infants with and without AD. In general, the role of Th2 and Th1 as pro-inflammatory and anti-inflammatory cytokines in allergic disorders is agreed. However, the results of the present study showed that the count of IL-13-producing cells was notably less in the milk of mothers of AD infants compared to healthy group. The multivariate analysis also showed that the determination of IL-13-secreting cells might be useful to predict the risk of AD. Moreover, the count of IL-4 producing cells was lower in the milk of mothers with AD infants compared to mothers with non-AD infants, although the difference was not significant.

The precise mechanisms by which the lower count of Th2-cytokine-producing cells may contribute to the development of AD infancy are not clear. It is known that atopic diseases are caused by Th2 cytokine response to allergens and increased levels of IL-4 and IL-13 have been shown in such conditions. The role of IL-13 in prognosis of AD appears to be complex. This cytokine may function as a pro-inflammatory and an anti-inflammatory cytokine. Since AD is caused by Th2 cytokine response dominance in acute phase and on the other hand, normal pregnancy is associated with the production of IL-13; based on these findings, infants at risk of atopic disease exhibit defective IL-13 production at birth. This may depict an inherent immaturity in the development of T cell-cytokine responses or could be an outcome of downregulation of responses by other factors. Furthermore, IL-13 response significantly decreases during the first year of life.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Univariate analysis, unadjusted OR (95% CI)</th>
<th>P</th>
<th>Multivariate analysis, adjusted OR (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-γ-producing cells level in milk</td>
<td>1.013 (0.998-1.029)</td>
<td>0.09</td>
<td>1.035 (1.006-1.065)</td>
<td>0.02</td>
</tr>
<tr>
<td>IL-13-producing cells level in milk</td>
<td>0.984 (0.968-1.001)</td>
<td>0.06</td>
<td>0.956 (0.924-0.989)</td>
<td>0.01</td>
</tr>
<tr>
<td>IL-4-producing cells level in milk</td>
<td>0.994 (0.986-1.003)</td>
<td>0.22</td>
<td>0.986 (0.971-1.001)</td>
<td>0.07</td>
</tr>
<tr>
<td>Mother’s age</td>
<td>1.16 (1.005-1.332)</td>
<td>0.04</td>
<td>1.321 (1.032-1.690)</td>
<td>0.03</td>
</tr>
<tr>
<td>Gender</td>
<td>0.243 (0.075-0.789)</td>
<td>0.02</td>
<td>0.239 (0.048-1.187)</td>
<td>0.08</td>
</tr>
<tr>
<td>Parents’ genetic relationship</td>
<td>0.285 (0.089-0.918)</td>
<td>0.03</td>
<td>0.253 (0.050-1.269)</td>
<td>0.09</td>
</tr>
</tbody>
</table>

*Due to low level of OR in our data, the level of cytokine-producing cells was calculated based on ten-fold increase. Variables that had a P<0.25 were significant in the univariate analysis, were entered in the logistic statistical model. First, each variable was individually and then all variables were examined together. Binary logistic regression assay was performed for analysis.

IFN-γ, interferon-gamma; IL, interleukin; OR, odds ratio; CI, confidence interval

Table II. Risk (unadjusted and adjusted odds ratio with 95% confidence interval) for atopic dermatitis development in infancy

<table>
<thead>
<tr>
<th>Cytokine-producing cells/ml milk</th>
<th>Mothers with AD infants</th>
<th>Mothers without AD infants</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-γ-producing cells</td>
<td>555.39±476.66</td>
<td>363.98±276.92</td>
</tr>
<tr>
<td>TNF-α-producing cells</td>
<td>502.86±384.84</td>
<td>443.73±330.68</td>
</tr>
<tr>
<td>IL-13-producing cells</td>
<td>324.91±255.45*</td>
<td>538.93±465.39</td>
</tr>
<tr>
<td>IL-4-producing cells</td>
<td>639.38±559.78</td>
<td>879.72±784.27</td>
</tr>
</tbody>
</table>

Values are given as mean±SD. *P<0.05 compared with mothers without AD infants. AD, atopic dermatitis; SD, standard deviation; IFN-γ, interferon-gamma; TNF-α, tumour necrosis factor-alpha; IL, interleukin

Table III. Comparison of milk counts of cytokine-producing cells/ml of milk between mothers with or without atopic dermatitis infants

Comparison to mothers of healthy infants (P<0.05). Furthermore, the risk of the disease was decreased to 4 per cent based on ten-fold increase in the milk level of IL-13-producing cells in allergic infants in comparison with non-allergic infants (Table II). No significant variance was observed between mothers with and without AD infants with respect to the IL-4-producing cells count. This parameter, however, was found to be lower in mothers with AD infants. For evaluating the accuracy of our results, the relation of the mean production level of each SFC was analyzed. There was no significant association between the mean count of IL-13- (434.02) with the IL-4- (761.90) and IFN-γ-producing cells (457.81) (P=0.47 and P=0.18, respectively). Conversely, there was a direct correlation between the mean count of IL-4- (761.90) with the IFN-γ mean count of cells (457.81) (P=0.004) (r=0.398) (not shown). Our study also indicated that most cytokine-producing cells were mononuclear and the viability percentage of cells was from 50 to 100 per cent.
The reduction in number of the two types of Th2-related cytokine-producing cells may contribute in the development of AD in infancy through impairment of mucosal immunity in the newborn intestine which leads to the local infections, and the infectious agents may have a role in the AD development. The association of a number of infections with AD development at infancy has been reported.

In accordance with our results, elevated levels of IFN-γ and TNF-α have been reported in allergic patients, however, there is a disagreement between our findings and other studies. The discrepancy may be because of difference in sample type (milk vs. blood) and phase of disease (early in infancy vs. elder age).

It should also be noted that both Th1 and Th2 cells are involved in the pathogenesis of AD. Actually, a biphasic immune response with a predominance of Th2 cell-related response during the acute phase and a dominant Th1 cell-related response in the chronic phase of AD has been reported. Accordingly, reduced number of mothers’ milk count of Th2 cell-related cytokines may result in an imbalance in the Th1/Th2 cell-associated immune responses with tendency toward the Th2 responses that may result in AD development and subsequent dominance of Th1-related pathways. However, modulation of the Th1 and Th2 cell-related immune responses is required for the control of AD.

Although we found no significant association between type of nutrition and AD, the impact of breast milk in preventing allergy has been reported controversially. These discrepancies may result from differences in study methods or disregarding the patients’ genetic dissimilarities and complex immunologic properties of breast milk.

Our study also showed that most cytokine-producing cells were mononuclear, and this was compatible with the predominance of these cells in breast milk. On the other hand, viability percentage of cells was from 50 to 100 per cent. Determination of viability percentage is of importance because high level of dead cells (30-50% and more) can represent excessive background staining and lack of special spots in the test.

This study had several limitations. The sample size was small to draw valid conclusions. Resolving limitations of such studies (e.g. small sample size, deficiency of random selection, short-term period of breastfeeding, absence of planning a single-blind study) and designing more interventional studies to elucidate efficacy or lack of efficacy of breastfeeding on allergic disorders can be beneficial.

In conclusion, our findings showed that small number of IL-13-producing cells in breast milk might be a potential biomarker for predicting AD in infancy. More research is necessary to validate the prediction models for AD in infancy based on evaluation of other cytokines originated from cells existing in the breast milk.

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Conflicts of Interest: None.

References


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High frequency of HPV16 European variant E350G among Mexican women from Sinaloa

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Background & objectives: Human papillomavirus (HPV) infections play a crucial role in the aetiology of cervical cancer (CC), and HPV16 is the primary viral genotype associated with CC. A number of variants of the HPV16 E6 gene are involved in the progression of CC, differing in their prevalence and biological and biochemical properties. This study was designed to determine the frequency of HPV types 16/18 and to identify the presence of HPV16 E6-variants in asymptomatic Mexican women.

Methods: A total of 189 cervical Pap smears were collected from women attending public health services in three different cities in Sinaloa, Mexico. Viral DNA was identified by amplification of E6 viral gene fragments using polymerase chain reaction (PCR). Identification of variants was done by sequencing a DNA fragment (321bp) of the HPV16 E6 gene.

Results: More than half of the women tested were HPV-positive (52.38%), with HPV16 being the most frequent genotype (21.16%), followed by HPV18 (8.99%). Sequence analysis of the E6-HPV16 PCR products showed that in all cases, the viruses corresponded to European variants. It was further observed that the E350G intra-variant was the most common (>76%).

Interpretation & conclusions: This study showed a predominance of European lineage variants of HPV16 among asymptomatic women from Sinaloa, Mexico, predominantly with of the E350G variant. This variant has been shown to be associated with an increased risk of early development of CC. The use of molecular identification of carcinogenic HPV and Pap test screening may be a good strategy for monitoring women to prevent CC.

Key words Cervical cancer - European variant - human papillomavirus - molecular diagnostics

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Cervical cancer (CC) is the fourth-most common cancer in women worldwide, affecting 500,000 individuals each year, primarily in the developing countries. Among Mexican women, CC is the leading cause of death from cancer with a mortality rate of 6.09 deaths per 100,000 women, as reported by the Health Ministry of Mexico. High mortality rates due to CC have been reported in Sinaloa, Mexico, where the mortality rate was 9.50 deaths per 100,000 >25 yr old women. The major cause of high-grade cervical squamous intraepithelial lesions (HG-SIL) is a persistent infection of the cervix with a high-risk human papillomavirus (HR-HPV) types, predominantly the HPV16 and/or HPV18 genotypes. The presence of HPV16 DNA has been reported in approximately 63-90 per cent of CC cases, being the most common worldwide. The HPV16 genotype is detected in almost 50 per cent of CC cases, followed by HPV18, HPV45 and HPV31. It has been reported that the prevalence of HPV infections in healthy women varies with age in different regions of the world. Most instances of CC occur 15-20 yr after the initial HPV infection. This period of latency could explain why CC is the most common in young and middle-aged women. The sequence variations in the HPV16 E6 gene have been observed to correctly classify HPV16 variants isolated from different continents. Therefore, most studies of HPV16 variants have focused on the E6 gene, which encodes the major transforming protein that inhibits apoptosis, promotes cellular proliferation and is associated with cancer aggressiveness. Worldwide, variants of the HPV16 genome in CC from different geographical areas have been classified into phylogenetic lineages, such as European (E) (A1-A4), Asian (As), Asian-America (AA) (D2 and D3), African-1 and -2 (Af1 and Af2) (B1, B2 and C) and North American (NA1) (D1). Some studies have shown that HPV16 variants promote disease progression, ranging from HG-SIL to the development of CC. In a previous study based on 47 sequenced HPV16 genomes, the European variant nt350G was observed to occur frequently in HG-SIL [odds ratio (OR)=3.8, P=0.03], and was present in 36 HPV16 isolates from women with minor cervical abnormalities [OR=4.8, P=0.07]. However, most strikingly, there was a clear association between the nt350G variant and HPV16 persistence over a period of two years. However, the distribution and genotyping of HPV16 among asymptomatic healthy women have been poorly studied in Mexico. The objective of this study was therefore, to identify the HPV16 variants present among healthy asymptomatic women with normal cervical cytology in the State of Sinaloa, Mexico.

Material & Methods

In this cross-sectional study, 189 cervical Pap smears were collected from equal number of women who attended the integral diagnostic service for early detection of CC from September 2013 to March 2014 in hospitals from the three primary cities of Sinaloa, Mexico (IMSS 032 Hospital from Guasave, IMSS 046 Hospital from Culiacan and a Public Hospital from Mazatlan). The study protocol was approved by the institutional Bioethics Committees for Clinical Research, Faculty of Chemical and Biological Sciences, Autonomous University of Sinaloa, Culiacan, Sinaloa, and National Institute of Cancer (INCan), Mexico City, and written informed consent was obtained from all participants.

A probabilistic sample size was obtained consecutively from a population of 2040 women with normal cytology, using a 90 per cent confidence level and 80 per cent of power with an accuracy of five per cent bilaterally, with an expected proportion of 16 per cent testing HPV16 positive, resulting in a sample size of 138. This study included women aged 16-69 yr who had attended a CC screening clinic, had normal cervical cytology, were not in their menstrual period and had not had intercourse 48 h before sampling. All consecutive women who fulfilled the inclusion criteria and consented to participate in the study were included.

All women with cervical neoplasia, dysplasia or who had a hysterectomy were excluded. Each woman was subjected to a cervical Pap smear, and an aliquot was stored at 4°C for analysis. Molecular HPV identification was performed using the consensus primers GP5+/GP6+ and HPV genotyping using HPV16 and HPV18 specific primers.

**Viral genotyping:** Cytological specimens were used for the detection of viral DNA by polymerase chain reaction (PCR) and for PCR-sequencing to identify HPV16 E6 variants. DNA extraction was performed using the salting out standard method. PCR was carried out with 150 ng of total DNA with the previously reported primers GP5+/GP6+ and HPV genotyping using HPV16 and HPV18 specific primers.
and HPV16 R (5´-CAA CAA GAC ATA CAT CGA CC-3´), and HPV18 F (5´-AAG GAT GCT GCA CCG GCT GA-3´), and HPV-18 R (5´-CAC GCA CAC GCT TGG CAG GT-3´) (Figure). When samples tested negative or the HPV type could not be identified, the primer pair GP5+/GP6+ was used, and the PCR products were sequenced.

Sequence analysis: HPV16 variants were characterized by full-length sequence analysis11 of a 321 bp fragment of the E6 gene in only 31 samples (Figure D). PCR products were purified using 75 per cent isopropanol Centri-Sep Spin Columns (Applied Biosystems, USA) and sequenced using a 310 ABI PRISM Genetic Analyser (Applied Biosystems, USA). All sequences were compared to known sequences for HPV types, available at the NCBI site (http://www.ncbi.nlm.nih.gov).

Statistical analysis: Nominal variables were reported as the number and percentages of cases. OR with 95 per cent confidence intervals (CIs) were used to estimate significance (χ² Mantel-Haenszel with P<0.05).

Results

Characteristics of 189 women included in this study are shown in Table I. Most women were young, had started being sexually active at approximately 17 yr of age, and had an average of two childbirths. Their average number of sex partners was two, ranged between 1 and 3. The results from cervical Pap smears showed that all women had normal cytology, but 102 (53.97%) exhibited low-to-moderate inflammation, with hyperaemia of stromal tissue, hyperplasia of basal cells, or leucocyte infiltration, despite all samples testing negative for cervical dysplasia. The PCR results showed that 99 of 189 samples (52.38%) were HPV positive, of these 40 (21.16%) were HPV16 positive and 17/189 (8.99%) tested positive for genotype 18; 10 (5.29%) were co-infected with both genotypes. Thirty one of 189 (16.40%) samples tested positive for other viral genotypes. Table II shows the association among risk factors, such as chronological age, age of the initiation of sexual activity, age at first childbirth, number of childbirths and number of sexual partners with HPV-infections and the presence of HPV16. With respect to HPV infection, only two factors showed a significant relationship. The age of women at their first childbirth being between the ages of 15 and 17 yr old (OR=3.1, CI 95%=1.19-8.24, P=0.03) and having three or more sex partners (OR=3.4, CI 95%=1.40-7.87, P=0.01) were associated with the high risk for testing HPV positive.

Concerning HPV16 infection, only one factor, the number of childbirths, was associated with a significantly increased risk of testing HPV16 positive (OR=7.2, CI 95%=1.69-30.55, P=0.01). There were limited results with respect to observations of HPV18 infections and HPV16/18 co-infections, precluded statistical analyses. Seventeen women were HPV18 positive, with two peaks observed in the number of positive cases, in 16-34 and 45-54 yr old women. There were 10 women with HPV18/16 co-infections, following a similar trend to that of HPV18 infections, albeit with a much lower number of cases. When age of the initiation of sexual activity was considered, there was a greater number of HPV18 infections and HPV16/18 co-infections in women older than 18 yr old. When reviewing the age at first childbirth, the highest incidence of HPV18 infections and HPV16/18 co-infections were observed in individuals between 18 and 24 yr old. In addition, women with <3 childbirths showed the highest occurrence of HPV18 infections and HPV16/18 co-infections.
Sequencing the E6 gene to identify HPV16 variants in 31 samples demonstrated that 100 per cent were of the European variant. Of these, 23 (74.2%) had a base pair substitution at the E350G position, with this change being the most prevalent. In total, 12 variations were observed in a 321 bp fragment of the E6 region. A similar distribution of the viral variants was observed in all samples.

Discussion

In this study, 52.4 per cent women with normal cytology were tested HPV positive. This differed from a previous study performed in two towns in southern Mexico that reported an HPV prevalence of 43.6 per cent. However, studies in India showed HPV prevalence rates over 60 per cent in asymptomatic populations, with a more commonly reported prevalence of HPV infection between 10 and 12 per cent. In our study, HPV16 genotype was present in 21.16 per cent of the women. This result was similar to previous reports of this genotype in asymptomatic populations elsewhere in the world and consistent with other studies conducted in Mexico. The frequency was lower than that reported in Mexican patients with CC in whom the frequency of HPV16 was 50.9 per cent.

In this study, 8.99 per cent women were tested positive for HPV18, and 5.29 per cent were co-infected with both the HPV16 and HPV18 genotypes. These data were in contrast with that reported in Puebla, Mexico, where 40 per cent of patients with a normal Pap smear had more than one viral genotype.

<table>
<thead>
<tr>
<th>Risk factors</th>
<th>HPV (n=189)</th>
<th>HPV 16 (n=189)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(+) n</td>
<td>(-) n</td>
</tr>
<tr>
<td>Age (yr)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16-24</td>
<td>21</td>
<td>15</td>
</tr>
<tr>
<td>25-34</td>
<td>35</td>
<td>20</td>
</tr>
<tr>
<td>35-44</td>
<td>22</td>
<td>31</td>
</tr>
<tr>
<td>45-54</td>
<td>15</td>
<td>17</td>
</tr>
<tr>
<td>55-67</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>Age of start sexual life (yr)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;18</td>
<td>52</td>
<td>46</td>
</tr>
<tr>
<td>&lt;18</td>
<td>47</td>
<td>44</td>
</tr>
<tr>
<td>Age of first childbirth (yr)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15-17</td>
<td>24</td>
<td>12</td>
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<tr>
<td>Number of childbirths</td>
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<tr>
<td>0</td>
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<td>18</td>
</tr>
<tr>
<td>4+</td>
<td>17</td>
<td>6</td>
</tr>
<tr>
<td>Number of sexual partners</td>
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<td></td>
</tr>
<tr>
<td>1</td>
<td>41</td>
<td>52</td>
</tr>
<tr>
<td>2</td>
<td>31</td>
<td>28</td>
</tr>
<tr>
<td>3+</td>
<td>27</td>
<td>10</td>
</tr>
</tbody>
</table>

OR, odds ratio; CI 95%, confidence interval at 95%
rates of co-infection with HPV16 and HPV18 were 15.3 per cent in Spain, 17.9 per cent in Denmark and 18.2 per cent in the Netherlands. It has been reported that there is a significant relationship between the presence of HPV16 and the development of HG-SIL. In addition, HPV18 is associated with low-grade lesions (LG-SIL). It is now believed that infection with HPV is necessary to cause, but is not sufficient for, the development of CC. Therefore, co-infection with other genotypes may pose a greater risk for the development of CC. Considering that we failed to determine the HPV genotypes in 16.40 per cent of HPV-positive women, further studies focused on identifying more HPV genotypes, and co-infections are necessary.

Although the identification of HPV16 variants in our study was done by assaying the E6 gene sequence, our results showed that all HPV16-positive samples were of the European variant, which was consistent with previously described results in America. A study from Brazil observed that 29.4 per cent of women with normal cytology were HPV positive, with HPV16 being the most commonly observed genotype (14%), followed by HPV-52 and HPV-45. In these studies, the observed HPV16 variants were classified predominantly as European (87.5%), but Asian, African and American variants were also reported. In our study, HPV16 was also the primary viral genotype detected, followed by HPV18.

While European HPV16 variants have been reported in both women with normal cytology and in women with CC, non-European HPV16 variants appear to be associated with a 2- to 9-fold increased risk of CC, as reported in biopsies of Mexican women with CC. The Asian-American HPV16 D2 variant has been shown to be associated with a high-risk of developing CC in Mexican women younger than 50 yr old. Here, we only observed the European HPV16 variant. However, the E350G (or L83V) intra-variant primarily found in our study was not associated with the development of CC in all European populations. The higher association of AA and E350G variants and cancer risk may be linked to a better ability to evade the host immune system and/or an increased oncogenic potential associated with intrinsic biological properties of viral proteins. In addition, functional studies demonstrated that the E350G E6 variant has biological advantages over the E-P (European-Prototype).

In other study conducted in Latin America in samples of healthy women, a 92 per cent of HPV16 European variant, followed by 4, and four per cent distribution of the AA and Af variants were reported, respectively. Moreover, similar distribution frequencies of HPV variants in women without cancer have been reported (87, 11 and 2% of infections with the E, AA and Af variants, respectively). It should be noted that in studies with CC biopsies performed in Mexico, non-European variants, such as AA and Af, were found in greater proportion (approximately 45%). Data showed an association between aggressiveness and the speed of onset of CC with the presence of the AA variant.

In contrast with the data reported in Mexico, in this work, all HPV16 samples having the E6 gene sequenced were of the European variant prototype. This could be explained by the fact that, in our study, samples of healthy women were used instead of CC biopsies. These variants are less aggressive than the AA and Af intratypic variants of HPV16 reported in Mexico (23.2% AA vs. 23.8% European).

One of the limitations of our study was that 53.97 per cent of the women had an inflammatory process and the presence of other pathogens responsible for sexually transmitted infections or immunosuppressed status not clinically confirmed, and we did not analyze other HPV-coinfections that could be present in these women.

In conclusion, a predominance of HPV16 European lineage variants was observed in asymptomatic women from in Sinaloa, Mexico. This variant is known to be associated with a higher risk of developing CC. Molecular diagnoses of HPV may be included in addition to cervical Pap smears as a measure to establish the prognosis of the development of CC in Mexican women.

Acknowledgment: The authors acknowledge all volunteering women participants and thank Dr Omar Fragosa Sosa for allowing us the use of the facilities for sampling and cervical Pap smear analyses.

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Conflicts of Interest: None.

References


Respiratory viruses in returning Hajj & Umrah pilgrims with acute respiratory illness in 2014-2015

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Received May 31, 2017

Background & objectives: Respiratory tract infections are common among Hajj and Umrah pilgrims which pose a public health risk of spread of respiratory infections. Influenza has been reported from Indian Hajj and Umrah returning pilgrims, but data on other respiratory pathogens are sparse in India. Here we report the presence of common respiratory viral pathogens in returning Hajj and Umrah pilgrims suffering from acute respiratory illness (ARI) in 2014-2015.

Methods: Respiratory specimens (nasopharyngeal and throat swabs) were collected from 300 consenting pilgrims with ARI in the past one week and tested for influenza and Middle East Respiratory Syndrome coronavirus (MERS-CoV) and other respiratory viruses using in-house standardized quantitative real-time reverse-transcription polymerase chain reaction. Clinical features among the pathogen positive and negative patients were compared. The patients received symptomatic treatment and antivirals where appropriate and were followed telephonically to collect data on illness outcome.

Results: Ninety seven (32.3%) of the 300 participants were tested positive for any virus, most common being influenza viruses (n=33, 11%). Other respiratory viruses that were detected included human coronaviruses [n=26, 8.7%; OC43 (n=19, 6.3%) and C229E (n=7, 2.3%)], rhinovirus (n=20, 6%), adenoviruses (n=8, 2.6%), parainfluenza viruses (n=7, 2.3%), respiratory syncytial virus (n=3, 1%) and bocaviruses (n=2, 0.6%). Clinical features observed in pathogen positive and pathogen negative patients did not differ significantly. Eighteen influenza positive patients were treated with oseltamivir.

Interpretation & conclusions: Pilgrims returning from mass gatherings are often afflicted with respiratory pathogens with a potential to facilitate transmission of respiratory pathogens across international borders. The study reinforces the need for better infection prevention and control measures such as vaccination, health education on cough etiquette and hand hygiene.

Key words Acute respiratory infection - coronavirus - Hajj pilgrims - influenza virus - respiratory syncytial virus - respiratory viruses
The annual pilgrimage of Hajj draws millions of pilgrims to Saudi Arabia during the 12th month of the lunar calendar, with pilgrims entering the country through any one of 16 ports of entry. The Hajj poses significant public health concern as a result of mandatory religious rituals that entail congregated situations for the pilgrims, particularly during the circumambulation of the holy Kaabah and the ritual of stoning the devil. A variety of bacterial and viral respiratory infections are common: 20-80 per cent of pilgrims may develop respiratory infection during the pilgrimage. Investigators have reported an overall mean prevalence for influenza of 2.1 per cent (range, 0.6-7.5%) among all arriving pilgrims and 3.6 per cent (range, 0.5-7.8%) among visitors departing the Hajj; however, some recent studies suggest influenza prevalence to be as high as 4-14 per cent among Hajj returnees. In the past few years, cases of Middle East Respiratory Syndrome coronavirus (MERS-CoV) have been reported from many countries, however, MERS-CoV among pilgrims travelling to Saudi Arabia has not been reported. The rapid spread in nosocomial settings suggests that person-to-person transmission can be efficient in certain settings. There are scanty data on the carriage of respiratory viruses among Indian pilgrims returning from Hajj and Umrah pilgrimage. We have recently reported on the absence of MERS-CoV and presence of influenza infection among symptomatic returning pilgrims from Hajj and Umrah of 2014-2015. In this study, the work was extended by testing the same Hajj pilgrims for additional pathogens of public health importance.

**Material & Methods**

The participants included pilgrims returning from Saudi Arabia after having completed Hajj or Umrah from October 2014 to April 2015. After in-flight announcement offering medical attention to those with fever or respiratory symptoms, consenting disembarking pilgrims were interviewed for respiratory symptoms, offered medical examination and testing for respiratory viruses by a medical team stationed in the arrival halls of the Srinagar International Airport, Jammu and Kashmir, India. The demographic and clinical features were recorded on a predefined case record form. Enrollment for testing was offered to those with respiratory symptoms (n=977); 300 consented. The study protocol was approved by the ethics committee of Sher-i-Kashmir Institute of Medical Sciences (SKIMS), Srinagar, India, and all 300 participants gave written informed consent. Twin swabs (nasopharyngeal and throat) were obtained from each participant, pooled in viral transport media; and then transported to the Influenza laboratory in SKIMS, within 3-4 h of collection. After testing for influenza and MERS-CoV at SKIMS, the samples were transported to the ICMR-National Institute of Virology, Pune, where testing for respiratory viruses was performed using in-house standardized duplex real-time reverse transcriptase polymerase chain reaction (RT-PCR) using previously published primers and probes. Briefly, eight tubes duplex PCR was performed for respiratory syncytial virus (RSV), human metapneumovirus (hMPV), rhinovirus and ribonuclease protein (RNP), parainfluenzaviruses (PIV) 1 and 2, PIV 3 and 4, adenovirus and bocavirus (MERS-CoV), OC 43 and NL 65, cox virus 229E and enterovirus. From 100 µl clinical specimen RNA was extracted using the MagMax automated RNA extractor as per the manufacturer details. Real-time RT PCR assays were carried out using the Invitrogen Superscript III one-step quantitative RT-PCR kit (Invitrogen, Thermo Fisher Scientific, USA). Real-time RT-PCR assays were performed on ABI 7500 machine (Applied Biosystems Inc, USA) in a 25 µl PCR reaction containing 10 picomol of each forward and reverse primer, 5 picomol of TaqMan probe, 12.5 µl x2 buffer, 0.5 µl Superscript III enzyme and 5 µl RNA extract. RT-PCR thermal cycling conditions were as follows: 50°C for 30 min, initial denaturation at 94°C for 10 min, 45 cycles of 15 sec at 94°C, 45 sec at 55°C. In vitro transcribed RNAs for all viruses to be detected were used as a positive control except enterovirus and bocavirus for which known positive clinical samples previously confirmed by additional conventional PCR and monoplex RT-PCR were used.

The patients were managed with symptomatic treatment and antiviral drugs where appropriate. Patients were followed telephonically at weekly intervals until two weeks to collect data on illness outcome.

**Statistical analysis:** Descriptive statistics was used to assess various prevalence rates. Comparison of continuous variables was performed using Student’s t test, whereas comparison of various proportions (clinical features and other variables among pathogen positive and pathogen negative participants) was performed by SPSS (Statistical Package for Social Sciences) Version 17 software (IBM Limited, USA). All values were expressed as percentages and P<0.05 was considered significant.
Table. Clinical symptoms and respiratory viruses detected among enrolled Hajj returnees (2014-2015, n=300)

<table>
<thead>
<tr>
<th>Symptoms</th>
<th>Any virus (n=97)</th>
<th>Coronaviruses (n=26)</th>
<th>Rhinoviruses (n=20)</th>
<th>No virus (n=203)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fever</td>
<td>55 (56.7)</td>
<td>12 (46.2)</td>
<td>10 (50.0)</td>
<td>102 (50.2)</td>
</tr>
<tr>
<td>Chills and rigors</td>
<td>43 (44.3)</td>
<td>8 (30.8)</td>
<td>10 (50.0)</td>
<td>71 (34.0)</td>
</tr>
<tr>
<td>Nasal discharge</td>
<td>85 (87.6)</td>
<td>24 (92.3)</td>
<td>14 (70.0)</td>
<td>174 (85.7)</td>
</tr>
<tr>
<td>Cough</td>
<td>84 (86.6)</td>
<td>23 (88.5)</td>
<td>16 (80.0)</td>
<td>185 (91.1)</td>
</tr>
<tr>
<td>Sore throat</td>
<td>61 (62.9)</td>
<td>20 (76.9)</td>
<td>13 (65.0)</td>
<td>135 (66.5)</td>
</tr>
<tr>
<td>Breathlessness</td>
<td>24 (24.7)</td>
<td>5 (19.2)</td>
<td>4 (20.0)</td>
<td>56 (27.6)</td>
</tr>
<tr>
<td>Expectoration</td>
<td>23 (23.7)</td>
<td>5 (19.2)</td>
<td>3 (15.0)</td>
<td>52 (25.6)</td>
</tr>
<tr>
<td>Headache</td>
<td>57 (58.8)</td>
<td>11 (42.3)</td>
<td>15 (75.0)</td>
<td>99 (48.8)</td>
</tr>
<tr>
<td>Body ache</td>
<td>59 (60.8)</td>
<td>14 (53.8)</td>
<td>15 (75.0)</td>
<td>106 (52.2)</td>
</tr>
<tr>
<td>Fatigue</td>
<td>51 (52.6)</td>
<td>13 (50.0)</td>
<td>15 (75.0)</td>
<td>97 (47.8)</td>
</tr>
<tr>
<td>ARI in roommates in past two weeks</td>
<td>3 (3.1)</td>
<td>0</td>
<td>1 (5.0)</td>
<td>6 (2.9)</td>
</tr>
<tr>
<td>Concomitant illness</td>
<td>3 (3.1)</td>
<td>0</td>
<td>0</td>
<td>5 (2.5)</td>
</tr>
<tr>
<td>Vomiting</td>
<td>3 (3.1)</td>
<td>0</td>
<td>0</td>
<td>11 (5.4)</td>
</tr>
<tr>
<td>Diarrhoea</td>
<td>4 (4.1)</td>
<td>0</td>
<td>0</td>
<td>6 (2.9)</td>
</tr>
<tr>
<td>Ear discharge</td>
<td>1 (1.0)</td>
<td>0</td>
<td>0</td>
<td>4 (1.9)</td>
</tr>
<tr>
<td>Seizures</td>
<td>1 (1.0)</td>
<td>0</td>
<td>0</td>
<td>2 (1.0)</td>
</tr>
</tbody>
</table>

Values in parentheses are percentages. ARI, acute respiratory illness.

Results

Of the 300 participants 140 were males and 160 females with age ranging from 26 to 60 yr (median age 60 yr). Ninety seven (32.3%) were tested positive for any virus (Table), influenza viruses [n=33, A (H3N2)=13], A [(H1N1pdm09)=9 and B (Yamagata)=11] were the most frequent (Figure)10. Of the other viruses, human coronaviruses (n=26, 8.7%) were the most common detected consisting in OC43 (n=19, 6.3%) and C229E (n=7, 2.3%) subtypes. MERS-CoV was not detected in any of the patients10. Human coronavirus (HCOV)-positive patients (15 males, age 32-75 yr, median 60 yr) presented with symptoms of 1-3 days (median 2 days) duration, with no significant differences in the age distribution and the symptomatology between HCOV-positive and negative patients. Co-infections of HCOV with other respiratory viruses were seen in five patients (aged 38-75 yr, median 60 yr, 4 males). These included HCOV OC43+ adenovirus (n=1), HCOV 229E + adenovirus + influenza B (Victoria) (n=1), HCOV 229E + influenza A (H3N2) (n=1), HCOV 229E + adenovirus (n=1) and HCOV 229E + influenza A (H1N1pdm09) (n=1). Human rhinovirus (HRV) was detected in 20 patients, and there were no significant differences between age distribution, co-morbidities and clinical features of HRV-positive

Figure. Frequency of respiratory pathogens detected in pilgrims returning from Hajj/Umrah (2014-2015). * Denotes data reported previously for these viruses10. HCOV, human coronavirus; PIV, para influenza virus; RSV, respiratory syncytial virus; Inf, influenza virus.
and negative patients. Other respiratory viruses that were detected included adenoviruses (n=8, 2.6%), parainfluenza viruses (n=7, 2.3%), RSV (n=3, 1%) and bocaviruses (n=2, 0.6%) (Figure). Co-infections were seen in two patients (aged 50 and 60 yr, both females): one each of enterovirus + adenovirus and adenovirus + PIV-1.

All patients were given symptomatic treatment and advised about cough etiquette, hand hygiene and other infection control measures. Oseltamivir was used in 18 patients with laboratory-confirmed influenza viral infection who presented within 1-5 days (interquartile range 2-4 days) of the onset of the illness. None of the patients required hospitalization, and all had an uneventful recovery in 2-5 days.

Of the 300 participants, 216 (72%) had received trivalent inactivated influenza vaccine [comprising of A/California/7/2009 (H1N1)pdm09-like virus; A/Texas/50/2012 (H3N2)-like virus and B/Massachusetts/2/2012 (Yamagata)-like virus], 14-21 days (median 16 days) before embarking on the pilgrimage. The corresponding influenza infection detected included influenza A/H3N2 (n=13), A/H1N1pdm09 (n=9) and B/Yamagata (n=11). The participants had received the influenza vaccine as a part of their travel advice. Twenty one (10%) of these developed influenza-positive respiratory illness in comparison to 14.3 per cent (n=12) of those not vaccinated before the pilgrimage. None had received pneumococcal vaccination.

Discussion

Our data showed that apart from influenza, coronavirus infections were the most commonly detected viruses in Indian pilgrims returning from Hajj and Umrah in 2015. Coronavirus OC43 and 229E have been known to be associated with upper respiratory infection. A systematic review of 31 studies showed that rhinoviruses were the most commonly isolated viruses from symptomatic patients during the Hajj (5.9-48.8% prevalence), followed by influenza viruses (4.5-13.9%) and coronaviruses (2.7-13.2%) with coronavirus 229E being the most common; other viruses were less frequently detected. The high prevalence of viruses such as influenza A (H1N1pdm09), influenza A (H3N2), rhinoviruses, coronaviruses (229E, HKU1 and OC43) in the study participants was similar to other such studies, however, the aetiopathogenetic contribution was not clear in the absence of assessing the asymptomatic carriage in a control group. The crowded conditions during pilgrimage likely facilitate transmission of these viruses and could contribute to the high frequency of respiratory illness, the most common symptoms among the pilgrims. Our data showed that respiratory virus infections were common among returning pilgrims with influenza, with corona and rhinoviruses being the most frequent and that clinical symptoms were not helpful in differentiating among respiratory infections.

Pilgrimage acquired viral infections are important for public health because these do not follow the traditional seasonality of any geography. We have earlier demonstrated that influenza and non-influenza respiratory viruses contribute significantly to acute exacerbations of COPD with a high level of activity during winter months in northern India, in a pattern that resembles the northern hemispherical pattern of circulation of influenza. However, since Hajj and Umrah are performed throughout the year with changing dates, the congregation of potentially infected individuals from all across the world with individual seasonality of circulation of these viruses makes the acquisition and transmission of the viruses possible. International gatherings have a potential of acquisition of communicable disease outbreaks and exportation of these pathogens and initiation of local chain of spread in home countries triggering local outbreaks. Although not reported in the context of Hajj, transmission of MERS-CoV has been reported in home countries after travelling to a MERS-CoV-affected country in several instances like in the UK, France, and South Korea.

Our study was limited by the fact that there was no information available about carriage of the viruses among those who did not have symptoms and the pre-pilgrimage status. However, all of the participants had been asymptomatic while before embarking on the pilgrimage. A control group of persons with a similar level of severity and without a recent history of travel, could have really brought out the difference in the viral spectrum, if any and given the quantitative impact of importation of viruses due to the pilgrimage. Furthermore, the patients represented a non-random selection that was, however, necessary in the unique circumstances of the recruitment.

Our study highlighted the potential for persons returning from mass gatherings to facilitate transmission of respiratory pathogens and reinforced the need for better infection prevention and control measures such as vaccination, health education on cough etiquette and
hand hygiene, or use of facemasks. There is a need for larger studies for surveillance for the viruses among the pilgrims returning from mass religious gatherings as also international and multi-sectorial coordination and communication for effective surveillance among Hajj and Umrah pilgrims and continued evaluation of the implementation of the recommended guidance for prevention of communicable health hazards among the pilgrims. An assessment of the burden of the viruses would also help public health and policy planners to devise appropriate public health response including infection control strategies during and after return from the pilgrimage.

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Conflicts of Interest: None.

References

Courtship activity, copulation & insemination success in a mosquito vector fed a herbal aphrodisiac: Implications for sterile insect technology

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Background & objectives: In sterile insect technology (SIT), mating competitiveness is a pre-condition for the reduction of target pest populations and a crucial parameter for judging efficacy. Still, current SIT trials are being hindered by decreased effectiveness due to reduced sexual performance of released males. Here, we explored the possible role of a herbal aphrodisiac in boosting the mating activity of Aedes aegypti.

Methods: Males were fed one of two diets in this study: experimental extract of Eurycoma longifolia (MSAs) and sugar only (MSOs). Differences in life span, courtship latency, copulation activity and mating success were examined between the two groups.

Results: No deaths occurred among MSA and MSO males. Life span of MSOs was similar to that of MSAs. The courtship latency of MSAs was shorter than that of MSOs (P<0.01). MSAs had greater copulation success than MSOs (P<0.001). In all female treatments, MSAs mated more than MSOs, but the differences in rate were significant only in the highest female density (P<0.05). In MSAs, mating success varied significantly with female density (P<0.01), with the 20-female group (P<0.01) having the lowest rate. Single MSA had better mating success at the two lowest female densities. In MSOs, there were no significant differences in mating success rate between the different female densities.

Interpretation & conclusions: Our results suggested that the herbal aphrodisiac, E. longifolia, stimulated the sexual activity of Ae. aegypti and may be useful for improving the mating competitiveness of sterile males, thus improving SIT programmes.

Key words Aedes aegypti - aphrodisiac - Eurycoma longifolia - mosquito vector - sexual behaviour - sterile insect technique

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Mosquito-transmitted illnesses have become a major global public health threat worldwide\(^1\). In 2016, the primary dengue vector, *Aedes aegypti*, was found to be responsible for many recent outbreaks of both dengue and Zika virus infections\(^2\). Insecticide use, the main strategy to combat dengue vectors has been ineffectual due to the development of resistance in mosquito populations\(^1\). The vector *Ae. aegypti* has developed resistance to nearly all classes of insecticide used to date\(^1\). Therefore, it is necessary to search for alternative vector control strategies.

The sterile insect technique (SIT) has been widely adopted as a sustainable method for control of mosquito-borne diseases\(^5\). The technique consists of mass-rearing, sterilization and release of sterile males into the wild, where they will compete for mates with their wild counterparts\(^5\). As they are sterile, such matings will produce no offspring and thus reduce the size of the target population\(^5\). However, most of these programmes also showed reduced sexual competitiveness of the released insects compared to their wild counterparts\(^6\). The Food and Agriculture Organization\(^7\) argued that the triumph or failure of this control strategy would be directly related to the ability of the released laboratory-produced insects to effectively mate with wild females. In SIT, larvae of the target insect are reared in substantial numbers\(^8\), a procedure that is known to affect adult quality and mating ability of the released adults\(^9\). Ionizing radiation, the main technique used for sterilization also reduces both competitiveness and life span\(^5\). Reduced longevity of released males can substantially decrease the effectiveness and increase the economic costs of SIT programmes\(^5\). Thus, the ability to produce laboratory insects with high sexual efficiency in the wild remains a major challenge in implementation of the SIT strategy.

SIT represents a promising strategy for dengue vector control\(^10\). Much of this optimism is based on the 72 per cent reduction in the wild population size obtained by Bellini *et al*\(^11\). There has been a marked increase in anti-dengue mosquito SIT trials in many parts of the world\(^12\).

For many centuries, aphrodisiacs have been used to increase libido and arousal, especially in individuals with sexual problems\(^13\). Herbs such as *Ginseng*, *Rehmannia*, *Epimedium*, *Cordyceps*, *Lepidium meyenii* (maca), *Muira puama*, *Turnera diffusa* (damiana), *Ginkgo*, *Tribulus terrestris* and *Yohimbine* derived from the bark of the *Pausinystalia yohimbe* tree, have long been used as aphrodisiacs\(^14,15\).

It has been shown that the scent of ripe and rotting fruit activates courtship-initiating brain pathways in the fruit fly, *Drosophila melanogaster*. These pathways, or networks of connected neurons, were previously shown to encourage male fruit flies to engage in courtship displays and begin mating\(^16\). Crickets infected with a viral aphrodisiac commence courtship at least twice as fast as healthy male crickets\(^17\). This study was undertaken to examine whether and to what extent the dengue vector, *Ae. aegypti*, survived after the uptake of a natural aphrodisiac. The impacts of such consumption on sexual behaviours, such as courtship latency, copulation activity and mating success, were also investigated.

**Material & Methods**

*Mosquito rearing and stocks*: Samples of eggs originating from a colony of *Ae. aegypti* from the University Sains Malaysia, Penang, Malaysia, were used to establish a colony at the Entomology laboratory (External Laboratories of the Faculty of Resource Science and Technology, Universiti Malaysia Sarawak, Kota Samarahan). Eggs were hatched in dechlorinated water and reared at a density of 200-250 in 4 l plastic trays (As One Corporation, Osaka, Japan) filled with 800 ml of aged tap water as delineated elsewhere\(^18\). Larval food consisted of powdered cat food pellets (ProDiet Cat Food, Malaysia) provided in an amount of 0.10-0.15 g every two days. The rearing medium was replaced with fresh medium before the third food supply. Pupae were collected from rearing trays and placed in 250 ml plastic vials containing 10-15 ml of water, which in turn were transferred into mosquito breeding cages measuring 30×30×30 cm (BugDorm; MegaView Science Co., Ltd., Taichung, Taiwan). Adults had access to a 10 per cent sucrose solution and females were blood-fed on hamsters 4-5 days post-emergence. Eggs were air-dried and stored as a stock colony as described\(^19\). The laboratory environmental conditions were 21-34°C, 60-86 per cent relative humidity and photoperiod 13:10 h (light:dark) with one hour of dusk.

*Production of experimental virgin subjects*: For colony establishment, egg samples taken from the stores were submerged in dechlorinated water. Twenty four hours later, newly hatched larvae were raised in a 4 l plastic tray (Tray 1) at a density of 1000 and supplied with 0.15 g of ProDiet cat food every two days, with the rearing medium being replaced with fresh before
the third food provision. To ensure the isolation of males from females, pupae were kept in individual 1.5 ml Eppendorf tubes containing 0.15 ml of water. On emergence, adults’ sex was determined under a stereomicroscope (SZ-LED; Kenis, Osaka, Japan). To prevent mating before bioassays, males and females were pooled in separate breeding cages (30×30×30 cm) and provided with 10 per cent sucrose solution. To produce blood-fed females, a restrained hamster was placed within the cage for one hour. Fully blood-fed females were transferred into another cage where they were allowed to digest the blood meals for 24 h. These females were referred to as blood-fed virgin females.

**Experimental aphrodisiac and experimental extract:** The natural aphrodisiac selected in this study was the root of the Borneo strain of *Eurycoma longifolia*. This *Simarouba* species is known as a supplement for enhancing sexual performance. Conventionally, the plant is used as a decoction. Several animal and human trials have been conducted to evaluate the properties of the roots using either water or ethanol extracts. A sample of the root of the plant obtained from the Faculty of Resource Science and Technology (Universiti Malaysia Sarawak) was cut into slices of 0.5-1 cm in thickness. A sample of 0.25 g weighed using a Vibra analytical balance (Shinko Denshi Co. Ltd., Tokyo, Japan) was soaked in 50 ml of water in a 250 ml plastic container and allowed to decay. After five days of disintegration, 10 g of sucrose was added to the filtered solution and the volume was adjusted to 50 ml. The solution thus obtained after complete dissolution of the sugar was designated as the experimental aphrodisiac extract.

**Male survival and longevity following uptake of *Eurycoma longifolia* extract:** As *E. longifolia* root extract can be toxic at certain doses, the impact of its uptake was examined on adult longevity. Briefly, 30 males that emerged on the same day were placed in a standard adult mosquito cage (30×30×30 cm) where they were allowed to feed on 10 per cent sucrose solution. After two days of sugar feeding, 15 males were singly placed in an experimental unit consisting of a glass tube (2×8 cm) with its cover modified to hold a 1.5 ml Eppendorf tube with the bottom cut out. All males had access to 10 per cent sucrose-aphrodisiac solution through cotton wicks placed through the cover crossing the Eppendorf tube. The remaining 15 males were individually transferred into similar tubes but with cotton wicks saturated with sugar solution (control). For convenience, the males fed sugar-aphrodisiac were designated as MSA and males fed sugar only were designated as MSO. All 30 tubes (15 MSA tubes and 15 MSO control tubes) were inspected after 24 h for adult death.

Another experiment was conducted to examine the longevity of males. Briefly, 24 males from the previous experiment were divided into two groups of 12 individuals each. Each group was placed in one of the subsequent environments: (i) cage holding a 250 ml feeding device containing 200 ml of sugar-aphrodisiac solution, or (ii) cage holding a 250 ml feeding device containing 200 ml of sugar solution. Six days later, the apparatuses (sugar and sugar-aphrodisiac) were removed from the cages and both groups were given continuous access to 10 per cent sucrose solution. The two cages were inspected daily and the number of dead individuals was recorded until all 24 individuals had died. Both survival and longevity experiments were conducted under the laboratory environmental conditions: 21-34°C, 60-86 per cent relative humidity and photoperiod 13:10 h (light:dark) with one hour of dusk.

**Courtship activity following uptake of *E. longifolia* extract:** Ten virgin MSAs (3-4 days old) were placed in a mating cup, which consisted of a 250 ml transparent plastic container with two opposite mesh-net screened windows (each 1 cm²) and covered with a lid at the middle having an aperture filled with a 1.5 ml Eppendorf tube; the bottom of the tube was cut out and filled with a cotton wick saturated with 10 per cent sucrose solution. After 10 min of adaptation to the plastic container environment, five sugar-fed virgin females (4-5 days old) were released together into the cup. Immediately upon release, an assistant sitting nearby began recording the time to first copulation attempt (courtship latency) and the number of successful copulations (copulations) within 30 min. Similarly, ten virgin MSOs (3-4 days old) and five sugar-fed virgin females (4-5 days old) were placed in another cup and observed as described above. For MSA and MSO, ten and eight replicates, respectively, were observed on different days. All of the observations were performed between 1600 and 1800 h.

**Mating success following uptake of *E. longifolia* extract:** One virgin MSA (3-4 days old) and five blood-fed virgin females (3-5 days old) that had digested blood meals for two days were placed together in a standard adult mosquito cage and allowed to
cohabit in the presence of 10 per cent sucrose solution. After one day of cohabitation, the females were singly placed in an oviposition device consisting of a glass tube (2×8 cm), identical to the experimental unit used in the male survival study, except it was lined with a section of filter paper as an egg deposition site. After a three-day oviposition period, the eggs were allowed to dry up in the laboratory environment for five days. The dried eggs were flooded by fully filling the glass tube with water (48 ml) enriched with 0.03 g of yeast, as described elsewhere. Flooding was repeated two more times with a five-day drying period between the two bouts of flooding. This bioassay was repeated a second time but with one old virgin MSO (3-4 days old) and five blood-fed virgin females (4-5 days old) (control). The bioassays ‘1 MSA×5 blood-fed females’ and ‘1 MSO×5 blood-fed females’ were replicated nine and eight times, respectively. Experimental procedures similar to those described above for ‘1 MSA×5 blood-fed females’ were also carried out for: (i) 1 MSA×10 blood-fed females/1 MSO×10 blood-fed females, and (ii) 1 MSA×20 blood-fed females/1 MSO×20 blood-fed females. There were 10 replicates for each of the bioassays ‘1 MSA×10 blood-fed females’ and ‘1 MSO×10 blood-fed females’; four and three replicates for ‘1 MSA×20 blood-fed females’ and ‘1 MSO×10 blood-fed females’, respectively. In all bioassays, the tubes were monitored for 24 h after flooding for the presence or absence of newly hatched larvae, which was used to score successful mating.

Data collection: In the survival bioassay, the status (live or dead) of both MSO and MSA individuals was recorded in each glass tube replicate. These numbers were used to compute the survival rate for MSO and MSA groups. This rate was considered as the total number of males that survived after one day of exposure to a given adult experimental feeding source/15×100. In the longevity bioassay, the number of days between egg hatching and adult death was scored as the life span. In the courtship behaviour study, immediately after the collective release of the five females into the cup, the assistant used a stopwatch to begin recording the courtship latency, which was defined according to Eastwood and Burnet as the time taken by a male to initiate courtship of a female. With reference to Roth, we considered initiation of courtship when a male tried to attach itself to a flying female and was noted for each cup replicate. Copulation was defined as any effective genital contact of a formed copula, which persisted for at least 10 sec, following Roth’s definition. The mean values of courtship latencies and numbers of copulations were used as parameters of courtship behaviour. In the mating success study, a female that had cohabited with either MSAs or MSOs of which at least one egg has hatched during one of the three flooding events was judged as mated. Such females were counted and the resulting numbers were used to calculate mating success rate as the number of females with at least one hatched egg/initial number of virgin females cohabited with males ×100.

Statistical analysis: The dissimilarities in survival, longevity, courtship latency, number of copulations and mating success were examined by analysis of variance (ANOVA) using Systat v.11 statistical software (Systat Software, Inc., Richmond, CA, USA). In the mating success study, means (±standard error) were analysed by Tukey’s honestly significant difference test where needed.

Results

No deaths occurred among the males that were allowed to feed on sugar (MSOs) or sugar- Eurycoma extract (MSAs) for 24 h. However, an additional six days exposure of these two groups of males to their respective food sources followed by continuous maintenance on sugar resulted in different life spans. Ae. aegypti MSOs had a life span of 84.08±5.25 days (range: 33-100 days), while the mean life span of their counterparts maintained on the herbal extract (MSAs) was 68.25±6.29 days (range: 33-95 days).

The courtship latency of Ae. aegypti males varied significantly with feeding history (P<0.01). The time taken by MSOs to initiate courtship of females presented in groups of five individuals was 78.90±13.46 sec (range: 30-182 sec). For MSAs, the time to courtship initiation was 25.10±5.28 sec and ranged between 8 and 53 sec.

The mean number of copulations of Ae. aegypti males fed on sugar (MSOs) was 15.62±3.76 and ranged between 5 and 37. In the MSA group, the number of copulatory events ranged from 10 to 37 and averaged 23.20±7.89. The difference in number sexual intercourse events between MSAs and MSOs was significant (P<0.001).

The mating success patterns of Ae. aegypti male types engaged with different numbers of females are shown in the Figure. In the five-female treatment groups, the mean mating rate of MSAs
MSAs had a mean mating rate 32 females under three different P 31 has been reported to improve sperm E. longifolia 29 10 28 33 34 20 MSAs. Many neurotransmission features P and those fed sugar+ densities (5, 10, and 20) by males fed sugar solution only (MSO) and those fed sugar+Eurycoma longifolia extract (MSA). Figure. Mating success. Mean (±standard error) of successful inseminations of Aedes aegypti females under three different densities (5, 10, and 20) by males fed sugar solution only (MSO) and those fed sugar+Eurycoma longifolia extract (MSA).

(62.22±8.58%, range: 20-100%) was higher than that of MSOs (48.12±13.94%, range 0-100%), but the difference was not significant. In the 10-female treatment groups, the cohabitation of MSAs and sugar-fed females (54.05±4.42%, range: 33.3-70%) tended to result in greater mating success compared to that of MSOs with the same type of females (44.08±3.30%, range: 33.3-62.5%), but the difference between MSA and MSO mating rates was not significant. In the 20-female treatment groups, Ae. aegypti MSAs had a mean mating rate of 16.72±2.11 per cent (range: 11.1-20%), whereas their MSO counterparts mated at a mean rate of 7.54±2.12 per cent (range: 5-11.76%); these two mean values were significantly different (P<0.05). In MSAs, the mean mating rate differed significantly between female densities (P<0.01). The mean mating rate recovered from cages hosting one MSA and five sugar-fed females was 62.22±8.58 per cent, which was higher than that from cages where one MSA cohabited with ten females, but the difference was not significant. The single MSA showed a lower rate of mating when mixed with 20 females (16.72±2.11%) than with five females (P<0.01). The mating rate was higher in cages with one MSA and 10 females than in those with one MSA and 20 females (P<0.01). In MSOs, there were no significant differences in mating success rate between the different female densities.

Discussion

In the present study, males fed with the aphrodisiac solution (MSAs) initiated courtship earlier than their sugar-fed counterparts (MSOs), indicating that the aphrodisiac stimulated courtship behaviours of Ae. aegypti MSAs. Many neurotransmission features and transcripts that are involved in the sexual behaviour control in mammals have homologues in flies26. Nearly all courtship rituals in flies require gene expression27. Ae. aegypti possesses a homologue of the fruitless gene named Aeafru, which is expressed in a manner similar to that in flies and is closely linked to the neurochemical control of courtship rituals28. There is a close relationship between fruitless gene expression level and courtship intensity. Males that lack the fruitless gene do not court females29 and low levels of fruitless gene expression hinder courtship activities30. In the present study, two solutions were orally administered to young virgin males and those that ingested 10 per cent sugar+E. longifolia showed markedly more courtship activities than those provided with the solution free of E. longifolia. The enhancing effects of E. longifolia on male mating motivation have been well established31. The root of E. longifolia contains many compounds, including quassinoids, which is known to be an important aphrodisiac molecule32. Although neurotransmission, gene expression or the chemistry of the Eurycoma extract were not assessed in the present study, the observed differences in courtship latency between MSAs and MSOs might be due to lower dopamine levels in MSOs. It was also likely that MSAs had higher expression levels of the Aeafru gene.

MSAs had more successful copulations than MSOs. In Ae. aegypti males, the act of copulation involves a series of behavioural actions before transfer of seminal material into the female32; and this cascade of behaviours has been well described33. Both MSAs and MSOs mated with females, but insemination and fertilization success rates were better for the former than the latter. For successful insemination, male mosquitoes must release sperm and seminal fluid into the reproductive tract of the female34. Many factors such as mate body size and age34 have been documented as major factors influencing sperm transfer. In the present study, experimental males were fed the same amount of larval food, emerged as adults on the same day and were 3-5 days old at the time of the bioassay. Therefore, differences in sperm count and quality between groups due to discrepancies in body size or age were unlikely. E. longifolia has been reported to improve sperm quality. Chan et al35, working with rodents, investigated the effects of Eurycoma extract containing quassinoids. They orally administered different doses of the extract.
to normal and infertile males for over one month and found that extract-fed males had significantly higher sperm count, which increased with concentrations of the extract. They reported that the infertile males fed the extract showed appreciable increase in sperm count and spermatozoa motility. In a related study performed in rats, Ambiy et al.16 evaluated the spermatogenic activity of a traditional Indian aphrodisiac, Ashwagandha (Withania somnifera) root. They orally administered Ashwagandha to oligospermic males and compared semen parameters between the treated group and a placebo control group. They observed a 167 per cent increase in sperm count, 53 per cent increase in semen volume and 57 per cent increase in sperm motility in the treated group. Increases in sperm number and motility have been reported to augment fertilization success35. Although sperm was not investigated in the present study, the increased insemination success rate observed in the MSA group was consistent with good sperm quality.

It was observed Eurycoma extract had little impact on survival or longevity of Ae. aegypti. The success of SIT programmes depends not only on mating performance but also on the released insects surviving at least as long as their wild counterparts5. SIT programmes require repeated release of sterile males into the target site. Sufficient longevity of the released insects would reduce the required frequency of release and therefore, decrease the associated economic costs5. In the present study, MSAs lived for about 68 days, which was just 16 days shorter than that in the group fed with 10 per cent sugar solution. The observed increases in courtship, copulation and mating capabilities of MSAs combined with their relative longevity will allow longer inter-release periods, thereby decreasing costs. Additional research is needed to determine whether sterilization by irradiation alters the effects of Eurycoma extract observed in the present study.

This study was carried out to evaluate the effects of the herbal aphrodisiac, E. longifolia, on the copulatory activities and mating success of Ae. aegypti with a view towards its prospective use to improve the mating ability of sterile males, thus benefiting SIT programmes against dengue vectors. Our results showed that the uptake of Eurycoma extract was not deleterious to the survival of Ae. aegypti males, as evidenced by their long life span. Furthermore, consuming the aphrodisiac shortened courtship latency. More importantly, males fed the aphrodisiac (MSAs) had more copulations and greater insemination success than those that did not (MSOs). In the field, increased courtship activities will tend to generate elevated sexual aggressiveness. With more sexual attacks, males have higher chances of acquiring mates and also increased chances of successful mating. The increased mating success will therefore, cause a reduction in the population size of the target insect as the eggs of the wild females will not hatch. Reduced courtship and mating activity remain major challenges in captive breeding, an important part of biodiversity conservation efforts. This approach is likely to reduce the costs associated with feeding as E. longifolia is a dietary supplement35 and has high energy30 and nutritional38 value.

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Conflicts of Interest: None.

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Do the clonally different *Escherichia coli* isolates causing different infections in a HIV positive patient affect the selection of antibiotics for their treatment?

Sir,

In HIV patients, bacterial infections are mostly caused by the pathogenic bacteria harbouring multidrug-resistance genes\(^1\). While carrying out a study (2014-2015) on infections caused by drug-resistant bacteria in HIV positive patients attending YRG Centre for AIDS Research and Education (YRG CARE), Chennai, Tamil Nadu, India, we found a 35 yr old male patient who had complaints of severe fever, irritation during micturition, vomiting and body chills. Hence, the urine and blood specimens were collected from this patient, and were subjected to bacterial culture and identification of the isolates. This patient was also infected with leptospirosis, *Pneumocystis jirovecii* pneumonia and pulmonary tuberculosis. He was under antiretroviral therapy with the following regimen: tenofovir, emtricitabine and efavirenz and was hospitalized for nine weeks. The CD4 cell count of this patient at the time of admission was 19 cells/µl. The bacterial isolates from both the specimens were identified as *Escherichia coli* based on the standard cultural and biochemical characteristics. Both these *E. coli* isolates were subjected to polymerase chain reaction-random amplified polymorphic DNA (PCR-RAPD) analysis to determine their clonal relationship and molecular detection of drug-resistance genes using PCR technique. Both the *E. coli* isolates showed different clonal patterns which indicated that the patient had blood and urinary tract infections caused by two different *E. coli* strains (Figure). The molecular characterization revealed that the *E. coli* isolate from urine sample harboured the genes *bla*\(_{TEM}\), *bla*\(_{CTX-M}\) and *bla*\(_{OXA}\) for ESBL and *sul1* and *sul2* genes for SMX and none for AmpC and Class 1 and Class 2 integrons. The phenotypic characterization showed that the isolate had resistance to ampicillin, doxycycline, gentamicin, cefpodoxime, cefoperazone, ceftriaxone, ciprofloxacin, trimethoprim-sulphamethoxazole, piperacillin, piperacillin-tazobactam, tetracyclin, trimethoprim, cefotaxime, cefazidine and cefoxitin and sensitivity to amikacin, chloramphenicol, ertapenem and imipenem. On the other hand, the *E. coli* isolate from blood sample was found to harbour the genes *bla*\(_{CTX-M}\) for ESBL, *bla*\(_{CT-M}\)

![Figure. *Escherichia coli* isolates from urine and blood samples of a HIV patient showing different gene patterns by polymerase chain reaction-random amplified polymorphic DNA analysis.](image-url)

M, marker; 138 - *E. coli* isolate from urine; 139 - *E. coli* isolate from blood.
for AmpC, sul1, sul2 and dfrA7 for TMP-SMX and also for Class 2 integron by molecular characterization and also showed resistance to ampicillin, aztreonam, cefpodoxime, cefoperazone, ceftriaxone, imipenem, piperacillin, piperacillin-tazobactam, trimethoprim, trimethoprim-sulphamethoxazole, cefotaxime, ceftazidime and cefoxitin and sensitivity to amikacin, doxycycline, chloramphenicol, ciprofloxacin, eritapenem, gentamicin and tetracycline by phenotypic characterization (Table). Both the isolates were negative for MBL-producing genes bla_{IMP}, bla_{VIM}, bla_{SIM}, bla_{SPM}, bla_{GIM} and bla_{NDM}, TMP resistance genes dfrA1, dfrA5 and dfrA17 and for Class 1 integron gene.

In our study, β-lactamases-producing genes from both the E. coli isolates were sequenced and identified as bla_{TEM-116}, bla_{CTX-M-15}, bla_{OXA-1}, and bla_{CMV-30} using BLAST and phylogenetic analyses. Similar to our study, the co-positivity of ESBL along with AmpC and other drug resistance genes in bacterial isolates from HIV patients was observed by Padmavathy et al. In this study, E. coli isolate from urine sample of HIV positive patient was found to be resistant to at least any one antibiotic of the classes β-lactams, aminoglycosides, tetracyclines, quinolones, pyrimidines and sulphonamides. It was also observed that the E. coli isolate from urine was phenotypically positive for ESBL, MBL and AmpC production. Vignesh et al reported that 80.6 per cent of the E. coli isolates from urine specimens from HIV patients were multirdrug resistant and among them 83.3 per cent showed resistance to TMP-SMX, 94.4 per cent to ampicillin and 100 per cent sensitivity to imipenem and 44.4 per cent sensitivity to amikacin. They also reported that 25 per cent of the isolates showed positive for β-lactamase production. Phe et al found that antibiotic resistant E. coli was the main contributor of bloodstream infection in HIV patients which corroborated this finding. In this study, E. coli isolate from urine sample was positive for bla_{TEM}, bla_{CTX-M} and bla_{OXA} genes related to ESBL production, and these

| Table. Demographic data, positivity of drug-resistance genes and antibiotic susceptibility patterns of clonally different Escherichia coli isolates from urine and blood samples of a HIV patient |
|---|---|
| Parameters studied | Positivity of drug-resistance genes and antibiotic susceptibility |
| Demographic data |  |
| Age and sex | 35 and male |
| CD4 cell count | 19 cells/µl |
| Sample | Urine |
| Organism | E. coli |
| Phenotypic production of β-lactamases | ESBL, MBL and AmpC |
| Resistance genes |  |
| ESBL | bla_{TEM,CTX-M,OXA} |
| AmpC | Not detected |
| MBL | Not detected |
| Sulphamethoxazole | sul1 and sul2 |
| Trimethoprim | Not detected |
| Integrons |  |
| Class 1 integron | Not detected |
| Class 2 integron | Detected |
| Resistance to antibiotics | Ampicillin, aztreonam, cefpodoxime, cefoperazone, ceftriaxone, ciprofloxacin, trimethoprim-sulphamethoxazole, piperacillin, piperacillin-tazobactam, tetracycline, trimethoprim, cefotaxime, ceftazidime and cefoxitin |
| Sensitive to antibiotics | Amikacin, doxycycline, chloramphenicol, eritapenem and imipenem |

ESBL, extended spectrum β-lactamases; MBL, metallo-beta-lactamases; AmpC, ampC beta-lactamases
findings were also in line with Lin et al16 who reported the coexistence of two or more ESBL genes in about 40 per cent of E. coli isolates. In our previous study17, we reported that Gram-negative bacteria harbouring β-lactamases-producing genes along with TMP-SMX resistance, and Class 1 and Class 2 integrons might make the treatment to bacterial infections more complicated in clinical settings. The probable source for the urinary tract and bloodstream infections of the HIV patient in this study may be from his own gut flora. An earlier study from India, reported that the endogenous translocation of gut flora was one of the major causes of infections of the urinary tract and bacteraemia18.

In conclusion, the present study showed the clonally different E. coli isolates causing blood and urinary tract infections in HIV patient from India and also the isolates harboured multiple drug-resistance genes. In this study, it is demonstrated that differences in antibiotic resistance and susceptibility profile of clonally different E. coli isolates causing different infections in an HIV patient may affect the selection of proper antibiotics for their treatment. This study also suggests that for effective treatment of bacterial infections, the proper antibiotic susceptibility testing should be carried out even for two bacterial isolates belonging to the same genus and species and isolated from two different infection sites of a patient.

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Reduction in prevalence of anaemia in pregnant women

Sir,

We read the article by Kalaivani and Ramachandran\(^1\) with great interest. The study analyzed the datasets of National Family Health Survey (NFHS)-II, III, IV, District Level Household Survey (DLHS) II, IV and Annual Health Survey (AHS)-Clinical and Anthropometric and Biochemical (AHS CAB). Authors reported that there has been a reduction in the prevalence of anaemia among pregnant women in the past 15 years.

We would like to discuss a few concerns about the interpretation of the predicted trend of reduction in the prevalence of anaemia using the National Survey data:

(i) The sampling procedure and inclusion criteria of Pregnant women differed greatly in NFHS-II (1998-1999), NFHS-III (2005-2006)\(^3\) and NFHS-IV (2015-2016)\(^4\) as compared to DLHS-II (2002-2004), DLHS-IV (2012-2013)\(^6\) and AHS CAB surveys (2014)\(^7\) presented in the study\(^1\).

(ii) NFHS II and III were designed to provide State-level estimates of anaemia. However, DLHS II, IV, AHS and AHS CAB and NFHS IV were designed to provide district level estimates of anaemia. There were large variations in the total number of pregnant mothers included for estimation of prevalence of anaemia in NFHS-II (n=2796), III (n=3788) and IV (n=30,320), DLHS II (n=38,710), IV (n=12,306) and AHS CAB (n=20,832).

(iii) The methods used for haemoglobin estimation were different in NFHS-II, NFHS-III and NFHS-IV as compared to DLHS-II, IV and AHS surveys. The NFHS used HemoCue analyzer for estimation of haemoglobin while DLHS-II, IV utilized cyanmethaemoglobin method. In addition, different models of HemoCue analyzers were used during NFHS-II, III and IV as consistent results of Hb estimations were not produced by the earlier models of the machine\(^8\).

(iv) The classification for grading of anaemia used in NFHS-II, NFHS-III and NFHS-IV as compared to DLHS-II, IV and AHS surveys also differed across the surveys. The NHFS II, III and IV graded anaemia according to the WHO grading of anaemia\(^9\); pregnant women with Hb levels ≥11 g/dl were graded as non-anaemic; those with Hb levels between 10.0 and 10.9 g/dl as mildly anaemic, those with Hb levels between 7.0 and 9.9 g/dl as moderately anaemic and those with Hb levels below 7.0 g/dl as severely anaemic. Whereas, DLHS 2 used the grading of anaemia as per the earlier published Indian data based on functional decompensation\(^10\) which has been associated with a fall in Hb levels. Pregnant women with Hb ≥11 g/dl were graded as not anaemic; while those with Hb levels between 8.0 and 10.9 g/dl as mildly anaemic, those with Hb levels between 5.0 and 7.9 g/dl as moderately anaemic and those with Hb levels below 5.0 g/dl as severely anaemic.

(v) All the NFHS surveys (II, III and IV)\(^2\)\(^-\)\(^4\) have documented lower prevalence of anaemia in pregnant women as compared to non-pregnant women. This is in contradiction to the existing knowledge according to which the prevalence of anaemia among pregnant women is higher due to haemo dilution during pregnancy\(^9\). The WHO also has recommended lower “cut-off” for Hb by 0.5 g/ dl for defining anaemia among pregnant mothers\(^8\).

(vi) High reduction in the prevalence of anaemia was recorded between NFHS-III and IV in Chhattisgarh (63-41%), Assam (72-44%), Haryana (71-51%), Odisha (68-47%) and Kerala (62-45%). However, the coverage of iron folic acid (IFA) supplementation (major intervention to reduce anaemia) among pregnant women was only 30.3 per cent in Chhattisgarh, 32.0 per cent in Assam, 32.5 per cent in Haryana, 36.5 per cent in

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Odisha and 67.1 per cent in Kerala in NFHS-IV. The distribution of IFA tablets and monitoring of their consumption were poorly undertaken due to various logistic reasons. Furthermore, anaemic pregnant women, possibly received only prophylactic dose of iron (instead of therapeutic dose) while the majority of them were suffering from anaemia. It has been suggested that only up to 50 per cent of women with anaemia in countries of South East Asia region are amenable to iron supplementation.

(vii) The drastic reduction in the prevalence of anaemia mentioned in this study between DLHS II to AHS conducted in Odisha (97-62%), Chhattisgarh (96-63%), Jharkhand (97-80%) and Madhya Pradesh (97-71%) could possibly be due to limitations in the process of estimation of haemoglobin rather than health interventions for reduction in anaemia.

In view of the above, combining the haemoglobin data of NFHS, DLHS and AHS surveys and concluding reduction in the prevalence of anaemia possibly does not provide true scenario. True trends in the prevalence of anaemia could be provided by utilizing data from similar sampling framework surveys with the same method for haemoglobin estimation.

Conflicts of Interest: None.

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Authors’ response

The issues raised by Gupta et al have been stated in our published article. Data from NFHS series showed that there was no consistent decline in prevalence of anaemia in pregnancy between 1998 and 2015 at State or national level. Data from DLHS 2, 4 and AHS CAB showed decline in prevalence and severity of anaemia in all States except Uttar Pradesh (where AHS CAB survey was not done in all districts). Factors associated with reduction in anaemia in different States were not explored in the published article.
Global and Indian studies have shown that HemoCue is not an accurate method for Hb estimation. Use of this convenient but not accurate method could have been the reason for the reported lower levels of anaemia, as well as lack of consistent decline in prevalence of anaemia in NFHS series. Cyanmethaemoglobin method is the gold standard method for Hb estimation; DLHS 2, 4 and AHS CAB used this method and showed that in 2002-2004 prevalence of anaemia was high and in 2014-2015 there was a reduction in prevalence of anaemia in pregnancy.

Data on prevalence of anaemia in pregnancy from NFHS series (NFHS 2, 3 and 4) were compared (not combined) with DLHS 2, 4, and AHS CAB. A national team of experts designed the sampling frames for all these massive surveys, so that State and national level estimates of parameters including prevalence of anaemia could be made.

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Procalcitonin-guided antibiotic usage - addressing heterogeneity in meta-analysis

Sir,

Shafiq et al., in their meta-analysis, have evaluated the utility of procalcitonin (PCT)-guided antibiotic dosing for decision-making among inpatients in three different settings-intensive care units (ICUs), wards and emergency care. The outcomes of interest assessed were total mortality, 28-day mortality, need for ICU admission, proportion of patients treated with antibiotics, duration of antibiotic therapy and stay in hospital and antibiotic use per 1000 days of follow up. The authors concluded that while hard endpoints of mortality and need for ICU admissions remained unaffected; there was a significant reduction in the use of antibiotics in the emergency, ICU setting and the ward setting due to the use of PCT-guided dosing.

Meta-analyses such as these are important for driving policy particularly in resource-constrained settings. We, however, wish to raise some concerns regarding the methodological aspects of the meta-analysis and their impact on the authors’ findings and conclusions.

The total number of studies included for the meta-analysis was 16 (n=8 studies in ICU, n=5 studies in the emergency setting and n=3 in the ward setting). None of the four figures depicting the Forest plots for the various outcome measures have all 16 studies included. For example, Fig. 2 (total mortality) shows only 12 studies, of which seven are in the ICU setting, four in the emergency setting and only one in the ward setting. In the ward setting, a single study is not actually amenable to a meta-analysis, and a minimum of two studies would be needed to draw any meaningful conclusion. Similarly, Fig. 5 (antibiotic use per 1000 days of follow up) has only two studies. This may be due to the fact that all 16 studies may not have reported all outcomes planned by the authors. Both the number of studies and their quality are important to the conclusions of a meta-analysis and the study by Shafiq et al. is limited by small number of studies in both the emergency and ward settings. In addition, although the authors mention “low risk of bias” for the study quality, the methodology used to ascertain bias is not presented. Neither are all 16 studies listed in a Table as per the requirement of the PRISMA statement.

An important methodological issue with the analysis is the clinical heterogeneity among the studies included. The spectrum of disease included ranges from simple fevers to chronic obstructive pulmonary disease and bronchial asthma. In addition, the patients included in the study have ages ranging from less than a month to more than 65 years. This likely explains the heterogeneity seen in the various outcome measures (ranging from 0-65%). Two other meta-analyses on this subject had tighter inclusions and consequently lower heterogeneity (below 50%). Several methods (albeit with caveats) have been proposed to address heterogeneity and two of these include the use of the random effects model (a model that assumes that different studies have different true effects) and meta-regression (a regression technique that assesses whether the treatment effect is related to one or more characteristics of the studies or patients). The authors have used the random effects model for the outcomes of antibiotic usage ($I^2$ 90%), antibiotic use per 1000 days of follow up ($I^2$ 85%) and the proportion of patients treated with antibiotics ($I^2$ 81%). The fixed effects model has been used for total mortality ($I^2$ 0%). The study does not mention any rationale for the choice of either model. An additional challenge in any meta-analysis lies with the test for detecting heterogeneity which has low power when the sample sizes are small or when only a few trials are included. Both emergency and ward settings in the study by Shafiq et al. have very small number of studies (2 or 3 studies only).

A major limitation of all meta-analyses worldwide in this area of PCT-guided treatment has been the
presence of significant heterogeneity among included trials and diverse PCT guidance strategies (including antibiotic initiation, discontinuation or combination of antibiotic initiation and discontinuation strategies)\. Shafiq et al\(^1\) besides having significant heterogeneity in their meta-analysis, also acknowledge the use of differential protocols for PCT estimations.

The challenge of lumping and splitting studies for any meta-analysis is one that will continue to plague researchers in this area given that there is little or no guidance for authors\. While high heterogeneity itself should not preclude a meta-analysis, the rationale for the choice of the model used for the meta-analysis and the impact of the heterogeneity on the summary effect and the consequent conclusions need to be explicitly stated.

**Conflicts of Interest:** None.

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**References**


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**Authors’ response**

We appreciate the interest shown by Birajdar et al\(^1\) in our meta-analysis\(^2\). They acknowledge that such meta-analyses are of considerable importance in guiding policy. In fact, it was driven by a question which arose during antimicrobial stewardship activities, i.e., significance of procalcitonin based decision in different settings within a hospital.

For the first point raised by Birajdar et al\(^1\) wherein they say that only one study was available for ward setting and hence was not amenable to meta-analyses, we could agree no less. However, readers would know that RevMan, the software used for the meta-analyses represents the data for subgroup analyses, whether or not data have been pooled for the subgroup. It can easily be deciphered from the Forest plot (Fig. 2), the confidence interval was the same as that shown in the individual study. The data for this single study, however, need to be entered and depicted to enable overall pooling which is represented at the end of the Figure. In fact, deleting this information would not only have flawed meta-analysis but also rendered the Forest plot incomplete. However, one does conclude that more ward based studies need to be done.

The reason for difference in the number of studies for different outcomes was because not all studies reported this outcome. This again, is more of a norm than exception. As regards to the method of quality assessment, the authors need to refer to the section on Quality Assessment\(^2\) wherein the method has been referenced and explained briefly.

Heterogeneity was assessed and wherever it was significant, appropriate model was used. The details of the same could have been added in the methods section. However, we thought that the Forest Plots would be self explanatory.

Sensitivity analyses based on the putative causes of heterogeneity were not planned *a priori* and was not presented in the paper. However, the suspected reasons for clinical heterogeneity have been commented upon in the discussion section\(^2\). As far as meta-regression
is concerned we would have needed to specifically address a factor or a set of factors for seeing impact on outcome. For our current analysis we did not undertake meta-regression knowing the shortcomings of post-hoc selection of variables. It would be interesting to see someone undertake this exercise.

Birajdar et al. referred to two meta-analyses with stricter inclusion criteria. One of these was available at the time of submission of our meta-analysis and was referenced. The other one was published later. In the latter meta-analysis, within critically ill patients, the focus of infection has been specified or not specified. There are more specific examples, which readers may have referred to. Studies with tighter inclusion criteria would affect heterogeneity favourably. Our meta-analysis was directed towards a very pragmatic decision making exercise during management in a hospital settings. Infections of various kinds are addressed in emergency, wards and intensive care units.

Regarding the Table of all included studies, we would agree as it is an important aspect of the study. However, in the past, we have had the experience of having been asked to either delete it or present it as an appendix as the journals are hard pressed for space. We have given the reference of the included studies.

Regarding the conclusive remark regarding challenge of ‘lumping and splitting studies for meta-analysis’, Ioannidis et al. who used these term explained at length the “difference in opinion of reviewers” to be an important determinant of whether to pool or not pool the data. In fact they made a case for pooling the data using appropriate methodology in case heterogeneity was present. We refrained ourselves from undertaking a meta-analysis when we are convinced any exercise in pooling would be logically and logistically flawed.

References

Clinical Image

Langerhans cell histiocytosis

A two years old girl† was admitted to the Pediatric ward of Command Hospital, Lucknow, India, in May 2013 with polyuria and polydipsia. Her height, weight and developmental milestones were appropriate for age. Plasma glucose (random, 86 mg/dl), serum sodium [132 mEq/l (normal range 135-145)], and serum osmolality [284 mOsm/kg (285-295)], were normal. However, urine osmolality was 34 mOsm/kg

†Consent obtained from father to publish clinical information and images.
(normal range 300-900). Magnetic resonance imaging (MRI) brain showed normal pituitary stalk thickness and absence of posterior pituitary bright spot (Fig. 1). Based on the symptoms, hypo-osmolar urine and findings of the MRI brain, she was diagnosed to have central diabetes insipidus (DI) and started on desmopressin nasal spray.

One year later, she developed induration over the right gluteal region which later developed into fistula (Fig. 2). Biopsy of the wall revealed numerous Langerhans cells (reniform nucleus and abundant pink cytoplasm) with characteristic immunoreactivity for CD1a antigen (Figs. 3 and 4). Skeletal survey and positron emission tomography-computed tomography (PET-CT) did not show any other organ involvement. She was diagnosed to have multisystem Langerhans cell histiocytosis (pituitary and skin) and treated with chemotherapy (prednisolone, vinblastine and 6-mercaptopurine) which led to the healing of cutaneous lesion. The patient has been on follow up for the last three years; though in clinical remission she is continued on desmopressin spray due to the persistence of DI.

Conflicts of Interest: None.

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Clinical Image

Chronic ulcer with rejected skin graft in a female: Pentazocine-induced skin ulcers revisited

A 37 yr old woman† was referred from the Plastic Surgery department to the department of Dermatology, Venereology and Leprosy, Post Graduate Institute of Medical Education and Research, Dr. Ram Manohar Lohia Hospital, New Delhi, India, in March 2016, with complaint of failure of the skin grafting on a chronic ulcer. On examination, single well-defined large ulcer of about 7 cm was present over the medial aspect of the right leg (Figure A), and scars of previous healed ulcers were found on both legs (Figure B). Multiple well-defined punched out ulcers ranging in size from 7 mm to 1.5 cm diameter were also seen over both arms and legs (Figure C & D). Skin grafting was done for the large ulcer which underwent rejection within six months. The multiple small ulcers were suggestive of extraneous cause and she admitted to getting injections from local doctor for cervical pain and to sleep better. Tests for antinuclear antibody (ANA), anti-dsDNA, human immunodeficiency virus (HIV), hepatitis B virus (HBV) and hepatitis C virus (HCV) were negative. A diagnosis of pentazocine-induced ulcers was made. Pentazocine abuse should be considered as a differential diagnosis in cases with non-healing ulcers, especially when asymmetrically distributed concomitant clean punched out ulcers are seen.

†Patient’s consent obtained to publish clinical information and images.
Histopathology can help rule out other disorders such as pyoderma gangrenosum, necrotizing vasculitis and mycobacterial infections.

The patient was referred to psychiatry department for deaddiction and was kept on gradually tapering doses of oral tapentadol and alprazolam. Alternate day dressing resulted in complete healing of small ulcers in three months. Repeat grafting was also planned. Drug cessation is crucial for effective cure of such drug-induced ulcers. Conservative treatment results in poor response if pentazocine is not stopped and skin grafting may be needed in large nonhealing ulcers.

Acknowledgment: Authors acknowledge the prompt and effective role of Dr Manoj Jha, Head, Department of Burns and Plastics Maxillofacial, Dr. Ram Manohar Lohia Hospital, New Delhi, in this case.

Conflicts of Interest: None.

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Gender-specific approach to medical disorders is gaining its rightful place in the medical practice globally. The incidence of a variety of neurological disorders, clinical profile, complication pattern and choice of therapy are greatly influenced by the sex of the individual. Reproductive age and pregnancy are the two important aspects that distinguish women from men with regard to medical disorders. The biological changes including hormonal, metabolic and immunological during pregnancy set the stage for these differences. Pharmacotherapy and other modalities of treatment during pregnancy also need to be tailored to suit the safety of mother and unborn baby.

This monograph fills in a major gap in the specific area of managing neurological disorders during pregnancy. Late Autumn Klein was a pioneer in the field of women’s neurology. The team she put-together carefully has comprehensively reviewed the common neurological disorders from a pregnancy perspective.

This book has 18 chapters. The first four chapters deal with clinical evaluation, hormonal and physiological changes during pregnancy, neuroimaging and neurological complications of anaesthesia. There are dedicated chapters for most of the common neurological disorders encountered during pregnancy. The chapters on stroke in pregnancy and puerperium provide a wide discussion on various vascular disorders specific for pregnancy such as reversible cerebral vasoconstriction syndrome, posterior reversible encephalopathy, amniotic fluid embolism and cerebral venous sinus thrombosis. The specific issues of various antiplatelet drugs, heparin and anticoagulants have been discussed briefly.

The chapter on epilepsy and pregnancy covers a wide range of topics related to epilepsy and reproductive issues including fertility, infant mortality and seizure control during pregnancy. This chapter also extensively discusses the risk of major congenital malformation and neurocognitive developmental problems related to antenatal exposure to antiepileptic drugs.

The brief chapter on neuourology of pregnancy has succinctly described the basic neuroanatomy and physiology of urinary bladder and the urethra in health and disease. Specific disorders related to protracted labour, parturition and caesarean delivery are also discussed well.

The authors have reviewed most of the current literature on the respective topics and have provided a balanced interpretation of the data in most situations. Additional reference to the guidelines brought out by the American Academy of Neurology (AAN), The National Institute for Health and Care Excellence (NICE) or The Scottish Intercollegiate Guidelines Network (SIGN) also would have been helpful to the practitioners.

Overall this monograph is well written and edited. It could have been organized better by segregating neurological disorders from pituitary disorders, contraception and other issues. It would have been greatly helpful to the reader if the editors had included a couple of dedicated chapters on topics such as eclampsia and in vitro fertilization were also included. More flow diagrams and charts would have also added value.

This book would be a good choice for practitioners who are specifically called on to manage
neurological problems in women of reproductive age. Residents and young consultants would also find this useful. It can be a good reference book in the medical libraries.

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This book is part of the ‘Infertility Management Series’ focusing on investigating infertility. Infertility is a common disorder of the reproductive system caused by the failure to achieve a clinical pregnancy after 12 months or more of regular unprotected sexual intercourse as described by the World Health Organization. Around 25 per cent of individuals may be conservatively estimated to be attempting parenthood at any given time, and approximately 13-19 million couples are likely to be infertile in India at any time. Almost one-third of the gynaecological outpatient department attendance is due to inability to bear a child, after a varying duration of married sexually active life. Many couples still seek medical help quite late even as long as 15-20 years later, after exhausting all primitive methods.

In recent years, there have been advances in the understanding of management of infertility, making it a speciality in its own. However, to manage appropriately, it is important to identify the reason for the same. The causes of infertility are wide ranging such as ovulatory disorders, tubal disease, endometriosis, chromosomal abnormalities, sperm factors, and unexplained infertility. Among other factors thought to affect human fertility are the physical, behavioural, genetic, socio-economic as well as environmental or occupational contaminants. Lifestyle factors, such as psychological stress, advanced age to start a family, nutrition, weight, physical exercise and occupational exposures, can also have substantial effects on fertility and outcome. Further, other personal lifestyle factors, such as tobacco smoking and chewing, illicit drug use, alcohol and excessive caffeine consumption, can also have negative influence on fertility.

Many specialized infertility and in vitro fertilization centres are only focusing on management of infertility, oxidative stress reported to have significant role in infertility; thus, this handbook will be a handy tool for those focusing on how to investigate an infertile couple.

The book is divided into 12 chapters, highlighting different factors contributing towards failure to conceive. The first chapter focuses on understanding the basics of human reproduction including the anatomy of the reproductive system both in men and women, the physiology of fertilization and implantation and the basics of the roles of neuroendocrinology, genetics and immunology in reproduction. The second chapter highlights the importance of history taking and clinical examination in an infertile couple. Preconception care is important; all high-risk factors are evaluated and treated as far as possible so that the couple is able to carry the pregnancy until the term safely. The third chapter highlights evidence-based preconception care and appropriate timing for the same.

Fourth and fifth chapters focus on evaluating ovulatory function and tubal patency, the two most common causes for failure to conceive in a woman. The sixth chapter highlights the mechanical issues of infertility in both female and male partners. These are emerging as important contributors for failure to conceive.

Once fertilization has occurred either in vivo or in vitro, the uterine endometrium has to be receptive for implantation to take place and let the embryo develop to its full potential, thus rightly the seventh chapter of the book focuses on endometrial receptivity, how to evaluate and improve it with an individualized approach. Several peritoneal and pelvic factors such as endometriosis, adhesions, fluid in peritoneal or uterine cavity and blockage also interfere infertility, and the eighth chapter provides information on evaluation of all these with clear-cut take-home messages.
Male partner contributes to almost 30-40 per cent of infertility, thus chapters nine and ten focus on the assessment of male partner and understanding of semen analysis and sperm function tests. Advanced genetics of infertility is discussed in chapter eleven. Last but not the least, the last chapter highlights when to stop investigating and start treating.

This is a multi-author book; thus, there is overlap between different chapters, such as. chapter 11 on genetics of fertility, also discussed under chapter one; approach to the evaluation of male factor also finds a place in other chapters besides the two dedicated chapters. The authors have approached the topic with their own perspective, and no uniform pattern has been followed. Some chapters end with a conclusion, some with summary and/or take home messages. Treatment options and modalities are also described in some chapters although the book focuses mainly on investigating infertility. It is hoped that these flaws will be taken care of during the editing and updating for the second edition.

Overall, those practicing infertility as well as medical postgraduates will find it a useful handbook.

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Contributors are welcome to submit their manuscripts online at

http://www.journalonweb.com/ijmr

Announcement

The Indian Journal of Medical Research invites contributions in the section on “Clinical Images”.

These are high quality image(s) of a clinical entity which is unique/rare along with a short and crisp write-up describing the condition and other information available, to be submitted by contributors from medical schools and other medical research institutions. Original, high quality (300 dpi resolution) images (JPEG or TIFF) will be considered for publication after peer evaluation.

Contributors kindly note:

1. There should be a title page with a clear title of 6-8 words, name(s) of contributor(s) – (not >2), with highest academic degree and affiliation(s).
2. Also provide a corresponding address and e-mail.
3. The write up should be about 100-125 words.

Specific Instructions:

1. Only two authors are allowed per clinical image, at least one of the authors should be a faculty associated with the department and hospital/institute where the case was first presented and diagnosed and followed up.
2. A duly signed patient’s consent form should be obtained by the authors (but not to be uploaded along with manuscript). In case the patient is deceased, the form should be signed by a family member or a close relative of the patient. The patient’s consent form should be as per the IJMR format (can be downloaded from our website).
3. The place (Department & Institute) and period of the study (month & year when the patient was first presented) should be incorporated within the first two lines of the text.
4. The treatment strategies employed should be briefly described with outcome.
5. Follow up details of the patient should be mentioned along with the duration of follow up and outcome.
6. Undertaking and copyright transfer forms should be submitted duly signed by both the authors.

Instructions for Figures & Videos:

1. Should be unique and innovative with more than one techniques involved.
2. Figures should preferably be represented as different panels of a single image (not mandatory)
3. Only CT/MRI scans, ultrasound images, Echo-cardiographs and images of ultra-sonography, endoscopy, etc. will have low preference for consideration.
4. The above may be accompanied with some other images such as
   • Histopathology: fluorescent micrographs, unique kinds of staining, electron micrographs, confocal microscopy, etc.
   • Genetic characterization techniques such as karyotyping, FISH, etc.
   • Cases where surgical intervention is involved should be represented with before and after images of the area under focus.
   • Conditions with morphological abnormalities/physical deformities may be depicted with relevant images of the same.
5. Videos (size not exceeding 1 MB) can be uploaded, preferably in MP4 or FLV format, not exceeding 30 seconds.

The contributions can be submitted online at www.journalonweb.com/ijmr.
Correspondences

Do the clonally different *Escherichia coli* isolates causing different infections in a HIV positive patient affect the selection of antibiotics for their treatment?

Marimuthu Ragavan Rameshkumar, Narasingam Arunagirinathan, Chinnambedu Ravichandran Swathirajan, Ramachandran Vignesh, Pachamuthu Balakrishnan & Sunil Suhas Solomon

DOI: 10.4103/ijmr.IJMR_730_17

Reduction in prevalence of anaemia in pregnant women

Aakriti Gupta, Radhika Kapil & Umesh Kapil

DOI: 10.4103/ijmr.IJMR_1429_18

Authors’ response

K. Kalaivani & Prema Ramachandran

DOI: 10.4103/0971-5916.245301

Procalcitonin-guided antibiotic usage - addressing heterogeneity in meta-analysis

Amit Ravindra Birajdar, Urmila M. Thatte & Nithya J. Gogtay

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Authors’ response


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Langerhans cell histiocytosis

Manish Kumar & Anil S. Menon

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Chronic ulcer with rejected skin graft in a female: Pentazocine-induced skin ulcers revisited

Sonali Bajaj & Kabir Sardana

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