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EDITORIAL

Hepatocyte selection medium eliminating induced pluripotent stem cells among primary human hepatocytes

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Abstract

Hepatic insufficiency is a fatal liver disease with a significant decrease in functioning hepatocytes. If hepatocytes could be generated from human induced pluripotent stem (hiPS) cells and transplanted into patients with hepatic insufficiency, the disease may become curable. However, a major limitation to this therapeutic strategy is due to the tumorigenicity of hiPS cells and their ability to form cancer. Current methods for eliminating unwanted hiPS cells use genetic manipulation or reagents that are potentially hazardous for hepatocytes; therefore, revised methods are necessary and anticipated. Glucose and arginine are essential cell culture medium ingredients for the survival of most cells, including hiPS cells. However, hepatocytes can produce its own glucose and arginine through galactokinase and ornithine transcarbamylase, respectively. Therefore, it was hypothesized that unwanted hiPS cells could be eliminated in a medium without glucose and arginine, and supplemented with galactose and ornithine instead. This modified medium has been established as hepatocyte selection medium (HSM). So far, attempts to generate a pure colony of mature hepatocytes from hiPS cells have not been successful. After establishment of co-culture in HSM,

primary human hepatocytes survive while hiPS cells die within three days. Our latest results regarding a modification of HSM will be introduced in this manuscript.

Key words: Ornithine transcarbamylase; Galactokinase; Arginine; Galactose; Urea cycle

Core tip: Human induced pluripotent stem (hiPS) cells have the potential to differentiate into mature hepatocytes. If undifferentiated hiPS cells persist among transplanted hepatocytes, hiPS cells may potentially develop into cancer. Glucose and arginine are essential for the survival of most cells; however, mature hepatocytes survive in the media because they can produce glucose and arginine using galactokinase and ornithine transcarbamylase, respectively. Therefore, we created a hepatocyte selection medium (HSM) that lacks glucose and arginine but is supplemented with galactose and ornithine. After establishment of co-culture in HSM, human primary hepatocytes survive while hiPS cells die within three days.

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INTRODUCTION

Human induced pluripotent stem (hiPS) cells have pluripotency and potential to differentiate to various types of somatic cells. HiPS cells are established with four reprogramming factors[1].

Hepatic failure is fatal in that numbers of functioning hepatocytes significantly decreases below a safe level to support life. If hepatocytes are generated from hiPS cells and transplanted to patients with hepatic failure, they would be cured[2]. One major problem of transplantation is graft-verses-host disease. The graft-verses-host disease could be avoided, if hepatocytes are differentiated from hiPS cells from the patients.

One problem arises with the transplantation. HiPS cells may reside in the transplanted hepatocytes. If the residual hiPS cells are transplanted to the patients, they potentially form cancer. Therefore, methods for eliminating hiPS cells among differentiated hepatocytes used for transplantation are needed for development. The methods should be non-hazardous to hepatocytes and specific for hiPS cells. As such, a new medium to eliminate hiPS cells "hepatocyte selection medium" (HSM), has been developed.

HEPATOCYTE DIFFERENTIATION FROM HIPS CELLS

HiPS cells differentiation to hepatocytes has been investigated[3-8]. They are divided into two categories: growth factors and transcription factors.

Currently, the most common protocols are stepwise addition of growth factors to simulate the process of *in vivo* hepatocyte differentiation during liver development[3-6]. Transcription factors are sequentially expressed during hepatocyte differentiation[7]. HiPS cells are difficult to transfect with plasmid DNA and as such, adenovirus vectors are more efficiently used to introduce transcription factors to hiPS cells. These transcriptions factors then induce differentiation of hiPS cells into hepatoblast-like or hepatocyte-like cells[6,8]. However, there are limitations using this method and cells exhibit only certain similarities to primary hepatocytes and are therefore called "hepatocyte-like cells". An organoid of liver is formed after mixing human mesenchymal stem cells, human umbilical vascular endothelial cells, and hepatocyte-like cells differentiated from hiPS cells[9]. One major problem is that most attempts to obtain mature hepatocytes from hiPS cells have not been successful.

Another limitation, as described in further detail below, is the possibility of tumor formation and cancer development due to the presence of undifferentiated hiPS cells among transplanted hepatocyte-like cells.

TUMORIGENICITY OF HIPS CELLS

HiPS cells proliferate rapidly and have active telomerase activity, which are closely associated with tumorigenicity[1,10]. Tumorigenicity is therefore a major concern with the transplantation of somatic cells differentiated from hiPS or other stem cells into patients[11]. In fact, teratoma is formed in the liver transplanted with mouse hepatocytes differentiated from embryonic stem (ES) cells[12]. This phenomenon strongly supports the tumorigenicity of the residual undifferentiated mouse ES cells among the hepatocytes. At an early stage, it was speculated that teratoma was caused by viral vectors integrated to the host genome[13]. To reduce this risk, two methods have been attempted. First one is the Sendai virus and plasmid vectors because they do not integrate to the genome[14,15]. Second one is to omit c-Myc from the four reprogramming factors[16]. With all the above trials, pluripotent stem cells still form teratoma. It is, then, speculated that tumorigenicity is strongly associated with pluripotency[10,17]. Methods should be investigated to eliminate hiPS cells from the transplanted cells.

ELIMINATION OF HIPS CELLS

Until now, several methods are reported on the elimination

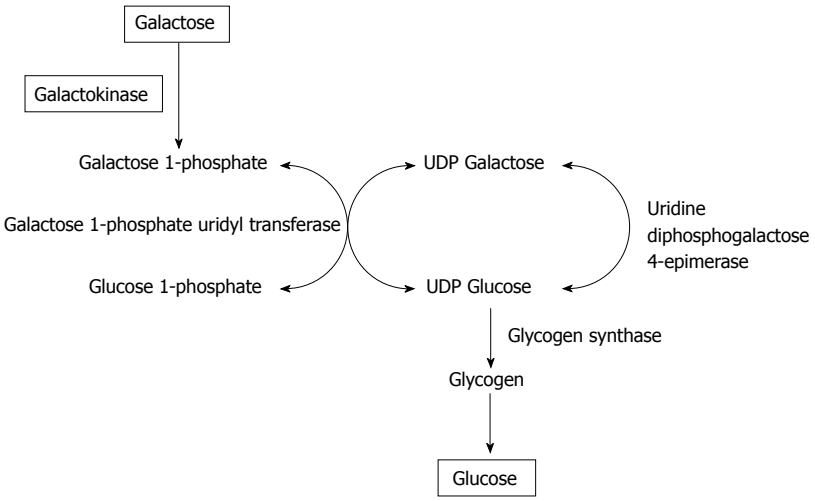

Figure 1 Gluconeogenesis from galactose. Galactose is converted to galactose 1-phosphate by galactokinase. The process of gluconeogenesis is shown and further described in the text. UDP: Uridine diphosphate.

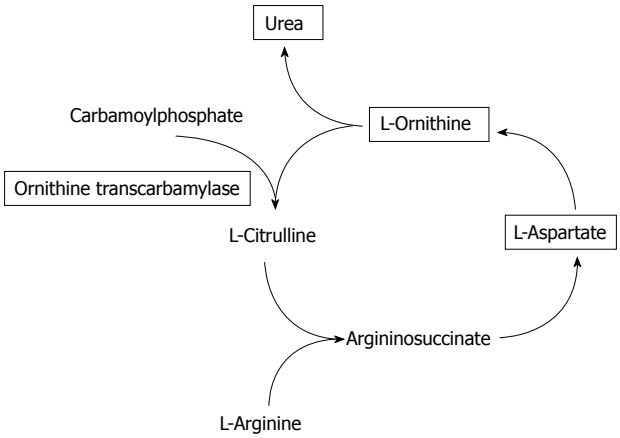

Figure 2 Urea cycle. L-ornithine is converted to L-citrulline by ornithine transcarbamylase. L-arginine is produced from L-ornithine by the urea cycle. Please see text for further detail.

of unwanted hiPS cells.

First attempt was introduction of thymidine kinase gene followed by addition of ganciclovir. Nanog is a homeodomain protein, and maintains pluripotency of ES cells[18]. Nanog promoter would drive thymidine kinase gene if the promoter is followed by the gene in ES cells. It would be expected that hiPS cell would be eliminated with ganciclovir. As expected, hiPS cells die after the addition of ganciclovir into the media[19]. Similarly, Lim *et al*[20] introduced the thymidine kinase gene with lentiviral vectors. However, the approach of selectively eliminating hiPS cells in this manner may be problematic because ganciclovir may be hazardous to hepatocytes.

Second attempt was small molecules. A library of small chemicals was screened to find molecules that had the potential of induction of apoptosis for mouse ES cells[21]. Benzethonium chloride and methylbenzethonium successfully induced apoptosis in hiPS.

N-oleoyl serinol (S18) is a ceramide analogue. S18 ablated pluripotent cells in EB differentiating toward neuronal lineage[22]. Unexpectedly, S18 promoted differentiation of embryoid bodies to neural lineage. S18 is unique in two aspects: elimination of hiPS cells and promotion of differentation toward neural lineage.

Altogether, the above methods require genetic modification or reagents that are hazardous to hiPS cells. However, genetic modifications and toxic reagents are not desirable for the transplantation of somatic cells differentiated from hiPS cells. Therefore, a method should be developed to eliminate hiPS cells using non-toxic materials.

GALACTOSE AS A SOURCE OF ENERGY

Glucose is indispensable for virtually all type of the cells to survive. Glucose is metabolized to pyruvate though glycolysis. Pyruvate produces energy through tricarboxylic acid cycle.

Figure 1 illustrates galactose metabolization to glucose. Galactose is catalyzed to galactose-1-phosphate by galackokinase (GALK). Galatcose-1-phosphate uridyl transferase changed galactose-1-phosphate to glucose-1-phosphate. Through this reaction, glycogen is synthesized. Glycogen enters glycolysis. Finally, glucose is produced from galactose. Glucose-1-phosphate is changed to glucose-6-phosphate. Glucose-6-phosphate is the first metabolite from glucose. Glycolysis follows glucose-6-phosphate. GALK is solely expressed in the liver and kidney[23,24]. In this sense, galactose is the same source of energy as glucose in hepatocytes due to GALK. Therefore, it is expected that hepatocytes survive in a medium without glucose or pyruvate, and added with galactose[25,26]. As expected, hepatocytes survive in a medium without glucose, and added with galactose[27].

GLUCOSE DEPRIVATION AND HIPS CELLS

Metabolomic profiling has shown that glycolysis is

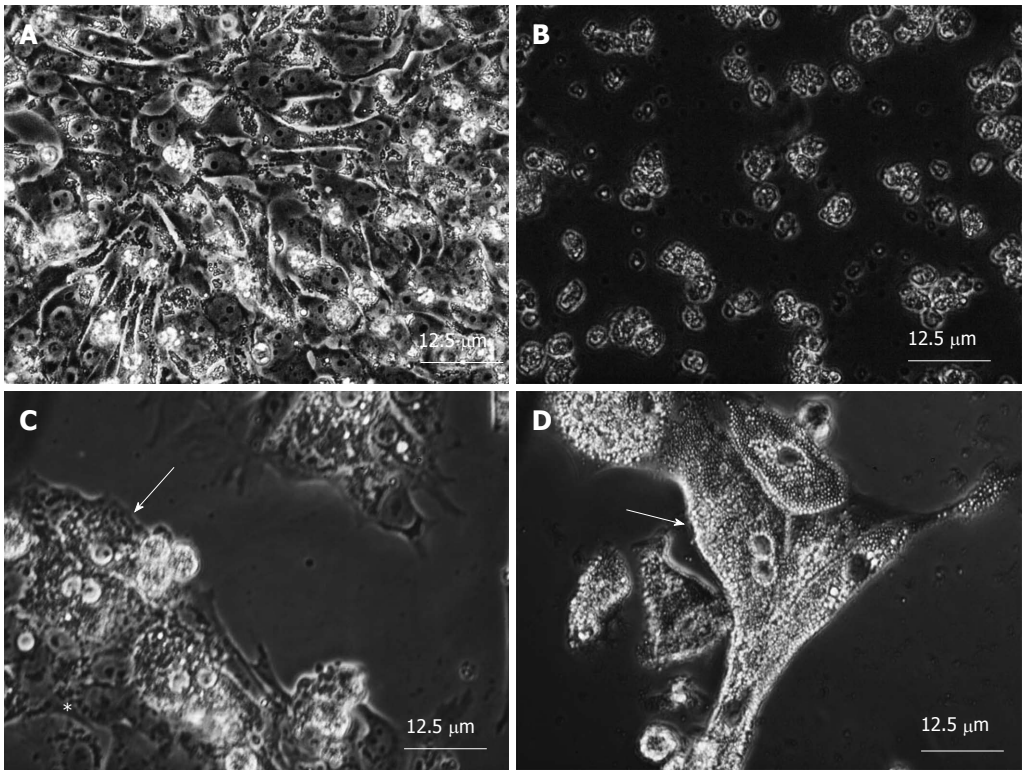

Figure 3 Co-culture of primary human hepatocytes and induced pluripotent stem cells. HiPS cells were cultured in Repro FF (Reprocell, Yokohama, Japan), a feeder free condition (A). The medium was changed to HSM, and cultured further for three days. All the iPS cells were eliminated (B). To address the possibility that hepatocytes survive and iPS cells were eliminated in HSM, co-culture of primary human hepatocytes (arrow) and hiPS cells (asterisk) were established (C). Currently, differentiation of hiPS cell to mature hepatocytes has not been successful. Primary human hepatocytes were used in place of hepatocytes from hiPS cells. HiPS cells were eliminated and primary human hepatocytes survived after three days culture in HSM (D). Original magnification: × 400. HiPS: Human induced pluripotent stem; HSM: Hepatocyte selection medium.

activated, glucose consumption is up-regulated, and lactate accumulation occurs in reprogrammed hiPS cells[28].

Glucose-depleted and lactate-enriched medium eliminated residual undifferentiated hiPS cells from induced differentiated cardiomyocytes[29]. Cardiomyocytes are able to obtain ATP from lactate while hiPS cells are not[30]. Using this non-genetic method, the authors have succeeded in selecting cardiomyocytes differentiated from hiPS cells with great purity and without forming tumors.

ARGININE AND THE UREA CYCLE

Arginine is important in production of nitric oxide polyamine[31]. Arginine is classified as a non-essential amino acid because the amino acid is produced by *de novo* synthesis. Cells require arginie to survive because its amount of production is not enough[32]. Cells are, therefore, hard to survive without argnine[33]. Culture media contain arginine for cells to survive and proliferate.

Arginine is produced through urea cycle. Arginine is normally produced in hepatocytes because urea cycle is expressed only in hepatocytes.

Urea cycle is important to detoxify ammonium ions produced from protein degradation[34]. Figure 2 maps metabolism of urea cycle. L-ornithine and carbamoylphosphate is metabolized to L-citrulline by ornithine transcarbamylase (OTC). OTC deficienty causes hyperammonaemic crises in neonates[35].

Niwa *et al*[27] cultured a rat hepatoma cell line, H4-IIE, in a medium without arginine. The cells were successfully cultured up to 30 passages. Interestingly, the surviving cells expressed all of the enzymes involved in the urea cycle. We could not find any literature addressing the involvement of the urea cycle in hiPS cells.

EXPRESSION LEVLES OF GALK AND OTC

In humans, two genes are involved in galactose metabolism: *GALK1* (NM_000154) and *GALK2* (NM_002044). *GALK1* is a major player of galactose metabolism, its deficiency causes cataracts in infants[36]. Whereas, *GALK2* was originally discovered to be involved in N-acetylgalactosamine metabolism[37]. However, in conditions with high concentrations of galactose, GALK2 demonstrates galactokinase activity.

HiPS cells express *GALK1*, *GALK2*, and *OTC* at significantly lower levels than fetal or adult liver[38]. It was therefore, expected that hiPS cells were eliminated in a medium without glucose or arginine.

HSM

Based on the discussions above, HSM was made to

eliminate mouse ES cells among cells differentiating toward hepatocyte lineage[39]. HSM was made from powder to omit glucose and arginine because the two ingredients are included in all the culture media commercially available. Formulation of HSM is based on Leibovits-15 medium that is suitable for the maintenance of function of cultured hepatocytes. HSM is not only deprived of glucose and arginine but also supplemted with galactose and ornithine. In addition, HSM is supplemented with proline for the synthesis of DNA in hepatocytes[40]. When HSM is applicable to human in the future, xeno-free condition is desirable. HSM, therefore, does not contain fetal bovine serum but knockout serum replacement (KSR) (Life Technologies, Grand Island, NY, United States) at 10%.

ELIMINATION OF UNWANTED HIPS CELLS AMONG PRIMARY HUMAN HEPATOCYTES

HSM was made as mentioned above. HiPS cells died within three days as expected in HSMs (Figure 3A and B)[38]. One concern arose: KSR. KSR might contain glucose, arginine, or both. To address this possibility, KSR was dialyzed, and compared with non-dialyzed one. HiPS cells died in HSM with or without dialysis. It was confirmed that hiPS cells die in HSM in three days. Theses encouraging results prompted us to culture hepatocytes in HSM. So far, differentiation has not been successful of hiPS cells to mature hepatocytes. Our HSM is not useful for generation of mature hepatocytes because the medium albated hiPS cells in three days culture. It, therefore, has not been successful to enrich mature hepatocytes differentiated from hiPS from undifferentiated hiPS cells. To overcome the limiting situation, primary human hepatocytes are subjected to co-culture experiments in place of hepatocytes successfully differentiated from hiPS cells. Figure 3C shows established co-culture of hiPS cells and primary human hepatocytes. Figure 3D clearly show that all the hiPS cells are eliminated and primary human hepatocytes survive in HSM[38].

POTENTIAL APPLICATION OF HSM

There are two ways of application of HSM. One is its initial aim to eliminate hPS cells among hepatocytes for transplantation. Another is application of HSM to hepatocyte differentiation.

One of the characteristics of HSM is that the medium does not have any toxic materials. Another characteristic of HSM is that it does not require genetic manipulation. HSM is, therefore, safe to eliminate unwanted hiPS cells. HSM is potentially necessary when patients with hepatic failure are transplanted with hepatocytes differentiated from hiPS cells to eliminated residual hiPS cells.

Kondo *et al*[41] report a medium that promotes hepatocyte differentiation from hiPS cells. The formulation of their medium is close to our HSM.

Recently, we have established a new medium based on HSM to initiate hepatocyte differentiation[42]. The medium is supplemented with an apoptosis inhibitor, oncostatin M, and small molecules. The report by Kondo *et al*[41] and our recent progress suggest that HSM may be a platform medium for differentiation of hiPS cells to hepatocytes.

CONCLUSION

HSM eliminates hiPS cells. HSM successfully isolates primary human hepatocytes from co-culture of hiPS cells and primary human hepatocytes. HSM may pave a way to a novel protocol to generate mature hepatocytes from hiPS cells.

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EDITORIAL

Metabolic bone disease in the preterm infant: Current state and future directions

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Abstract

Neonatal osteopenia is an important area of interest for neonatologists due to continuing increased survival of preterm infants. It can occur in high-risk infants such as preterm infants, infants on long-term diuretics or corticosteroids, and those with neuromuscular disorders. Complications such as rickets, pathological fractures, impaired respiratory function and poor growth in childhood can develop and may be the first clinical evidence of the condition. It is important for neonatologists managing such high-risk patients to regularly monitor biochemical markers for evidence of abnormal bone turnover and inadequate mineral intake in order to detect the early phases of impaired bone mineralization. Dual-energy X-ray absorptiometry has become an increasingly used research tool for assessing bone mineral density in children and neonates, but more studies are still needed before it can be used as a useful clinical tool. Prevention and early detection of osteopenia are key to the successful management of this condition and oral phosphate supplements should be started as soon as is feasible.

Key words: Premature; Osteopenia; Bone metabolism; Calcium; Alkaline phosphatase; Phosphorus; Nutrition

Core tip: Osteopenia of prematurity remains an important challenge in neonatal medicine due to continuing increased survival of preterm infants. The risk is higher with long-term diuretics or corticosteroids. It is important when managing such infants to regularly monitor biochemical markers for evidence of abnormal bone turnover and inadequate mineral intake. Dual-energy X-ray absorptiometry is increasingly used in research for assessing bone mass density in neonates. Prevention and early detection of osteopenia are key to the successful management of this condition and oral phosphate supplements should be started as soon as it is feasible.

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INTRODUCTION

Neonatal metabolic bone disease (MBD), osteopenia of prematurity (OOP), neonatal rickets or rickets of prematurity, are terms used to describe a reduction in bone mineral content (BMC) of the preterm infant. Although its exact prevalence is difficult to quantify because of the different methods used to screen infants at risk and also because of the difficulty in the interpretation of these results, it has been steadily increasing with the survival of more immature neonates as a result of advances in neonatal care. Born before a term pregnancy and thus deprived of a period of intrauterine supply of minerals, these infants already suffer at birth from suboptimal bone mineralization. The prevalence of MBD is inversely associated with birth weight and gestational age, with up to a third of infants weighing less than one kilogram at birth being osteopenic, more so if they are breastfed[1]. Other factors impeding normal bone mineralization include inadequate postnatal intake of vitamin D, calcium (Ca) and phosphorus (P), extended periods of total parenteral nutrition, lengthy duration of immobilization and as also a side effects of diuretics and corticosteroids prescribed to these infants[2,3]. Depending on the severity of the demineralization, osteopenia can remain clinically silent or develop as rickets, and, if severe, can even result in fractures[4].

As it is an important determinant of skeletal strength structure and density of the skeletal system throughout life, bone mineral density (BMD) in infants is an important topic for neonatologists, pediatricians and also endocrinologists. Guidelines for preventing, screening and treating MBD are not always consistent nor are they universally agreed upon, as still illustrated in a recently published review of this topic[5].

BURDEN OF OOP

Although the burden is not easy to quantify and available data remains conflicting, the known short-term complications are dominated by fractures of the long bones and ribs in the neonatal period. These respond well to therapy and there have no known residual long-term complications. The duration of hospital stay is unaffected by the diagnosis of OOP and preterm infants are routinely given mineral to prevent or treat neonatal rickets[6]. Growth alteration of the skull (dolichocephalic flattening) has been reported in association with poor BMC.

The weight, height, body mass index, lumbar BMC and BMD in 7-year-old children born prematurely and weighing less than 1500 g at birth are lower than those of the reference population[7]. Dual-energy X-ray absorptiometry (DEXA) assessment of areal BMD (aBMD; measured as grams per square centimeter) shows lower values at the level of the radial metaphysis, femoral neck and total hip in ex-preterm girls, but similar values at the radial and femoral diaphysis, with femoral neck aBMD remaining lower 12 mo later[8]. After adjusting for age, weight, height and jump power, prepubertal boys born at term have greater bone size and mass on DEXA scan at the age of at 5.7-8.3 years than those born before 34 wk of gestation[9]. It is still unknown if these changes in BMD in infancy and childhood increase the risk of developing early osteoporosis in adulthood.

PATHOPHYSIOLOGY AND RISK FACTORS

Antenatally

To develop normally, the skeleton of the growing fetus requires considerable active materno-fetal transfer of energy, protein, Ca and P. Serum Ca and *P* levels in the fetus are 20% more elevated than in the mother in the second trimester. Bone mineralization which occurs predominantly during the third trimester, will be inadequate if the fetal increased demands in Ca and P are not met. During pregnancy, augmented maternal intestinal absorption and increased skeletal mobilization increase maternal Ca supply to the fetus. The reduction in the Ca supply by the placenta results in a postnatal increase of parathyroid hormone (PTH) level that continues 48 h after birth when the peak serum Ca and the stabilization of serum Ca and *P* levels are attained[10,11].

Vitamin D also affects BMC and maternal hypovitaminosis D negatively affects the development of the fetal skeleton[12]. It is transferred across the placenta predominantly as 25-hydroxyvitamin D before conversion in the fetal kidney to the active form 1-25-dihydroxy-vitamin D.

Chronic damage to the placenta, with the resulting altered phosphate transport, also contributes to poor bone mineralization and explains the high postnatal incidence of rickets in neonates born with intrauterine growth retardation[13]. Such placental pathologies include pre-eclampsia[14] and also chorioamnionitis and placental infections[15].

Mechanical force patterns, such as fetal movements, including kicking against the wall of the uterus, also stimulate the growth of cortical bone[16]. As a result, preterm infants have a decrease in cortical bone growth leading to a reduction in bone strength. This, added to the reduction in transplacental accretion of Ca and P in the fetus, increases the risk of osteopenia in premature infants.

Postnatally

In infants who are exclusively breast fed, OOP is not

Table 1 Suggested guidelines for the prevention, monitoring and management of neonatal metabolic bone disease

Infants at risk	Prevention	Monitoring	Management
Born with birth weight below 1500 g Born before 28 wk of gestation	Early enteral nutritional intervention Maintain a sufficient supply of Ca and P. Start oral P supplements as soon as its feasible. The P absorption rate is very good in the presence of Ca, with absorption rates exceeding 90% with both human and formula milk. The Ca absorption rate increases from 35 to 60 mg/kg per day when both Ca and P are supplemented and to 90 mg/kg per day when the appropriate dietary Ca/P ratio is attained. High Ca and P retention rates are attained with high-mineral preterm milk formulae or with fortified human milk	Biochemical Monitor weekly serum "bone profile" (Ca, P and ALP): maintain serum Ca concentration between 2.05-2.75 mmol/L and serum P between 1.87-2.91 mmol/L If serum *P* < 1.8 mmol/L and ALP > 500 IU/L, renal TRP should be measured and, if it exceeds 95%, P supplementation should be started If serum P levels fail to increase and if serum ALP levels keep on rising, consider ergocalciferol or alphacalcidol therapy	If the biomarkers of MBD do not normalize, consider either vitamin D supplementation with up to 600 IU/d (although not well supported by evidence) or initiate instead ergocalciferol or alphacalcidol therapy in which case regular monitoring of urinary calcium/creatinine ratio is necessary to detect hypercalciuria
Having received total parenteral nutrition for more than four weeks	Vitamin D supplementation	DEXA	
On long-term diuretics or corticosteroid therapy	Ensure a minimum daily supplement of 400 IU vitamin D. Doses above 400 IU/d do not improve Ca and P absorption	Being increasingly used for assessing BMD in neonates, but not recommended as yet as a clinical tool	
Suffering from neuromuscular disorders	Parenteral nutrition Preparations providing 1.45 to 1.9 mmol/kg per day of Ca and 1.23 to 1.74 mmol/kg per day of P result in Ca and P retention rates of 88%-94% and 83%-97% respectively. The optimal Ca/P ratio in the intravenous solution fluid is between 1.3:1 and 1.7:1.54 If needed, parenteral P delivery can also be enhanced by using special preparations of organic P Exercises Daily exercises such as gentle compression and movements of the limbs Regular review of medications in use Discontinuation of diuretics and steroids when appropriate	Monitor for metabolic acidosis and hypercalciuria which may result from an increase in parenteral mineral delivery during parenteral nutrition	

ALP: Alkaline phosphatase; BMD: Bone mass density; Ca: Calcium; IU: International units; P: Phosphorus; TRP: Tubular reabsorption of phosphate. DEXA: Dual-energy X-ray absorptiometry.

correlated with the degree of the prematurity[17]. Very low birth weight infants (VLBW) whose full enteral feedings have been delayed delayed and who are on long term parenteral nutrition are at increased risk of OOP. Poor bone mineralization is also associated with common neonatal conditions. These include sepsis, broncho-pulmonary dysplasia, cerebral pathology, neuromuscular conditions leading to prolonged immobilisation, acidosis, necrotizing enterocolitis and also cholestasis. Frequently used medications such as diuretics, corticosteroids and methylxanthines also increase the risk of inadequate bone mineralization. Factors associated with increased BMD included higher birth weight, short duration of parenteral nutrition, absence of intraventricular hemorrhage, exclusive feeding of fortified breast milk, and older age at discharge[18].

Candidate genes associated with adult osteoporosis have recently been evaluated in VLBW infants where MBD was found to be associated with a lower number of thymidine-adenine (TA) repeats polymorphism of the estrogen receptor gene, compared to a higher number in those without MBD[19].

SCREENING AND MONITORING

As MBD is usually asymptomatic in most infants, its diagnosis depends essentially on screening. This is based on a set of criteria defined by the presence of clinical manifestations, radiologic findings, biochemical markers and BMC measurements. The recognized clinical-radiological associations include, bone demineralization, periosteal reactions and, in severe cases of osteopenia, rickets and pathological fractures may be present[20]. Infants at high risk of osteopenia, including VLBW infants or neonates on long term diuretic therapy should be regularly monitored for that condition as serious complications can be avoided by early diagnosis with appropriate management. Measuring BMC and BMD relies on a few surrogate markers (Table 1).

Serum biomarkers

As a normal serum Ca level can still be maintained to the detriment of Ca loss from the bone, it should not be used to screen infants at risk. Furthermore, serum Ca may also be affected by unrelated conditions such

as hypophosphataemia[16,21]. Serum P concentration is correlated with BMD, is highly specific but is not sensitive enough to identify infants with ostepenia. While serum P concentration adequately reflects *P* levels in the bone, serum Ca concentration remains maintained at the cost of Ca content in the skeleton.

Serum alkaline phosphatase (ALP) is a marker of bony turnover. Elevated levels indicate increased bone cellular activity and when exceeding 700 to 750 IU/L, they are associated with osteopenia, which is still asymptomatic at that stage[22,23]. The diagnosis of MBD in the preterm infant is usually suggested by the presence of low serum P levels in association with elevated serum ALP levels[1]. The association of serum ALP levels exceeding 900 UI/L with a serum *P* level less than 1.8 mmol/L is 100% sensitive and 70% specific to diagnose OOP[24]. A serum ALP level exceeding five times the upper limit of the normal range in adults is also associated with an increased risk of rickets[25]. The diagnosis of OOP cannot be made however with certainty by elevated serum ALP concentrations, because DEXA scan measurements of BMC did not find an association between ALP levels and OOP in some studies[26] and also because healthy preterm and osteopenic infants have higher serum ALP concentrations than full term infants. Associating multiple measurements of serum ALP with a wrist radiograph, with or without that of the knee, has been suggested for the identification of rickets in VLBW infants if the levels exceed 800 IU/L[27]. Because it is located on osteoblast surfaces, bone-specific ALP is a more specific biomarker of bone turnover, useful to confirm OOP, when high levels of total serum ALP are found[28,29]. Despite its limitations and, despite the absence of a clear cut-off diagnostic level, serum ALP measurement is frequently used to screen high risk infants for MBD. It is a readily available measurement in most laboratories and serial serum levels provide a trend very useful for follow up. Using it in conjunction with serum P levels as a screening tool significantly increases the sensitivity of identifying infants at risk of MBD.

Serum osteocalcin (OC), a non-collagenous protein of the bony matrix, is also a biomarker of osteoblastic activity. It is synthesized by osteoblasts and is partly regulated by 1,25-dihydroxyvitamin D levels. Its serum concentrations are elevated whenever bone turnover is increased, making it a possible useful tool to diagnose OOP[1]. However, despite its specificity, there is no correlation between serum OC levels and BMC in the first four months of age[30].

Urinary biomarkers

Urinary Ca and P excretion have also been used as biomarkers of postnatal skeletal mineralization. Urinary excretion of Ca exceeding 1.2 mmol/L and P exceeding 0.4 mmol/L are strongly associated with high bone mineralization. Infants born between 23 and 25 wk of gestation have a significantly lower renal P excretion threshold than other preterm neonates, resulting in elevated urinary P excretion even when serum *P* levels are low[31]. As, unlike Ca, P is not bound in the plasma, it has been suggested that a better biomarker for OOP is the percentage of renal tubular reabsorption of phosphate (TRP), with TRP > 95% indicating inadequate supplementation, bearing in mind that renal tubular leak of P can also be associated with inadequate Ca intake and increased serum PTH concentration[32]. Similarly urinary Ca or P to creatinine ratios may also be useful as biomarkers for OOP; normal reference ranges in preterm infants have already been established for these ratios[33,34]. However these urinary ratio results need to be carefully interpreted as they are highly dependent on the dietary intake (resulting in uncertainty if the standard reference range) and are also affected by the administration of drug such as furosemide or theophylline[35].

Radiological markers

Old fractures and cortical thinning may be seen on plain radiographs, reflecting poor bone mineralization, but are usually very late signs because they are not usually apparent unless the BMC decreases to 40%[36].

DEXA is currently the most widely used modality to assess BMD. It correlates well with fracture risk and, in both term and preterm infants, it can be used to estimate BMC[37]. Measuring BMD prior to adulthood however is hindered by the "areal" nature of the derived measurement. In addition, the establishment of robust, reliable neonatal, ethnic and gender specific normograms is urgently needed. Barriers to the routine use of DEXA as a screening tool for OOP include its high cost, its limited availability, the dimensions of the equipment used, the lengthy time required for imaging, as well as its sensitivity to movement artifact.

Quantitative Ultra sound (QUS), with already established reference values for both preterm and term infants, is a new inexpensive and portable modality of investigating OOP[38-40]. This simple, non-invasive and inexpensive bedside method measures the broadband ultrasound speed attenuation, and is usually performed on the tibia. Although the measurements it provides correlate well with bone density and structure, the association is a poor with the thickness of the bony cortex[41]. QUS is significantly lower in preterm infants than term infants and a significant correlation of QUS exists with serum ALP, supplementation with Ca, P and vitamin D as well as risk factors for reduced BMD[42]. The combination of longitudinal QUS measurements with serum ALP and *P* levels are helpful to identify infants at increased risk of OOP[43].

Although ultrasound reference values are available for term and preterm infants, there is limited information showing its usefulness.

PREVENTION AND TREATMENT

These are summarized in Table 1. The prevalence and also the severity of OOP can be reduced by early nutritional intervention. Maintaining a sufficient supply of

Ca and P for the growth of VLBW infants' skeleton is challenging because of their relatively high physiological requirements. In addition, although preterm infants are capable of absorbing up to 70% of Ca from human milk, the P content affects the Ca retention rate. Supplementing milk with both Ca and P is more effective: while the Ca absorption rate is 35 mg/kg per day in the presence of P supplementation alone, it increases to 60 mg/kg per day when both Ca and P are supplemented. Ca absorption is also affected by the dietary Ca/P ratio with the retention rate reaching up to 90 mg/kg per day when the appropriate ratio is attained. The neonatal intestinal absorption of P is very good in the presence of Ca, with absorption rates exceeding 90% with both human and formula milk[44]. Ca and P retention rates similar to those observed in utero are attained with high-mineral preterm milk formulae or with fortified human milk[45].

It is imperative to monitor closely serum Ca, P and ALP in such high-risk infants. To prevent OOP, serum Ca concentration should be maintained between 2.05-2.75 mmol/L and serum P between 1.87-2.91 mmol/L. Although VLBW infants are routinely given vitamin D supplementation to increase intestinal absorption of Ca and P, doses above 400 IU/d do not improve their absorption[46].

Parenteral nutrition preparations providing 1.45 to 1.9 mmol/kg per day of Ca and 1.23 to 1.74 mmol/kg per day of P result in Ca and P retention rates of 88%-94% and 83%-97% respectively, equivalent to 60% to 70% of the expected in utero Ca and P accretion rates[47,48]. Ca and P delivery by parenteral nutrition are affected not only by their respective concentrations in the intravenous solution, but also by the ratio of their concentrations. The optimal Ca/P ratio in the intravenous solution fluid is between 1.3:1 and 1.7:1.54[49-51]. The supply of these minerals to infants is limited by the poor solubility of both Ca and P in parenteral nutrition solution, resulting in an increase in the risk of OOP when enteral feeding is not possible for an extended period. Further research is required to improve Ca and P delivery with parenteral nutrition. Vigilance is required during parenteral nutrition as the increase in parenteral mineral delivery may result in metabolic acidosis and hypercalciuria[52]. If needed, parenteral P delivery can also be enhanced by using special preparations of organic P.

Because of the crucial role of mechanical forces on the development of the skeleton, daily exercises such as gentle compression and movements of the limbs are recommended in infants at risk of OOP if greater increase in body weight, forearm bone length, bone area and BMC are to be achieved[53-55].

CURRENT RECOMMENDATIONS

Guidelines for screening and treating infants at risk of OOP have been developed[56]. As summarized in Table 1, it is recommended to monitor all infants for MBD if their birth weight is below 1500 g, or if born before 28 wk of gestation, or if they have received total parenteral nutrition for more than four weeks or in case of diuretic or corticosteroid therapy. Monitoring consists of weekly serum "bone profile" (Ca, P and ALP). If serum P < 1.8 mmol/L and ALP > 500 IU/L, renal TRP should be measured and, if it exceeds 95%, P supplementation should be started. If serum P levels fail to increase and if serum ALP levels keep on rising, ergocalciferol or alphacalcidol therapy should be then considered. The American Academy of Pediatrics recommends that all breast-fed, partially breast-fed and non-breast-fed infants consuming less than 1000 mL of vitamin D fortified milk daily should be supplemented daily with a minimum of 400 IU vitamin D[57]. If the biomarkers of MBD do not normalize, vitamin D supplementation with up to 600 IU/d has been suggested, but without much supporting evidence. In addition, daily passive exercises should be encouraged and the medications in use should be regularly reviewed with discontinuation of diuretics and steroids when appropriate.

CONCLUSION

Preterm infants, those on long-term diuretics or corticosteroids, and those with neuromuscular disorders are at high risk of developing osteopenia. Complications such as rickets and pathological fractures may be the first manifestation of the condition. To detect the early asymptomatic phases of impaired bone mineralization and allow early intervention, all neonates at high risk of MBD appropriate biochemical markers of insufficient intake minerals and of abnormal bone turnover should be regularly monitored. DEXA is being increasingly used for assessing BMD in neonates, but more studies are still needed before it can be used as a useful clinical tool. Prevention and early diagnosis of MBD are key to the successful management of this condition and oral P supplements should be started as soon as is feasible.

Prospective studies of cohorts of preterm infants with OOP are needed with close long-term follow up for later outcomes. More research into urinary Ca and P to creatinine ratios is needed before they can reliably replace direct measurement of BMC. Similarly DEXA needs to be studied further to better define the "areal" nature of the measurement derived for BMD estimation in the newborn and also to establish reliable neonatal, ethnic and sex specific normograms. The possible role of QUS in routine screening for OOP needs also to be studied. As the poor solubility of Ca and P in parenteral nutrition solution hampers the adequacy of their supply to the growing newborn, further research in this area is required to increase their delivery.

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EDITORIAL

Methodological challenges to control for immortal time bias in addressing drug effects in type 2 diabetes

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Abstract

There are multiple biases in using observational studies to examine treatment effects such as those from prevalent drug users, immortal time and drug indications. We used renin angiotensin system (RAS) inhibitors and statins as reference drugs with proven efficacies in randomized clinical trials (RCTs) and examined their effectiveness in the prospective Hong Kong Diabetes Registry using adjustment methods proposed in the literature. Using time-dependent exposures to drug treatments yielded greatly inflated hazard ratios (HR) regarding the treatment effects of these drugs for cardiovascular disease (CVD) in type 2 diabetes. These errors were probably due to changing indications to use these drugs during follow up periods, especially at the time of drug commencement making time-dependent analysis extremely problematic. Using time-fixed analysis with exclusion of immortal time and adjustment for confounders at baseline and/or during follow-up periods, the HR of RAS inhibitors for CVD was comparable to that in RCT. The result supported the use of the Registry for performing pharmacoepidemiological analysis which revealed an attenuated low low-density lipoprotein cholesterol related cancer risk with RAS inhibitors. On the other hand, time-fixed analysis with including immortal time and adjustment for confounders at baseline and/or during follow-up periods, the HR of statins for CVD was similar to that in the RCT. Our results highlight the complexity and difficulty in removing these biases. We call for validations of the methods to cope with immortal time and drug use indications before applying them to particular research questions, so to avoid making erroneous conclusions.

Key words: Pharmacoepidemiological analysis; Immortal time bias; Drug effects; Prevalent drug user bias; Drug indication bias; Type 2 diabetes

Core tip: There are multiple biases in using observational studies to examine treatment effects. These biases include those due to prevalent drug users, immortal time and drug indications that must be taken into consideration. In this regard, we used drugs with proven effects in randomized controlled trials and applied those proposed methods by other groups to estimate their effects in a prospective cohort of patients with type 2 diabetes. Our results highlighted the importance of validating adjustment methods for immortal time and drug use indications before applying them to addressing research questions, so to avoid making erroneous conclusions.

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INTRODUCTION

In pharmacoepidemiological analysis, there are multiple biases in using observational studies to examine treatment effects. These biases may be due to prevalent drug users, immortal time and drug indications[1]. Given these biases, the question to ask is, do we have a way to judge whether the quality of the database of an observational study or the method of analysis is free from these three major biases?

The prevalent user bias is easy to discern and can be readily excluded during data analysis. In type 2 diabetes (T2D), biases due to drug indication depend upon whether the subphenotypes associated with the drug usage (indications) may have selected a patient subgroup inherently at high risk for a clinical outcome, *e.g.,* cardiovascular disease (CVD) or cancer. Immortal time bias is not difficult to detect because we can suspect such a bias as long as immortal time is reported, *i.e.,* non-drug exposure periods have been classified as exposure periods. Several methods have been proposed to control for immortal bias but it is uncertain whether these methods can adequately remove these biases[2-4]. Our recent work has shed light on these important issues[5,6].

INDICATIONS OF DRUG USE AND CONFOUNDERS

Hyperglycemia is the reason why a person is prescribed an antidiabetic regimen which can include various combinations of oral antidiabetic drugs (OAD), insulin and other injectables, such as glucagon like peptide 1[7]. Besides, many factors such as disease severity, predominant disease mechanisms (*e.g.,* insulin deficiency versus insulin resistance), prescribing habits, formulary restrictions, willingness to pay for or accept treatment, referral and volunteer biases can also affect the choices of drug combinations as first, second or third lines of treatments during the clinical course. Of note, some of these factors which can influence drug choices may not be captured in observational studies. Hence, randomized clinical trials (RCT) remain to be the gold standard by evenly distributing these unmeasured confounders in different experimental and control groups to reduce these biases.

Several studies including ours have reported an association of hyperglycemia with cancer in diabetes[8,9]. Our group also reported a linear association between glycated hemoglobin (HbA_{1c}) and all-site cancer in T2D with 1% increase in HbA_{1c} associated with 18% increase in the risk of cancer[10]. These observations were supported by a meta-analysis of RCT data where 0.5% reduction in HbA_{1c} was associated with a non-significant hazard ratio (HR) of 0.91 for cancer risk in T2D[11]. In a recent large randomized trial[12], treatment with saxagliptin, a dipeptidyl peptidase 4, was associated with 0.3% reduction in HbA_{1c} accompanied by a 50% reduction in the risk of pancreatic cancer, albeit short of significance[12]. Although the underlying mechanism linking hyperglycemia and cancer remains to be elucidated, the overarching premise is that users of OADs and insulin are high risk subjects for cancer. Unless these drug indications are captured and removed, these drug users are likely to be found to increase cancer risk, which might be erroneously attributed to drug effects.

In epidemiological analysis, propensity score is often used to control for indications of drug use[13]. The robustness of these scores in removing selection bias is indicated by the area under receiver's operating characteristics curve (AUC) where values $\geqslant 0.90$, $\geqslant 0.80$ to < 0.90, and $\geqslant 0.70$ to < 0.80 indicate excellent, good and fair performance, respectively[1]. Apart from including propensity score, multivariable analysis with inclusion of subphenotypes associated with a clinical event, *e.g.,* cancer, can also attenuate bias due to drug indications[1]. In prospective cohort analysis of the Hong Kong Diabetes Registry, we had identified a group of subphenotypes for cancer risk in T2D[14], in addition to age and hyperglycemia. These included (1) body mass index $\geqslant 27.6$ kg/m^2 and $<$ 24 kg/m^2[15]; (2) low-density lipoprotein cholesterol (LDL-C) $\geqslant 3.8$ mmol/L[16]; (3) co-presence of LDL-C < 2.8 mmol/L and albuminuria[17] which was further enhanced in the presence of increased high density lipoprotein cholesterol (HDL-C) $\geqslant 1.0$ mmol/L[18]; (4) co-presence of LDL-C < 2.8 mmol/L and triglyceride < 1.7 mmol/L[18]; and (5) HDL-C < 1.0 mmol/L[19].

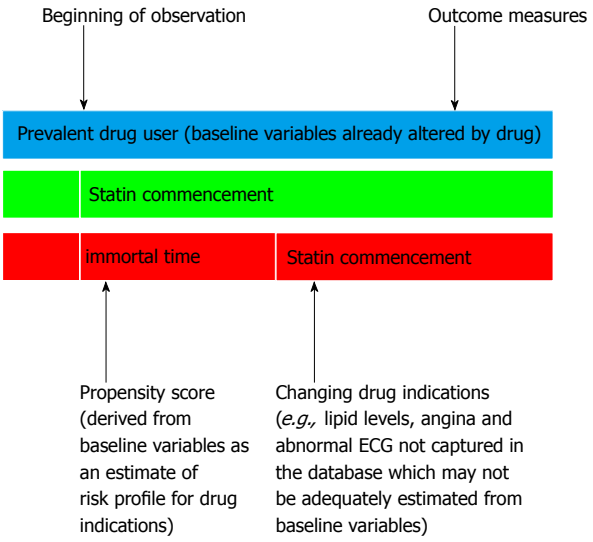

Figure 1 Schematic diagram to explain the multiple biases in epidemiological analysis of cardiovascular disease with use of statins in observational cohorts. While baseline variables may be used to derive a propensity score to adjust for drug indications, this will not apply to prevalent drug users whose risk profile has already been influenced by drug use (blue panel). For patients who were started on the drug after a period of immortal time (non-drug exposure), insufficient data capturing during the observation period may not allow full adjustment for confounders resulting in an inflated hazard ratio of cardiovascular disease, while inclusion of immortal time in a time-dependent Cox model may lead to a reduced hazard ratio by including a period of non-drug exposure during estimation of event rates (red panel). In patients with detailed documentation of risk factors followed by drug commencement as in a clinical trial setting, time fixed analysis may yield hazards unbiased by immortal time (green panel). ECG: Electrocardiogram.

IMMORTAL TIME AND IMMORTAL TIME BIAS

Immortal time refers to a period in cohort studies when non-exposure to a drug treatment from the baseline to the time of initiation of the drug treatment in the "drug exposure group" is misclassified as exposure to the drug treatment[1]. This misclassification may lead to a deflated HR of the treatment for the endpoint due to addition of the non-drug exposure period into the drug exposure period. This can lead to a false conclusion that the drug reduces the risk of the event of interest. Several researchers recommended the use of time-varying or time-dependent drug exposure Cox proportional hazard regression to cope with immortal time bias[3,4]. The use of this method assumes that exposure to the drug treatment or the drug commencement is at random[20]. However, this is rarely the case in real world practice since patients usually start on a drug treatment for a new or changed indication which may not be systematically captured in analysis of cohort study data. If these confounders are not available, the use of a time-dependent model may lead to an increased HR of the treatment for the endpoint (Figure 1).

In order to test the validity of these methods[3,4], we used a referent drug with proven benefits [*e.g.,* statin or renin angiotensin system (RAS) inhibitors] and applied the methods to the Hong Kong Diabetes Registry[5,6] to find out if the estimated effect size fell within the bounds of that reported in RCTs with regard to their associations with CVD. We tested various combinations of exclusion/inclusion of immortal time and adjustment for drug indications at baseline or at the end of immortal time when drug was commenced Consistently, time-dependent drug-exposure Cox models severely inflated the HR of these two drugs for CVD risk[5,6], despite their proven cardioprotective effects in RCTs. In the statin-CVD validation[6], compared to a HR of 0.63 (95%CI: 0.48 to 0.83) in a RCT[21], exclusion of immortal time and adjustment for estimated covariables at the end of the immortal time when statins were commenced, resulted in a 52.3% inflation in the HR of statins for CVD (0.96, 0.72 to 1.27), which was above the higher bound of the 95%CI. On the other hand, inclusion of immortal time, *i.e.*, ignoring immortal time bias, and adjustment for covariables at baseline generated the least inflated HR of 0.64 (0.48 to 0.84) which was within the HR estimates in clinical trial and inflated by 1.59% compared to the absolute HR of 0.63.

In the RAS inhibitors-CVD validation[5], exclusion of immortal time and adjustment for covariables when RAS inhibitors was commenced resulted in a HR of 0.89 (0.68 to 1.17) which was within the estimates of 0.92 (0.84-1.0) reported in RCTs with 3% deflated risk compared to the absolute hazard. By contrast, inclusion of immortal time and adjustment for covariables at baseline yielded a HR of 0.66 (0.51 to 0.86) which was outside the estimates with a 28.3% deflation rate (or inflation rate: -28.3%) compared to the absolute HR.

In most observational or administrative databases, the events preceding the commencement of drugs like statins (*e.g.,* high LDL-C, angina, abnormal imaging) were often not available in the dataset. In a time-dependent model which includes immortal time, inadequate adjustment for indications at the time of drug commencement can lead to overinflated hazards. In this situation, a non-time-dependent analysis but ignoring immortal time and adjusting for propensity score using covariables at baseline might yield the least bias. It is also possible that inflated hazards due to inadequate removal of drug indications and reduced hazards by including the immortal time might have cancelled out one another, giving a HR close to that in a RCT. In the case of drugs with more general indications such as RAS inhibitors, a time-fixed Cox model with exclusion of immortal time and adjustment of covariables at the end of the immortal time, estimated from the baseline variables, might remove most, if not all, of these biases.

Our results highlight the challenges in removing bias from drug indications and immortal time simultaneously if these biases have not been systematically captured. In this new era of big data, clearly, more research is needed to develop methods for removing immortal time bias. This is especially relevant to drugs such as statins and insulin, often prescribed for clinical conditions (*e.g.,* angina, poor glycemic control), the information of which

may not be documented in the database. Pending better methodologies, we recommend the use of non-time-dependent model with exclusion of immortal time and adjustment for propensity score or subphenotypes associated with the event of interest to reduce potential biases.

On the other hand, by selecting high quality datasets with documentation of drug usage and prognostic variables, pharmacoepidemiological analysis may uncover novel hypothesis for further testing. In an analysis of the diabetes-cancer link, in light of the phenotypic heterogeneity, we first used multivariable analysis to identify risk factors or subphenotypes associated with cancer. By adjusting for a low LDL-C related cancer-subphenotype at drug commencement, we discovered a novel drug-subphenotype interaction where RAS inhibitors specifically attenuated low LDL-C related cancer risk in T2D[22]. These pharmacoepidemiological findings, coupled with pathophysiological knowledge and evidence from mechanistic investigations, have provided the basis for a hypothesis where the complex cross-talk between the RAS and the insulin-like growth factor 1-cholesterol pathway might explain the diabetes-cancer link, for further testing[14].

CONCLUSION

In pharmacoepidemiological analysis, there are methodological challenges in removing biases from immortal time and drug indications simultaneously. Hence, risk associations between drug use and clinical events based on observational studies must be interpreted with great caution. To avoid misinterpretation, researchers should take these biases into consideration at the stage of study design, *e.g.,* by documenting indications or variables at the time when drugs are introduced or changed. Our validation studies indicated that exclusion of immortal time in an analysis testing effects of RAS inhibitors while inclusion of immortal time in an analysis testing effects of statins on CVD, respectively yielded effect sizes in T2D close to those obtained in RCTs. Our findings call for further research in developing methodology to simultaneously remove immortal time bias and drug use indication bias. Meanwhile, in the absence of methods which can address effects of different drugs in multiple databases, it will be prudent to use reference drugs and test the quality of databases and adjustment methods for immortal time and drug indications before testing of other drug associations with clinical outcomes to avoid erroneous conclusions.

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REVIEW

Endoscopic management of adenomatous ampullary lesions

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Abstract

Lesions of the ampulla of Vater represent an uncommon group of gastrointestinal malignancies. The majority of lesions of the ampulla of Vater are either adenomas or adenocarcinomas. Ampullary lesions are often incidental findings. Accurate preoperative diagnosis and staging of ampullary tumors is imperative for predicting prognosis and determining the most appropriate therapeutic approach. Endoscopic ampullectomy is a safe and efficacious therapeutic procedure that can obviate the need for potentially major surgical intervention. This review will provide the framework for the diagnosis and management of ampullary lesions from the perspective of the practicing gastroenterologist. Strategies for safe and successful endoscopic ampullectomy with a focus on accurate preoperative diagnosis and staging, resection technique, and management of complications are presented.

Key words: Papillary tumors; Endoscopic ampullectomy; Endoscopic ultrasound; Ampullary adenoma; Pancreatitis

Core tip: Adenomatous ampullary lesions are rare. Endoscopic retrograde cholangiopancreatography and endoscopic ultrasound (EUS) have changed the management of patients with these lesions. Endoscopic ampullectomy is a technique that has revolutionized the treatment of these lesions avoiding potential complications of surgery. We herein discuss the epidemiology, the role of EUS in the local staging and the role of endoscopy in the treatment of the adenomatous ampullary neoplasms.

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INTRODUCTION

The anatomy of the ampulla of Vater is complex. Ampullary adenomas (AA) are an uncommon group of gastrointestinal malignances. With the advances in esophagogastroduodenoscopy and ultrasonography, detection of ampullary neoplasms has increased. Most periampullary lesions are malignant tumors appearing from the ampulla, duodenum, or pancreas. Benign neoplasms entail in this region only < 10% of neoplasms[1-3]. Advances in endoscopic retrograde cholangiopancreatography (ERCP) and endoscopic ultrasound (EUS) have changed the clinical management of these patients. Endoscopic ampullectomy may be considered in patients with smaller lesions that do not contain invasive carcinoma, and in patients who are poor surgical candidates[4-6]. Many series have reported low morbidity and mortality with endoscopic therapy[4,7-19]. Detailed preoperative assessment and staging is needed in other to decide on the best therapeutic option. We review the epidemiology, the role of EUS, ERCP and endoscopy in the approach of ampullary neoplasms.

EPIDEMIOLOGY

Ampullary neoplasms comprise several lesions: adenoma, adenocarcinoma, adenoendocrine carcinoma, small cell carcinoma, adenosquamous carcinoma, and undifferentiated carcinoma[20]. Adenomas or adenocarcinomas representing > 95% of these lesions[21,22]. AA are benign lesions but, can potencially develop into ampullary carcinomas in a comparable progression to that of colorectal cancer[2,3,23-29]. AA can be sporadic or in the context of a familial polyposis syndromes [*e.g.*, familial adenomatous polyposis (FAP)]. FAP is a risk factor; 80% of affected patients develop duodenal adenomas, which are often multiple[30]. In this polyposis syndrome, the lifetime incidence of peri-AA is 50%-100%. The prevalence of AA has increased in the last years with the extensive availability of endoscopy.

CLINICAL MANIFESTATIONS

Ampullary lesions are often found incidentally on cross-sectional imaging or by endoscopic examination. Presenting symptoms are usually non-specific, reflecting biliary or pancreatic obstruction. The most common presentation is with painless jaundice, which is present in 50%-75% of patients[21,31-34]. Cholangitis or acute pancreatitis are rare manifestations[35-38]. Nausea, vomiting, biliary colic, and weight loss may also occur[21,33].

ACCURATE PREOPERATIVE DIAGNOSIS AND STAGING

Accurate preoperative diagnosis and staging is critical to decide on the best treatment option and establish a prognosis.

Endoscopy

The best endoscopic examination of the papilla of Vater is performed with a side-viewing endoscope[20]. This endoscope allows an adequate assessment of the morphological features of the lesion. Thus the following features are suggestive of benign disease: (1) a regular margin; (2) absence of ulceration or spontaneous bleeding; and (3) a soft consistency[39]. Furthermore, the side-viewing endoscope enables an easy acquisition of tissue by biopsy, at the time of procedure. However, on this respect, we know that sensitivity with forceps biopsies for demonstrating the presence of adenoma is > 90%; this is lower for adenocarcinoma, and there is up to 30% of miss diagnosis[11,40-42]. Thus, a negative histological diagnosis of carcinoma on endoscopic biopsy of an ampullary adenoma does not exclude a possible focus of adenocarcinoma[42-47]. The accuracy of endoscopic biopsies can be enhanced when additional techniques are employed. Thus, taking biopsies several days after sphincterotomy[48], and taking at least six biopsies, minimizes the chance of false negative results[49] . Despite its gaps, endoscopic forceps biopsy is the mainstay of pre-excisional histological assessment in lesions of the ampulla. However, we ought to remember that resection of all AA might be the best approach for excluding the presence of carcinoma.

Endoscopic retrograde cholangiopancreatography

ERCP has a central role in the staging and management of obstructive jaundice in AA. Adenoma ingrowth into the pancreatic or biliary ducts does not always indicate malignancy, but may hinder endoscopic excision and considerably decreases the chance of complete endoscopic resection. ERCP at the time of endoscopic papillectomy permits: (1) evaluate the intraductal extension; (2) the placement of a pancreatic stent in order to reduce the risk of pancreatitis; and (3) deploy, if required, a biliary duct stent for the palliation of obstructive jaundice.

EUS

EUS, in conjunction with ERCP, allows to assess for infiltration of the periampullary wall layers and pancreatobiliary ducts but, it does not have to be universally incorpored into the diagnostic evaluation of an ampullary adenoma[45,50-57]. The use of EUS in the assessment of AA is undefined. There is no consensus on the requirement or not for EUS prior to consideration of treatment on all patients with AA. It has been suggested by some experts that EUS is not required if the lesion is less than

1 cm in diameter or there are no endoscopic signs to suggest malignancy[58]. Others claim that, if accessible, EUS testing ought to be taken into consideration prior to endoscopic or surgical resection[59]. EUS has been reported to be of help in recognizing non-invasive lesions amenable to local resection, but as yet there are no preoperative test which are as accurate as clinical judgment and intraoperative pathological diagnosis[45,60]. A recent retrospective review concluded that EUS is useful in predicting the depth of mucosal invasion in the preoperative evaluation of suspected peri-ampullary and duodenal adenomas (specificity: 88%; negative predictive value: 90%)[53]. However, EUS is an invasive technique, operator dependent, with different rates of over-diagnosis and under-diagnosis. In this context, peritumoral inflammatory changes can lead to over-staging and likewise focal pancreatic infiltration to under-staging[61,62]. A recent meta-analisis of 14 studies and a systematic review, concluded that the results obtained by EUS were comparable to the histological results with moderate strength of agreement in the following: preoperative staging of papillary neoplasm, predicting lymph node involvement and tumor invasion[63]. The modest EUS sensitivity (77%) and specificity (78%) in predicting T1 neoplasms makes it not optimal in choosing the right patients for endoscopic papillectomy. EUS sensitivity and specificity for detecting nodal invasion was 70% and 74%, respectively. We believe, as other authors that if the clinical suspicion for invasive carcinoma is low (*e.g.*, absence of jaundice, endoscopic features of noncancerous lesion), and the lesion appears amenable to endoscopic resection, then EUS may not impact the endoscopist's decision to stage the lesion *via* ampullectomy. Few studies have been reported comparing efficacy of EUS and intraductal ultrasound (IDUS) for ampullary neoplasms[54,60,64]. IDUS was superior to EUS in terms of tumor visualization and staging (staging accuracy: 78%-93%). Therefore, IDUS can be particularly appropriate in deciding which patients should undergo endoscopic ampullectomy. However, the availability of this technique is limited and therefore the number of patients undergoing IDUS is small.

Magnetic resonance imaging and computed tomography

Magnetic resonance imaging and computed tomography (CT) use is limited to staging of know ampullary cancers, for nodal staging and metastatic evaluation. CT is less precise than EUS for T staging of ampullary cancer[56,65].

ENDOSCOPIC AMPULLECTOMY

Patients diagnosed with an ampullary adenoma have three treatment options: pancreaticoduodenectomy (Whipple procedure), surgical local excision (surgical ampullectomy), or endoscopic ampullectomy. There are no clear guidelines about the surgical or endoscopic management of AA and, if they should undergo postprocedure surveillance[66]. Surgical excision is typically recommended for patients with larger lesions, lesions that contain carcinoma, lesions with lymph node involvement on preprocedure imaging, or for patients who do not have access to an experienced endoscopist in ampullectomy. Pancreaticoduodenectomy is more likely to achieve complete excision compared with local excision, but it is associated with higher operative morbidity and mortality rates (25%-65% and 0%-10%, respectively)[67,68]. Perioperative mortality rates were lowest (< 4%) in centers with a high procedure volume. Surgical ampullectomy has lower morbidity and mortality, but has the disadvantage of having more recurrence rate. Randomized trials comparing surgical ampullectomy with pancreaticoduodenectomy have not been performed. Endoscopic ampullectomy was first described in 1983 by Suzuki *et al*[59] and ten years later Binmoeller *et al*[4] described a considerable case series. More recently, many other series have reported low morbidity and mortality with endoscopic therapy[7-19]. However, the role of endoscopic ampullectomy is still debatable and it is largely performed only in reference hospitals with skill in therapeutic endoscopy. Endoscopic ampullectomy may be considered in smaller lesions (< 30 mm) that do not contain carcinoma and in patients with severe diseases. Lesions with endoscopic characteristics suggestive of posible malignancy (*e.g.,* nonlifting, firmness, ulceration, friability) should be offered surgical resection[6].

ENDOSCOPIC AMPULLECTOMY TECHNIQUE

General principles

Endoscopic ampullectomy is a therapeutic modality which must be undertaken by an endoscopist with enough training and expertise. The goal with AA is for total en-bloc removal of the neoplasm. Initially, the endoscopist must determine whether resection of the entire lesion in one piece ("*en bloc*") is feasible and locate the margins of the lesion. This method has several advantages: (1) it increases the likelihood of complete removal; (2) it provides clear margins for histopathologic evaluation; and (3) it reduces the procedure time. However, *en bloc* excision may not be technically feasible if the adenoma is of a large size, and/or there is a limited endoscopic accessibility. Piecemeal excision is usually reserved for these cases, frequently with adjuvant ablative therapy[69]. It has been postulated that this technique can reduce recurrence rates, bleeding and perforation. However, comparative trials are lacking[13] (Figure 1).

Submucosal lifting

The role of submucosal injection of saline, which may be combined with epinephrine or methylene blue before ampullectomy, is controversial[6,62,66]. Epinephrine

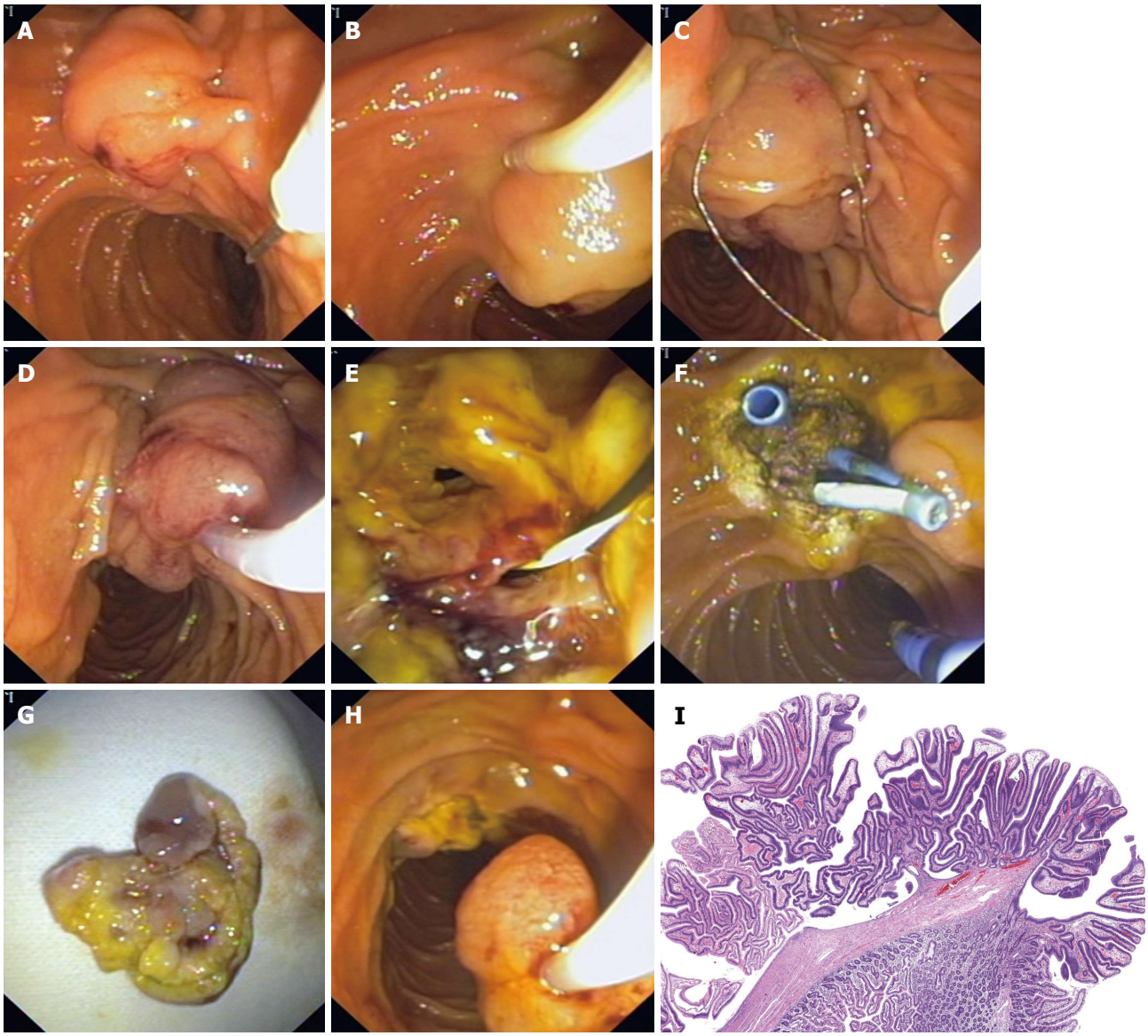

Figure 1 Technique of en-bloc ampullectomy. A: Lesion is identified; B: Submucosal (saline + epinephrine) injection; C: With the snare tip anchored above the papillary mound the entire papilla is snared; D: Check mobility and ensure the snare is firmly closed; E: En-bloc ampullary resection. Biliary and pancreatic (guidewire) orifice is identified; F: Biliary and pancreatic stents are placed. Adjuvant APC therapy is applied; G: Tissue retrieval with the snare; H: Ampullectomy specimen; I: Ampullary adenoma: tubulovillous architecture that shows neoplastic epithelial cells with pseudostratified and enlarged hyperchromatic nuclei. Adjacent there is normal duodenal mucosa. (HE: 20 ×). (Courtesy of Mercedes Hernando, MD). APC: Argon plasma coagulation.

and methylene blue may help minimize bleeding and enhance endoscopic visualization of the lesions margins, respectively[13]. Local saline injection may increase technical success and decrease complications similar to mucosectomy[13,70]. However, this technique is not recommended by other authors because submucosal saline injection may involve certain disadvantages: (1) the ampullary lesion may not lift due to tethering by the biliary and pancreatic ducts; (2) The dome effect created by submucosal injection may cause difficulty in the placement of the snare for effective *en bloc* resection[13,70-72]; and (3) increased risk of postresection pancreatitis has been reported. Currently, the evidence to support submucosal injection before ampullectomy is not significant. A possible indication may be adenomas with lateral extraampullary spread[72].

Endoscopic resection

There is no specific type of snare for endoscopic ampullectomy. For the majority of usual adenomas both hexagonal or oval snares of 3 cm are recommended. Standard braided polypectomy snares are typically used. The use of a thin wire snare is advised by some authors, limiting dispersion of the energy and risk of injury to the pancreatic orifice[72]. Occasionally, a peripheral circumferential incision to the adenoma with a needle knife device may make easier the snare capture[6]. To resect the lesion, the tip of the snare is placed on the top of adenoma; then, the snare is closed maximally and, after previously checking for papilla mobility, the lesion is

sectioned by continuous application of current.

Optimal current

There is no general recommendation regarding the optimal current and power output for endoscopic ampulectomy. Some investigators recommend pure-cutting current for this purpose[4,15,73] to preclude the edema originated by the coagulation mode, although, a pure cutting current has been related to bleeding. Others, using a blended electrosurgical current[4,6,9] or alternating cut/coagulation modes[6,62,74]. Power output oscillates between 30 W and 150 W[6,9,13,73,75]. Most experts, advocate a blended current[76]. We prefer to use Erbe electrosurgical generators (Endocut, effect 2)[77].

Retrieval of resected specimen

Retrieval of the specimen is essential for total evaluation and detection of small malignant foci. An anti-peristaltic agent administration (*e.g.*, glucagon or hyoscine butylbromide) to avoid intestinal migration is recommended. Retrieval should be performed immediately after excision since there is a tendency for the excised specimen to migrate distally into the jejunum. For this purpose, the snare that was used for the excision or a retrieval net is ideal. Endoscopic suction can also prevent the tissue migration. However, the specimen should not be aspirated through the accessory channel of the duodenoscope into a trap because this could lead to fragmentation of the specimen. Once retrieved, the specimen can be pinned to a polystyrene block to aid orientation and facilitate margin analysis.

Residual tissue ablation

After specimen retrieval, the duodenoscope is reintroduced to examine the resection site for: (1) active bleeding or bleeding stigmata; (2) residual tissue ablation. Usually, ablation therapy is used as adjunctive therapy to treat residual adenomatous tissue remaining after, *en bloc* or piecemeal, snare resection. With piecemeal excision, the tissue near the duct holes may be hard to excise completely. However, the benefits of this adjunctive therapy remain controversial. The overall success rate was comparable in patients with and without adjuvant thermal ablation (81% *vs* 78%, respectively)[9]. Ablation can be performed with monopolar or bipolar coagulation[49,70], and others devices[11,13,70,78]. We often use argon plasma coagulation (APC) (setting of 40 to 50 watts) to ablate residual tissue. We carry out a biliary sphincterotomy prior to fulguration, and we place a pancreatic stent before thermally coagulating around the pancreatic orifice.

Sphincterotomy and stent placement

The aim with a pancreatic or biliar sphincterotomy and stent placement is to enhance the technical success and decrease the complications of endoscopic ampullectomy[4,13,70,79-81]. However, a preresection sphincterotomy has some drawbacks. First, *en bloc* resection can be more difficult and will hinder total histologic evaluation of the resected specimen as result of thermal injury. Secondly, it may increase risks of bleeding, perforation and tumor seeding[82].

Usually, a meticulous inspection of the ampullectomy site allows identification of focal biliary and pancreatic orifices within the duodenal wall. Otherwise, secretin administration can produce juice flow to identify the pancreatic orifice. A 5 French pancreatic stent placement is advised to decrease the incidence and severity of pancreatitis[6,9,81,83,84]. Therefore, pancreatic duct stenting after endoscopic ampullectomy appears recomendable[74]. If ERCP or prior MRCP have demonstrated a pancreas divisum, pancreatic duct stenting is usually not necessary. Acute cholangitis after papillectomy is uncommon[76], and prophylactic biliary stent placement is generally unnecessary. However, we often perform either a biliary sphincterotomy or a prophylactic biliary stent is placed to minimize this probability. Biliary stenting may ensure the correct bile drainage if major bleeding occurs. The pancreatic and biliary stents are generally removed two or three weeks later, at which time any suspicious-appearing residual polypoid tissue can be removed to ensure complete excision.

COMPLICATIONS OF AMPULLECTOMY

Complications after endoscopic ampullectomy include bleeding (0%-25%), pancreatitis (0%-25%), perforation (0%-4%), papillary stenosis (0%-8%) and cholangitis (0%-2%)[4,6,9,11,13,62,85-87]. Pancreatitis, perforation and delayed bleeding are the most severe complications[62]. The overall complication rate is around 15%[4,11,49,70,80]. Ampullectomy-related mortality is exceptional, occurring in 0.3%[76].

Bleeding

The duodenal wall has a high vascularization. Bleeding can habitually stopped by hemostatic procedures (*e.g.*, adrenaline injection, APC, clipping)[88]. If substantial bleeding is expected then, biliary stent placement is useful to avoid cholagitis. If massive bleeding occurs, urgent arteriography is probably the best diagnostic and treatment option. In patients with a high risk of cardiovascular incidents aspirin may be continued; however, anti-coagulants agents should be discontinued.

Perforation

Perforation is usually retroperitoneal. Therefore, if perforation is suspected (endoscopic features, ongoing pain) a CT is more sensitive than simple radiology. Not all cases of perforation need surgical treatment, selected patients can be treated conservatively (intravenous antibiotics, gut rest)[6,14]. In anycase, a multi-disciplinary management is imperative to reach the best result.

ENDOSCOPIC OUTCOMES

The success rates for endoscopic resection of AA is high (range: 45%-92%), with recurrence rates of

Table 1 Recommended intervals for endoscopic surveillance after ampullectomy

	Surveillance
No residual polyp after the primary resection	3 mo later
If negative result for residual adenoma	1 yr later
Beyond this	every 3-5 yr
Patients with FAP	every 3 yr

FAP: Familial adenomatous polyposis.

0%-33%[9,89]. Intraductal adenoma growth had less favorable outcomes compared with adenomas without intraductal growth[15]. Predictors of success include: (1) lack of a genetic predisposition to adenoma formation (*e.g.*, FAP); (2) age > 48 years; (3) male sex; and (4) lesion size < 2.4 cm[70].

ENDOSCOPIC FOLLOW UP AND SURVEILLANCE

After ampullectomy patients should remain fasting for 4-12 h. Then, they are discharged home on a liquid diet and later continue with a normal diet. To reduce the risk of ductal lesion, the pancreatic stent should be removed in 2 wk.

Adenoma recurrence can occur in up to 25% of cases despite of complete removal during the index procedure[6,9,76]. In the absence of symptoms, surveillance endoscopy can be accomplished using a side-viewing duodendoscope without ERCP. Intervals change based on the histology and margin status of the resected lesion, history of FAP, patient age and comorbidities.

Recommended intervals (Table 1): (1) If there was no residual polyp after the primary resection: endoscopy 3 mo later; (2) If the result is negative for residual adenoma: surveillance 1 year later; (3) Beyond this, the yield of long-term surveillance in sporadic AA is unknown. We usually perform surveillance every 3-5 years; and (4) Given the risk for metachronous duodenal lesions, patients with FAP should undergo routine surveillance every 3 years.

CONCLUSION

Advances in endoscopy, EUS and ERCP have influenced the management to patients with ampullary lesions. Endoscopic ampullectomy has replaced surgical interventions for the treatment of AA without ductal extension. Endoscopic ampullectomy has lower morbidity and mortality rates than surgical approaches. Disadvantages include: difficult technique, few experienced endoscopists, several procedures to achieve total resection, moderate recurrence rates (30%), and, as with surgical ampullectomy, the need for postprocedure endoscopic surveillance. The best technique for endoscopic ampullectomy is subject to the adenoma size. *En bloc* resection is recomended for lesions confined to the papilla. Endoscopic ampullectomy is an effective and safe treatment for AA in experienced endoscopist but, the endoscopist must be alert to potential complications. Long-term follow-up information is required to clarify the appropiate surveillance interval for patients with sporadic AA.

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REVIEW

"How many times must a man look up before he can really see the sky?" Rheumatic cardiovascular disease in the era of multimodality imaging

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Abstract

Cardiovascular involvement in rheumatic diseases (RD) is the result of various pathophysiologic mechanisms including inflammation, accelerated atherosclerosis, myocardial ischemia, due to micro- or macro-vascular lesions and fibrosis. Noninvasive cardiovascular imaging, including echocardiography, nuclear techniques, cardiovascular computed tomography and cardiovascular magnetic resonance, represents the main diagnostic tool for early, non-invasive diagnosis of heart disease in RD. However, in the era of multimodality imaging and financial crisis there is an imperative need for rational use of imaging techniques in order to obtain the maximum benefit at the lowest possible cost for the health insurance system. The oligo-asymptomatic cardiovascular presentation and the high cardiovascular mortality of RD necessitate a reliable and reproducible diagnostic approach to catch early cardiovascular involvement. Echocardiography remains the routine cornerstone of cardiovascular evaluation. However, a normal echocardiogram can not always exclude cardiac involvement and/or identify heart disease acuity and pathophysiology. Therefore, cardiovascular magnetic resonance is a necessary adjunct complementary to echocardiography, especially in new onset heart failure and when there are conflicting data from clinical, electrocardiographic and echocardiographic evaluation of RD patients.

Key words: Echocardiography; Cardiovascular magnetic resonance; Nuclear imaging; Cardiovascular computed tomography; Myocardial perfusion-fibrosis; Coronary artery disease; Vasculitis; Rheumatic cardiovascular disease; Myocarditis

Core tip: The oligo-asymptomatic cardiovascular presentation and the high cardiovascular mortality of rheumatic diseases (RD) necessitate a reliable and

reproducible diagnostic approach to catch early cardiovascular involvement. Echocardiography, although being the cornerstone of cardiac evaluation, can not always exclude cardiac involvement and/or identify acuity and pathophysiology of cardiac lesions. Therefore, cardiovascular magnetic resonance is a necessary adjunct, complementary to echocardiography, especially in new onset heart failure and when there are conflicting data from clinical, electrocardiographic and echocardiographic evaluation of RD patients.

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INTRODUCTION

Cardiovascular involvement in rheumatic diseases (RD) is the result of various pathophysiologic mechanisms including systemic, myocardial, vascular inflammatory process, atherosclerosis, cardiac ischemia, due to micro and/or macrovascular lesions, abnormal coronary vasoreactivity and fibrosis[1,2]. RD patients may present with valvular, myocardial, pericardial, coronary artery or microvascular disease, vasculitis, systolic and diastolic heart failure, as well as pulmonary arterial hypertension. Symptoms of heart involvement in RD are usually subtle and underestimated, because they are attributed to the underlying systemic disease. The application of targeted treatment in RD led to a significant reduction of disease-associated mortality; however, the life expectancy of RD patients still remains lower, compared to general population[3], predominantly due to high incidence of cardiovascular disease[4-8].

RD with cardiovascular involvement include: (1) Rheumatoid arthritis and the spondyloarthropathies; (2) Systemic lupus erythematosus (SLE); (3) Systemic vasculitides; (4) Inflammatory myopathies; (5) Systemic sclerosis; (6) Mixed connective tissue diseases (MCTD); and (7) Sarcoidosis (SRC).

Non-invasive cardiovascular imaging, including echocardiography, nuclear techniques, cardiovascular computed tomography and cardiovascular magnetic resonance, represents useful diagnostic tool for early, non-invasive assessment of cardiac disease in RD. However, in the era of multimodality imaging and financial crisis there is an imperative need for rational use of the above mentioned techniques in order to obtain the maximum diagnostic benefit at the lowest possible cost for the health insurance system.

Applying the Bob Dylan's lyrics to cardiovascular evaluation of RD, it is clear that a careful and cost-effective evaluation of all available imaging techniques is needed, before to achieve the best diagnostic approach. The aim of this review is to present the value of various imaging techniques and propose an efficient diagnostic algorithm for early detection of cardiovascular involvement in RD.

NON-INVASIVE IMAGING TECHNIQUES

Echocardiography

Currently, the most commonly noninvasive, imaging technique, used in cardiovascular imaging, is echocardiography, due to high availability, portability, low cost, lack of radiation and high expertise among cardiologists. Transthoracic echocardiography (TE) allows an accurate evaluation of valvular morphology and function, assessment of pericardium and abnormalities of ventricular wall motion; by adding Doppler analysis, valuable information about left ventricular diastolic function, valvular flow and pulmonary pressure can be also obtained. Recently, it was documented that the definition of pulmonary hypertension obtained by echocardiography is useful to predict the 6-year mortality in SLE[9]. In another study evaluating patients with antiphospholid syndrome (APS), pulmonary hypertension was the most common finding in APS and was associated with thromboembolic disease; in contrary left ventricular disease and cardiac thrombi were rare[10]. Furthermore, in APS and SLE (with or without aPL), SLE/APS and disease duration were independent predictors for valvular disease progression and ventricular diastolic dysfunction in a 10-year follow-up echocardiographic evaluation[11]. In a meta-analysis, the presence of aPL in SLE was associated with high risk for heart valvular disease, including Libman-Sacks endocarditis. Therefore, systematic echocardiography evaluation in SLE with aPL should be always scheduled[12]. Echocardiography has been successfully used in both antiphospholipid syndrome[11,12] and asymptomatic patients with juvenile-onset SLE, who presented evidence of declining ventricular diastolic function with time[13]. Rexhepaj *et al*[14] found significant differences in early diastolic flow velocity (E), atrial flow velocity (A) and E/A ratios in rheumatoid arthritis (RA) compared with normals, suggesting that a subclinical lesion of left and right ventricular function is present in RA patients, although left ventricular parameters were still normal. Improvement of cardiac function was also shown by conventional echocardiography in RA after treatment with infliximab[15].

Another application is transthoracic dipyridamole stress echocardiography and coronary flow reserve (CFR) evaluation. CFR is assessed in the distal left anterior descending coronary artery expressed as ratio between peak diastolic velocity during stress and at baseline. It is an extremely sensitive marker (> 90%) for coronary artery disease (CAD)[16,17] and, if it was considered together with regional wall motion abnormalities, is even more specific[18]. A CFR < 2 is a very accurate index to predict the presence of CAD[17]. If coronary arteries are normal, CFR abnormalities show impairment of coronary microcirculation, as in

arterial hypertension with or without left ventricular hypertrophy, diabetes mellitus, hypercholesterolemia, syndrome X, hypertrophic cardiomyopathy and other diseases[19]. A reduced CFR reflects bad CAD prognosis[20]. Finally, we should emphasize that not only the binary (normal-abnormal) response in CFR, but also the continuous spectrum of CFR values is a strong independent predictor in known or suspected CAD[21]. Hirata *et al*[22] found a serious reduction of CFR in premenopausal SLE women compared with matched controls, due to microvascular disease that leads to decreased vasodilation during pharmacological stress. Turiel *et al*[23] detected a significant impairment of CFR in early RA without any anti-rheumatic therapy and disease duration < 1 year. The reduced CFR in the absence of wall motion abnormalities indicated abnormalities of coronary microcirculation, due to endothelial dysfunction.

Furthermore, exercise echocardiography (EE) using dobutamine was proven of great value for the evaluation of myocardial ischemia in RD. In a study by Saghir *et al*[24], RA was associated with a 2-fold higher risk for cardiac ischemia on EE and the risk was depending on RA disease duration; mortality rate was also higher in RA with ischemic EE study[24]. Furthermore, asymptomatic RA patients may present cardiac ischaemia at similar levels to DM patients but with low prevalence of obstructive coronary artery lesions and higher incidence of microvascular disease, due to increased inflammatory response[25].

Tissue Doppler imaging (TDI) is a new echocardiographic technique that allows the measurement of myocardial velocities and myocardial deformation. It is limited by the angle used, because only deformation along the ultrasound beam can be used from velocities evaluation; however, myocardium presents simultaneous deformation in 3 dimensions[24]. Birdane *et al*[26] demonstrated that RA patients had a significant impairment of TDI biventricular diastolic function compared with controls that was depended on age and steroids treatment. To overcome TDI limitations, speckle tracking analysis has been applied to evaluate myocardial strain along the longitudinal, circumferential and radial axes[27]. Recently, it was demonstrated that interleukin-1 inhibition contributes to a greater amelioration in endothelial, coronary and aortic function in addition to left ventricular myocardial deformation and twisting in RA patients with CAD than in those without[28]. Additionally, global longitudinal LV/RV strain was reduced in RA patients compared with controls and strain abnormalities were correlated with RA disease severity[29]. Furthermore, 3D-speckle tracking is a new method to detect early abnormalities in SLE patients with normal LV systolic function assessed by 2D echocardiography[30].

Another application of echocardiography which is very useful in systemic autoimmune diseases is transesophageal echocardiography (TOE). TOE is more sensitive compared with TE for the detection of valvular lesions and cardiac masses[31]. Turiel *et al*[32] detected a high prevalence (61%) of valvular abnormalities or vegetations as potential embolic causes using TOE in 56 patients with primary APS. Recently, the development of 3D TOE allows cross-sectional visualization of the mitral, aortic and tricuspid valves, improving the diagnostic sensitivity compared to traditional 2D imaging[33]. Its superiority over 2D echocardiography includes more accurate and reproducible calculation of LV volumes, mass and ejection fraction, more accurate identification of wall motion changes, more reliable evaluation of right ventricle and better assessment of valvular and subvalvular abnormalities[34].

Echocardiography represents the most versatile and popular cardiac imaging modality easily applicable in any patient from outpatient clinic to intensive care unit; however, it carries some limitations. It is operator dependent, has the limitation of the acoustic window, cannot perform detailed tissue characterization and cannot define the type of tissue lesions in patients with preserved diastolic or systolic function[35].

Nuclear techniques

Single-photon emission computed tomography: It is the most widely used nuclear technique to evaluate stress/rest cardiac perfusion. Maximal exercise or pharmacological stress can be used as a stressing factor and diffusible radiotracers injected during the peak stress allow the detection of myocardial stress perfusion defects[36]. Myocardial blood flow during stress increases about 3 to 5 fold compared with the values during rest. If there is a significant coronary stenosis, myocardial perfusion will not increase adequately in the territory supplied by the stenotic artery conducting to perfusion defect. The currently used single-photon emission computed tomography (SPECT) radiotracers are characterized by a myocardial uptake proportional to blood flow[37]. SPECT is considered as a very sensitive technique for the detection of myocardial ischemia[38]; however, its specificity is relatively lower[39], mainly due to soft-tissue attenuation artifacts. Additional disadvantages of SPECT include high cost and the use of radioactive materials[40].

Positron emission tomography: It has higher spatial resolution compared with SPECT and provides absolute quantitative information about the physiologic parameters of myocardium; moreover, it has high sensitivity and specificity for assessment of myocardial ischemia compared with SPECT. Recently, positron emission tomography (PET) with flurpiridaz F 18 was proven safe and superior to SPECT, due to better image quality, higher certainty during interpretation and generally better CAD diagnosis[41].

Myocardial perfusion by nuclear techniques is a useful tool for the detection of subclinical CAD in RD[42]. Silent myocardial infarction has been also diagnosed by myocardial perfusion SPECT in SLE[43]. Additionally, abnormal perfusion was identified in asymptomatic,

low risk for CAD in SLE patients using a technetium-99m sestamibi[44]. Finally, in SLE patients with cardiac symptoms an abnormal glucose metabolism of the myocardium was detected, shown as a pathological 18FDG scan, whereas perfusion appeared normal (reversed mismatch)[45]. In inflammatory myopathies, Technetium-99m pyrophosphate (99mTc-PYP) and gallium-67 scans had similar sensitivity, specificity and accuracy in the detection of skeletal muscle disease, compared with serum enzymes (70%, 100% and 80%, respectively). Compared with clinical parameters, 99mTc-PYP presented 70% and 67Ga 65% accuracy. Abnormal PYP and 67Ga cardiac uptake was observed in 57% and 15% of patients, respectively[46].

However, nuclear imaging techniques have the disadvantages of high cost, radiation, inability to perform tissue characterization and low spatial resolution, not allowing the assessment of subepicardial, intramyocardial or subendocardial fibrotic lesions, frequently found in RD[33].

Multislice computed tomography

Coronary artery calcification (CAC) occurs due to atherosclerotic process and reflects the total coronary atherosclerotic burden[47]. The Agatston coronary calcium score identifies the extent of calcification in the coronary arteries[48]. Electron-beam computed tomography (EBCT) is a very sensitive technique able to detect small depositions of calcium in the coronary arteries. The radiation dose during an EBCT is considerably lower compared with X ray coronary angiography[49]. Recently published studies, using multislice computed tomography (CT) with iodinate contrast agents to visualize the coronary artery lumen, demonstrated high accuracy in the early diagnosis of CAD[50]. This technique plays a diagnostic role not only for the detection of significant coronary artery stenosis, but also for tissue characterization of the atherosclerotic plaque. Moreover, it allows coronary calcium assessment along the coronary arteries[51]. CT requires iodinated contrast agents, which could provoke symptoms of intolerance and/or renal impairment. Furthermore, during the CT examination patients undergo ionizing radiation exposure. The prevalence of CAD using CTA in asymptomatic high-risk patients is high. If coronary artery calcium score is zero, it can not exclude CAD; however, a normal CTA is extremely accurate to exclude CAD. Total coronary plaque burden, even if only one segment is involved, are associated with high risk for cardiac events[52]. Finally, according to the recently published CONFIRM study, coronary CT angiography has incremental prognostic value for prediction of mortality and non-fatal myocardial infarction in asymptomatic patients with moderately high coronary artery calcium score (CACS), but not in lower or higher CACS[53].

Using CT, it was documented that SLE patients had significantly higher prevalence and/or extent of arterial calcification, compared with matched controls[54] and the disease activity was a potentially modifiable risk factor[55]. Finally, another CT study demonstrated that the calcification of cardiac valves is more prevalent in RA and SLE, compared with controls. The presence of mitral valve, but not aortic valve calcifications, independently predicted premature atherosclerosis in RA and SLE[56]. Furthermore, RA patients without CAD had higher prevalence and severity of all types of coronary plaque. Residual disease activity associates with higher incidence of non-calcified and mixed plaques contributing to future cardiac events[57]. In another CT study, it was also documented that coronary atherosclerosis was not uncommon in asymptomatic SSc patients[58]. Finally, CT scan is the technique of choice for assessment of pulmonary embolism and pulmonary hypertension secondary due to recurrent pulmonary emboli[59].

Cardiovascular magnetic resonance

Cardiovascular magnetic resonance (CMR) is a non-invasive, nonradiating, operator independent technique that can offer reliable and reproducible information about myocardial function, inflammation, perfusion, fibrosis and heart disease acuity; additionally, vascular disease acuity and vascular inflammation and/or stenosis can be also assessed.

Table 1 summarises the most frequent findings, advantages and disadvantages of each methodology.

The evaluation of rheumatic diseases by CMR can offer: (1) Angiography, imaging of vessel wall and cardiac evaluation (function, oedema, early, late gadolinium enhancement and stress CMR) in vasculitis. Techniques for angiography include both contrast-enhanced MR angiography (CE-MRA) as well as non-contrast methods. Pre-contrast T1W and T2W dark blood imaging but also post-gadolinium T1W imaging can reveal presence of inflammation, even when the disease is clinically under remission[60]; (2) Function, oedema, early, late gadolinium enhancement and stress CMR for RA, SLE, SSc and MTCD. Evidence of myocardial inflammation and/or fibrosis can be identified by STIR T2, early and late gadolinium enhancement, even if the rheumatic disease is under remission[61,62]; (3) Additionally, it is the gatekeeper for differential diagnosis between various types of scar: scar due to CAD that should motivate coronary artery evaluation (subendocardial or transmural scar following the distribution of coronary arteries in CAD) and scar due to inflammation or vasculitis (subepicardial or intramural scar not following the distribution of coronary arteries in inflammation and diffuse subendocardial fibrosis in case of diffuse subendocardial vasculitis)[61-63]; (4) Function, oedema, early and late gadolinium enhancement in inflammatory myopathies using SSFP, STIR T2, early and late gadolinium enhancement, even if the disease is under remission[64,65]; (5) Carotid angiography and vessel wall imaging in RA and SLE[60]; (6) Coronary angiography, oedema, early, late gadolinium enhancement, stress CMR and scar detection for Kawasaki disease[66]; and (7) Assessment of PAH includes information about

Table 1 Clinical findings, advantages and disadvantages of each technique

Noninvasive imaging techniques	Evaluation of	Advantages	Disadvantages
Rest echocardiography	Cardiac valves	Cheap	Operator dependent
	Pericardium	Widely available	Limitation due to poor acoustic window
	Ventricular function	Bedside	No tissue characterization
	Wall motion	No radiation	
	Pulmonary pressure		
Tissue doppler imaging	Measurement of myocardial velocities	The same as in rest echocardiography	Limited by angle-dependency
Stress echocardiography	CFR in LAD	The same as in rest echocardiography	The same as in rest echocardiography
	Myocardial ischemia		
Transesophageal echocardiography	Valvular lesions	The same as in rest echocardiography	The same as in rest echocardiography
	Intracardiac masses		Semi-invasive
SPECT	Myocardial ischemia	Widely available	Radiation
	Ventricular function	Reasonable sensitive	High cost
		Not very specific	Low spatial resolution
PET	Myocardial ischemia	Very sensitive	Radiation
	Ventricular function	Very specific	High cost
			Low spatial resolution
			Not widely available
CT	Great vessels	Fast	Radiation
	Coronary arteries/grafts	Widely available	High cost
			Iodinated contrast agent
CMR	Ventricular function	Highly reproducible	Not widely available
	Inflammation	Operator independent	High Cost
	Perfusion	No radiation	Claustrophobia
	Fibrosis	Tissue characterisation	Non MRI compatible devices can not be scanned
	Heart disease acuity	High spatial resolution	Low temporal resolution
	Vascular disease acuity		
	Vascular inflammation and/or stenosis		

SPECT: Single-photon emission computed tomography; PET: Positron emission tomography; CMR: Cardiovascular magnetic resonance; CFR: Coronary flow reserve; CT: Computed tomography; LAD: Left anterior descending.

right ventricular (RV) mass index, RV volumes-ejection fraction, late gadolinium enhancement (LGE) and phase contrast imaging, including average velocity (cm/s), retrograde flow (L/min) and percentage retrograde flow (%)[67]. These indexes have been shown to be prognostic of long-term outcomes. LGE at ventricular insertion points in PAH is due to altered intraventricular septal motion and not to elevated RV pressure or remodelling[68].

TOWARDS AN ALGORITHM ABOUT THE APPLICATION OF NONINVASIVE CARDIOVASCULAR IMAGING IN RHEUMATIC DISEASES

The first line and cornerstone of routine cardiac assessment in RD is echocardiography. However, it is unable to detect cardiac disease acuity, myocardial or vascular inflammation and scar in cases with normal LV function[69]. Nuclear techniques are also unable to detect small perfusion defects, commonly found in RD, due to low spatial resolution, to identify disease acuity and the exact location of the lesion (subendocardial, transmural or subepicardial) and to further guide risk stratification[70]. CT coronary angiography cannot be included in the routine assessment of cardiac involvement in RD, because it cannot answer all the relevant queries, raised in these diseases. Furthermore, high cost, the need of repetitive radiation in both nuclear techniques and CT and the use of iodinated contrast agents in CT constitute serious limitations for its routine use in diagnosis and follow up.

CMR is a non-invasive, nonradiating, highly reproducible technique, capable to answer queries about cardiac disease acuity, etiology of cardiac lesion, necessity for cardiac catheterization and persistence of myocardial involvement, although the systemic disease seems quiescent[69].

CONCLUSION

The oligo-asymptomatic cardiovascular presentation and the high cardiovascular mortality of RD necessitate a reliable and reproducible diagnostic approach to catch early cardiovascular involvement. Echocardiography remains the routine cornerstone of cardiac evaluation. However, a normal echocardiogram cannot always exclude cardiac involvement and/or identify acuity and pathophysiology of cardiac lesions. Therefore, CMR is a necessary adjunct, complementary to echocardiography, especially in new onset heart failure and when there are conflicting data from clinical, electrocardiographic and echocardiographic evaluation of RD patients.

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Lamb's head: The model for novice education in endoscopic sinus surgery

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Abstract

Structured training in endonasal endoscopic sinus surgery (EESS) and skull base surgery is essential considering serious potential complications. We have developed a detailed concept on training these surgical skills on the lamb's head. This simple and extremely cheap model offers the possibility of training even more demanding and advanced procedures in human endonasal endoscopic surgery such as: frontal sinus surgery, orbital decompression, cerebrospinal fluid-leak repair followed also by the naso-septal flap, *etc.* Unfortunately, the sphenoid sinus surgery cannot be practiced since quadrupeds do not have this sinus. Still, despite this anatomical limitation, it seems that the lamb's head can be very useful even for the surgeons already practicing EESS, but in a limited edition because of a lack of the experience and dexterity. Only after gaining the essential surgical skills of this demanding field it makes sense to go for the expensive trainings on the human cadaveric model.

Key words: Endonasal; Endoscopic; Sinus surgery; Skull base; Learning; Training; Lamb's head

Core tip: Structured training in endonasal endoscopic sinus surgery (EESS) and skull base surgery is essential considering serious potential complications. We have developed a detailed concept on training these surgical skills on the lamb's head. This simple and extremely cheap model offers the possibility of training even more demanding and advanced procedures in human EESS such as: frontal sinus surgery, orbital decompression, cerebrospinal fluid-leak repair followed also by the naso-septal flap, *etc.* Unfortunately, the sphenoid sinus surgery cannot be practiced since quadrupeds

do not have this sinus. Still, despite this morphological limitation, it seems that the lamb's head can be very useful model.

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INTRODUCTION

Endonasal endoscopic sinus surgery (EESS) procedures are one of the most common surgical procedures performed in nowadays otorhinolaryngology. They are considered the gold-standard treatment for several entities regarding the paranasal sinuses and nasal cavity[1,2]. The orientation within the anatomic structures and a proper use of the surgical instruments during the procedures is challenging for the inexperienced surgeon, owing to the complexity of the intranasal anatomy and the intimate relation with noble structures, such as the brain, the carotid arteries, the eye-ball, orbit and the optic nerve itself[1-4]. Nowadays, the training of the novices in endonasal sinus and skull base surgery is conducted in the operating room, upon the real patients, under the surveillance of more experienced surgeons[1,2]. The complication rate in endonasal sinus and skull base surgery may vary from 5% to 8%[2]. The low and flat learning curve of the particular novices may increase the risk of serious complications. To prevent additional problems to both novices and their supervisors as well as to protect patients, all activities that objectively can help to over-bridge the gaps in theoretical and practical expertise should be employed. One of these activities undoubtedly is the lamb's head dissection training according to our model.

In terms of that, a comprehensive booklet on this matter has been written by our team and published so far in Croatian, English, Italian and Russian language by Karl Storz GmbH (Germany)[5]. In collaboration with the same manufacturer we have produced also an attractive DVD on complete dissection in a "step-by-step" manner.

LAMB'S HEAD ANATOMY

The first distinctive feature that arises when entering the lamb's nose is an abundant similarity to the human nasal cavity. Lamb's septum does not have vomer at all, thus a typical triangular lack of septum can be seen endoscopicaly in the deepest septal regions.

The inferior turbinate could be most confusing detail at the beginning since it resembles very much the middle turbinate in man. In veterinary terminology it is named *concha ventralis,* meaning anterior turbinate. According to the veterinary anatomical nomenclature, the term "middle turbinate" (*concha nasalis media*) in lamb is used to denominate a structure that is located much deeper in the lamb's nose, and can be clearly seen only if almost all of the inferior turbinate has been removed. The lamb's inferior turbinate has two main portions: the superior one, named *pars dorsalis* and the inferior one, named *pars ventralis.*

The nasal septum is straight as in all other quadrupeds. The maxillary sinus consist of two "sub-sinuses" since a perpendicular crest, arising from the bottom of this sinus, divides it in a laterally positioned, spacious cavity, so called *maxillary sinus proper*, and medially positioned, poky sinus named *palatinal sinus*. The superior, free edge of the crest that divides maxillary cavity into two sinuses is characterized by the course of the infraorbital nerve within its bone. The formation of the middle antrostomy is easy, simple, instructive and motivating for the next steps of the dissection. Posterior wall of both palatal sinus and maxillary sinus proper is at the same time the anterior orbital wall thus making an endoscopic approach to the orbital decompression relatively simple. The frontal sinus consists of queue of chambers positioned semicircular in the frontal bone thus forming a structure that resembles very much a crown. Frontal sinus is also relatively easy to approach since the surgeon has a reliable signpost: the superior turbinate gradually, as getting deeper and deeper with the instruments and endoscope, gets a tube-like form. At the bottom of this tube the surgeon finds himself in the first, most anteriorly positioned, so called supraorbital frontal sinus cell. The sphenoid sinus surgery can't be practiced in lamb's head model since quadrupeds, because of the lack of the skull base angulation (*Huxley's angle*) do not have this sinus.

LAMB'S HEAD PREPARATION

The lamb heads are purchased fresh at the butcher's shop (approximate price is about two US$) and the muzzle is always cut off (cartilaginous part) as to make the entrance to the nasal cavities much easier and practical. After that, the heads are washed under the tap water and then put into a tree-liters bowl of water containing three tablespoons of alcoholic vinegar. After 24 h of soaking the heads are usually left to drain in the sink for 30 min, than wiped with a clean cloth and finally frozen at -18 ℃ until the moment they will be used for the dissection. On the day of dissection the heads are unfrozen, washed and wiped. Heads prepared in this manner are far more easy to use, without an excess of grease and fluids. For the dissection purposes the heads are mounted in a special Lamb's Head Holder (Karl Storz GmbH) (Figure 1).

DISSECTION TECHNIQUE

The dissection usually consists of ten classical steps. Step 1 concerns to the very simple task: removal of the inferior turbinate; Step 2 concerns to the clear

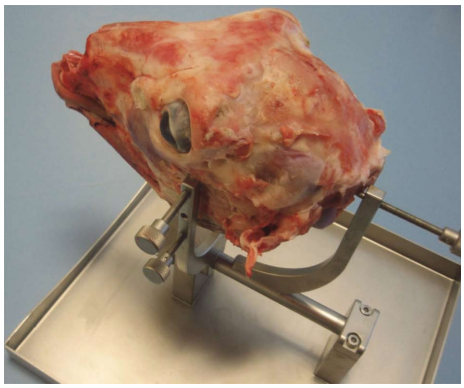

Figure 1 The head holder. Six sideward-screws serve to fix the lamb's head in desired position while dissecting.

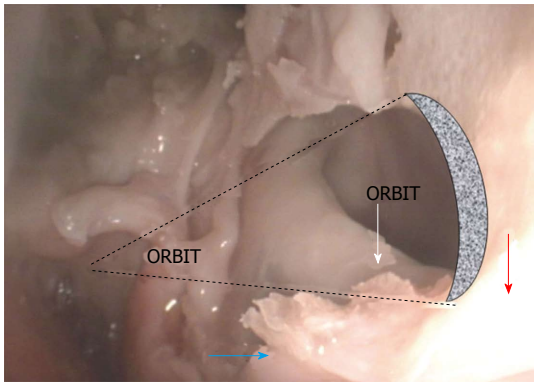

Figure 2 Endoscopic view to the left maxillary complex. The perpendicular crest (white arrow) divides the cavity within the maxilla into maxillary sinus proper (red arrow) and palatine sinus (blue arrow).

presentation of the adenoids and Eustachian tube openings bilaterally, while Step 3 regards to the clear presentation of the middle turbinate, uncinectomy and middle antrostomy followed by as clear as possible presentation of the perpendicular crest within its cavity that divides it into the maxillary sinus proper (lateral compartment), and palatine sinus (medial compartment) (Figure 2). The superior edge of this crest contains the infraorbital nerve canal; Step 4 means the identification of the posterior wall of palatine sinus which is, at the same time, the medial half of the anterior orbital wall, followed by alignment with its lateral half, *i.e*., posterior wall of the maxillary sinus proper; Step 5 is performed in sense of the endonasal endoscopic orbital decompression; Step 6 belongs to the ethmoidectomy, whereas Step 7 mean the formation of the artificial skull base defect, presentation of dura, followed by insertion of the artificial patch in the underlay manner; Step 8 means an elevation and adequate positioning of the nasoseptal flap to be used to cover the closed skull base defect in an overlay manner. The next, 9th Step, belongs to the removal of the tube-like formation, so called dorsal turbinate (plica recta), positioned at the anterior part of the roof of the

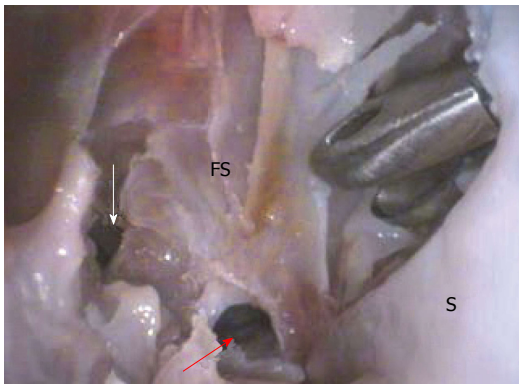

Figure 3 Endoscopic 30° view at the region of the bottom of the frontal sinus. The tip of the Kerisson's punch juts from the left nasal cavity through the artificially made septal (S) defect. Red and white arrows indicate the frontal sinus lateral and anterior cells. FS: Frontal sinus.

nasal cavity, which transforms gradually, getting more posterior, into dorsal turbinate and leads directly to the opening of the supraorbital frontal sinus chamber first. Once opened, this cell leads the surgeon to the more anteriorly and medially positioned frontal sinus cells. Finally, the Step 10 concerns to the removal of the most superior part of the nasal septum as to make the bottom of the frontal sinus visible from both sides and thus bilaterally approachable. Afterwards, the same procedure as described in Step 9 is repeated on the opposite side in order to open the contra lateral frontal sinus chambers. Finally one has to drill out the bony bottom of the frontal sinus thus connecting the previously opened cells in one, large cavity - Draf Ⅲ procedure (Figure 3).

DISCUSSION

Safe endonasal endoscopic sinus and skull base surgery requires a good and reliable knowledge on surgical anatomy and, at the same time, the safe usage of the endoscopes and instruments. Many institutions around the world insist on trainings performed upon the human cadaveric model before the surgeon undertakes his/her first surgery on a real patient[6-8]. Training courses and workshops in EESS provide a well structured supervised approach to endonasal anatomy and technical training. Unfortunately, these trainings can be achieved once or, at the most, twice during the particular novice's education because of very high registration fees for the courses. Even more, they do not offer the calm that the novice, confused and scared from all sides, can't get. Such courses, regardless of the fact that almost always are perfectly organized, have the disadvantage, not only of being such expensive, but also of being dependent on the strict medico-legal regulations regarding the question of collecting, storage and disposal of the human cadaveric heads which, nowadays, represents a rapidly growing problem in the increasing number of countries all over. Artificial models of human heads were also designed and available for training[9] but are

highly priced. Furthermore, endoscopic sinus surgery simulators have also been introduced as teaching tools for EESS[1,10,11].

However, in our opinion, the initial goal for the novices in the field of endoscopic endonasal sinus surgery is to gain the surgical skills and to become familiar with the complexity of simultaneous bimanual work in operating field, distant from that what they actually see on the monitor during their work. So, the trainees should be firstly trained on the animal models as to become more skilled with the endoscopes of various angles, orientation and understanding the surgical space and field, and to get skilled in the use of the instruments. The study of human anatomy on the cadaveric dissections should be only the second step in any case. In this way the learning curve for EESS gets more attractive thus giving the novices, *i.e.*, trainees more confidence in operating area and better understanding of anatomical details they are about to dissect. Animal model of a comparable sino-nasal appearance to human seems to be the logical choice for the initial training of surgical skills.

Paying a great respect to Gardiner's introduction of a sheep model for training in EESS[12], we have developed a detailed study for training on the lamb's head, combining radiology findings, frozen three-axis sections and a meticulous research on lamb's sino-nasal surgical anatomy as to facilitate the first steps in EESS for all those who approach this field[5].

This was the result of long lasting attempts to find a low-cost and suitable animal model that will fit two most important demands: (1) high degree of the anatomical similarity to human sino-nasal anatomy; and (2) the appropriate dimensions of the sino-nasal unit that will allow the easy use of the standard endoscopic sinus surgery instruments otherwise used in human medicine. We have tried with dogs, pigs, sheep and goats, but, at the end, we found everything we needed with the lamb's head[13]. Some years ago we also developed a special head holder for the lamb's head, produced by Karl Storz, Germany. The lamb's head as a simple and extremely cheap model offers the possibility of training even more demanding and advanced procedures in human endonasal endoscopic surgery such as endoscopic endonasal orbital decompression, Draf Ⅲ procedure or cerebrospinal fluid leak repair, including the naso-septal flap[14]. Dacryocystorhinostomy can't be performed since the lamb does not have the lacrimal sac at all[4]. The sphenoid sinus surgery can't be practiced either, since quadrupeds do not have this sinus because of the natural lack of the skull base angulation. Still, despite these two morphological limitations, it seems that the lamb's head model can be very useful also for the surgeons that want to amend their technique and take it to a higher level. In terms of that, it was mandatory to have also the navigational system for the lamb's head and it was built by Karl Storz GmbH as well.

Regarding maxillary sinus, it's amazingly easy to perform middle antrostomy Frontal sinus surgery resembling Draf 1, 2 and 3 procedures can be trained with the ease and calm as well.

In our opinion the lamb's head is an effective, extremely cheap and user-friendly animal model for learning and training the endonasal endoscopic sinus and skull base surgery techniques.

CONCLUSION

We have developed a practical and detailed program for the training of surgical techniques in EESS on the lamb's head, combining radiology findings, frozen three-axis sections and a meticulous research on lamb's sinonasal surgical anatomy. Lamb's head proved to be an excellent model with comparable anatomy to that in humans and thus very appropriate to practice the usual EESS techniques with ease using the standard EESS instruments.

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MINIREVIEWS

Refractory chronic cough due to gastroesophageal reflux: Definition, mechanism and management

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Abstract

Refractory chronic cough due to gastroesophageal reflux is a troublesome condition unresponsive to the standard medical anti-reflux therapy. Its underlying mechanisms may include incomplete acid suppression, non-acid reflux, transient lower esophageal sphincter relaxations and esophageal hypersensitivity. The diagnosis of this disorder depends on both the findings of multi-channel intraluminal impedance-pH monitoring and the subsequent intensified anti-reflux therapy. The strategies of pharmacological treatment for refractory chronic cough due to reflux include the optimization of proton pump inhibitors and add-on therapies with histamine H_2 receptor antagonists, baclofen and gabapentin. However, the further study is needed to satisfy its management.

Key words: Esophageal pH monitoring; Chronic cough; Anti-reflux therapy; Refractory cough; Gastroesophageal reflux

Core tip: Refractory cough due to reflux can be defined as a reflux-induced cough resistant to standard medical anti-reflux treatment but responsive to the subsequent intensified anti-reflux therapy. It may be associated with the residual acid or non-acid reflux, transient lower esophageal sphincter relaxations and esophageal hypersensitivity. The definite diagnosis of the disorder depends on the positive findings of multi-channel intraluminal impedance-pH monitoring as well as favorable response to the intensified anti-reflux therapy. The current therapeutic strategies include the complete acid suppression and add-on uses of baclofen or gabapentin.

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INTRODUCTION

Gastroesophageal reflux-induced chronic cough (GERC) is a special form of gastroesophageal reflux disease with predominant cough symptom[1] and along with cough variant asthma, upper airway cough syndrome or eosinophilic bronchitis, is considered as a common cause of chronic cough[2,3]. Like gastroesophageal reflux disease, proton pump inhibitors (PPIs) alone or in combination with prokinetic agents are a standard medical therapy for GERC and can resolve the cough in most patients[1]. However, a small percentage of patients with GERC are resistant to the standard anti-reflux treatment and this condition is also defined as refractory GERC[4]. This review summarizes our understanding about the definition, mechanism and management of refractory GERC.

DEFINITION OF REFRACTORY GERC

How to define refractory GERC remains to be controversial. There is no consensus on the refractory gastroesophageal reflux disease which GERC can refer to. The generally accepted definition of refractory gastroesophageal reflux disease is the persistent classical reflux-related symptoms such as regurgitation and heartburn despite the treatment with PPIs twice daily for at least 4-8 wk[5]. Recently, Sifrim *et al*[6] proposed that refractory gastroesophageal reflux disease should be defined as the condition in which symptoms (heartburn and/or regurgitation) are not responsive to a stable double dose of PPIs during a treatment period of at least 12 wk and patients continue to report troublesome symptoms while "on" PPIs at least thrice weekly for the last 3 mo[6]. As one of extraesophageal symptoms, cough can be caused by many diseases other than GERC. A cause-effect association between reflux and cough is more difficult to establish than regurgitation and heartburn, and too long-lasting trial with PPIs may delay the diagnosis and treatment of the other etiologies of chronic cough. Therefore, we have defined refractory GERC as a condition of chronic cough with the objective evidence of abnormal reflux as demonstrated by multichannel intraluminal impedance-pH monitoring (MII-pH), and resistant to a 8-wk standard medical anti-reflux treatment but responsive to the subsequent intensified anti-reflux therapy[4,7]. This definition is consistent with the principles recommended in several guidelines for the management of chronic cough[1,8] as well as the generally accepted definition of refractory gastroesophageal reflux disease[5].

The exact prevalence of refractory GERC is still unclear. It is estimated that 10%-40% patients with gastroesophageal reflux disease do not or only partially respond to the standard dose of PPIs[9]. Unlike erosive esophagitis, nonerosive reflux disease has basically normal esophageal mucosa under an endoscope and normal or slightly abnormal esophageal acid exposure as indicated by MII-pH, accounts for 70% of gastroesophageal reflux disease and is poorly responsive to PPIs treatment[10,11]. Therefore, nonerosive reflux disease is responsible for the majority of refractory gastroesophageal reflux disease. Our preliminary results have shown that refractory GERC accounts for about one third of GERC[12], and is comparable with the prevalence of refractory gastroesophageal reflux disease.

MECHANISMS OF REFRACTORY GERC

It is well known that GERC may be caused by microaspiration of the refluxate into the airways (reflux hypothesis) and esophageal-tracheobronchial reflexes mediated by the afferent nerves in the distal esophagus (reflex hypothesis)[1]. However, the mechanisms underlying the refractory GERC is poorly understood. It may be associated with the incomplete acid suppression, non-acid reflux, transient lower esophageal sphincter relaxations (TLESRs) and esophageal hypersensitivity.

INCOMPLETE ACID SUPPRESSION

Incomplete acid suppression has been documented in patients with persistent symptoms despite the therapy with PPIs at a standard dose. Several studies have shown 4%-17% patients presented with abnormal acid reflux[13,14] and 7%-11% patients had a positive symptom index[15,16] as revealed by 24-h esophageal pH monitoring when they were "on" PPIs. The residual acid reflux can continue to elicit cough through microaspiration or esophageal-tracheobronchial reflex[1]. In addition to poor compliance such as not taking PPIs in time or at the suitable time, ineffective acid suppression may also be related to the difference in the responsiveness to therapy among patients. For example, the rapid metabolism of PPIs in some patients may result in that the high serum PPIs level can not be achieved for the adequate acid suppression[17]. Nocturnal acid breakthrough, a phenomenon of gastric pH below 4 for at least 1 h during the night, was also suggested as a cause of the failure to PPIs treatment by promoting gastroesophageal reflux during sleep[18]. However, accumulating evidence does not support a significant role of nocturnal acid breakthrough in the failure of PPIs treatment.

NON-ACID REFLUX

Non-acid reflux, an important constituent of reflux, includes weakly acidic (refluxate pH = 4-7) and weakly alkaline (refluxate pH > 7) reflux, with 95% belonging to weakly acidic reflux and only 5% belonging to weakly alkaline reflux[19]. Non-acid reflux accounts for 50% and 95% of reflux in the patients with gastroesophageal reflux disease "off" and "on" PPIs, respectively[20]. It is reported that cough-related reflux consists of acid (65%), weakly acidic (29%) and weakly alkaline (6%) reflux in the GERC patients "off" acid suppressive therapy[21]. In contrast, reflux-related cough is caused

by weakly acidic (74%), weakly alkaline (17%) and acid (4%) reflux in patients with refractory GERC[22]. The increase in weakly acidic reflux episode may derive from the relative increase in the percentage of original weakly acidic reflux due to the inhibition of acid reflux or in a considerable part, the transition of the original acid reflux due to pH value shift in the refluxates after PPIs treatment. The cough induced by weakly acidic reflux may be associated with esophageal distension due to increased reflux volume, persistent impaired mucosal integrity and esophageal hypersensitivity[23].

TLESRS

TLESRs refer to the spontaneous (not preceded by a swallow) relaxations of the lower esophageal sphincter lasting 10-60 s[24], which is a vagally mediated event induced by the volume distension of the stomach. Physiologically, it plays a role in venting air from the stomach after meals and also represents a main mechanism underlying all types of reflux[25]. In patients with established gastroesophageal reflux disease, TLESRs have a high prevalence and is two times more likely to be related to the reflux[26]. In general, PPIs can reduce the acidity and volume of refluxate in the esophagus, but have no ability to rectify the dysfunction of the lower esophageal sphincter and decrease reflux episodes.

ESOPHAGEAL HYPERSENSITIVITY

Esophageal hypersensitivity is defined as an exaggerated response of esophageal mucosa to normal or subthreshold stimuli and involved in the pathogenesis of GERC. It is unclear whether esophageal sensitivity in refractory GERC is higher than naive GERC. However, several lines of evidence have demonstrated that patients with nonerosive reflux disease are more sensitive to intraesophageal acid infusion, balloon distension and electrical stimulation than patients with erosive esophagitis[27,28].

The function of peripheral sensory terminals may be modified by inflammatory mediators released from the injured and inflammatory esophageal mocosa caused by reflux. Consequently, the transduction threshold is decreased in the primary sensory afferents, resulting in hypersensitivity at the site of injury or inflammation, and a heightened response to subthreshold or innocuous chemical, mechanical and electrical stimuli. It has been shown that the expression of acid-sensing receptors and transient receptor potential vanilloid 1 are up-regulated in the esophageal mucosa of patients with gastroesophageal reflux disease[28]. Patients with refractory GERC have the dilated intercellular spaces in the esophageal epithelium due to repeated exposure to acid and enzymes, which permits the penetration of some noxious or sensitizing substances through the epithelial barrier, exposes and activates subepithelial nerves, and prompts the transduction of acid signals from the peripheral afferents to cough center[29]. Once central sensitization is established, it can continue to potentiate cough even though the initial peripheral stimulus is discontinued.

DIAGNOSTIC APPROACH

According to the algorithms recommended in several guidelines for the management of chronic cough, a complete laboratory workup including sinus or chest imaging, pulmonary function test, bronchial provocation and induced sputum cytology should be performed in sequence or simultaneously to identify the common causes of chronic cough such as cough variant asthma, upper airway cough syndrome and eosinophilic bronchitis[1,8]. Possible GERC is considered when the patients have the concomitant typical reflux-related symptoms, the other common causes of chronic cough are excluded and the treatment specific to current etiologies fails to resolve cough completely[30]. If the laboratory findings reveal the abnormal reflux, the favorable response to the subsequent standard medical anti-reflux treatment will confirm the diagnosis of GERC. Otherwise, refractory GERC has to be assumed (Figure 1).

The difficulty in diagnosing refractory GERC is to confirm the cause-effect association between reflux and cough. The resistance to medical anti-reflux therapy may be refractory GERC or the ongoing cough is not related to any continuing reflux[4]. Of note, the false refractoriness to PPIs treatment due to poor compliance to treatment should be excluded. It is found that 40% of the patients with gastroesophageal reflux disease are not compliant to PPIs therapy in a large population based study[31]. Except for omeprazole sodium bicarbonate and dexlansoprazole, the traditional delayed release PPIs should be administered 30-60 min before meals to assure the maximal gastric acid inhibition[32]. When the factors are addressed, the possibility of refractory GERC may be increased.

As shown in Table 1, MII-pH is currently a major laboratory examination for the diagnosis of refractory GERC while barium radiographs and upper gastrointestinal endoscopy are not recommended or used only when MII-pH is unavailable because their overall sensitivity is extremely low[32]. With the impedance probes located at different sites of the esophagus, MII-pH can record the changes in the electric impedance induced by the movement of various types of bolus in the esophagus, recognize gas, liquid and mixed reflux based on the impedance value of bolus, and classify reflux as acid and non-acid according to the pH value of refluxate[19,33]. Theoretically, MII-pH has an ability to detect all types of reflux, identify the characters of refluxate and establish a temporal association between acid or non-acid reflux and cough. MII-pH is superior to the ambulatory 24-h esophageal pH monitoring for the diagnosis of GERC in that it provides additional information of non-acid reflux[30]. Recently, it has been reported that the presence of a pathological acid

Table 1 Diagnostic value of laboratory investigation for chronic cough due to gastroesophageal reflux

Diagnostic tests	Indications	Advantages	Drawbacks
Barium radiographs	Not recommended for diagnosis of GERC unless evaluating for dysphagia	High specificity	Extremely low sensitivity
Upper gastrointestinal endoscopy	Not recommended for diagnosis of GERC. Only useful for the detection of erosive esophagitis but not for non-erosive reflux disease	High specificity	Low sensitivity
Ambulatory 24-h esophageal pH monitoring	Able to detect acid reflux but not non-acid (weakly acidic and alkaline) reflux	Relatively high sensitivity	Modest specificity
Multi-channel intraluminal impedance-pH monitoring	Able to detect both acid and non-acid reflux	High sensitivity	Modest specificity

GERC: Gastroesophageal reflux-induced chronic cough.

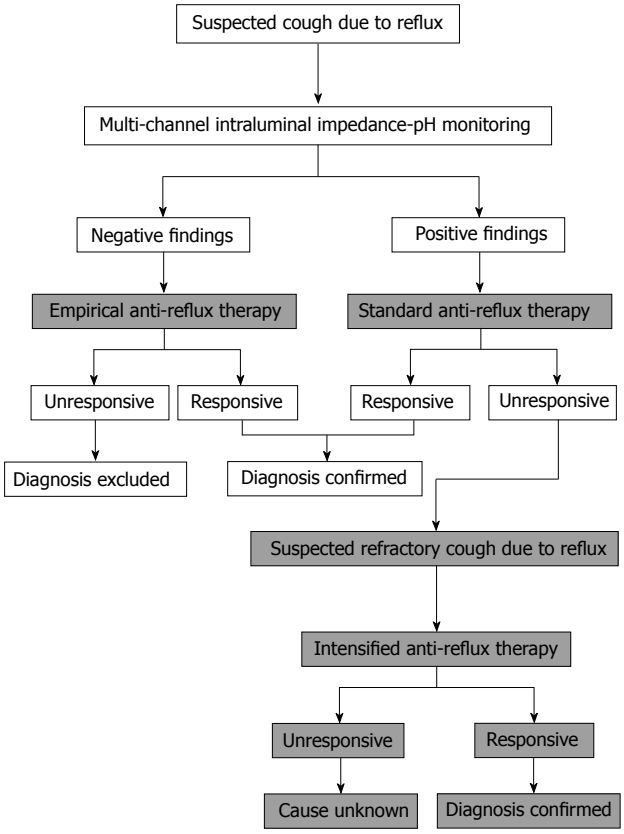

Figure 1 Diagnostic algorithm for refractory chronic cough due to reflux.

exposure time or pathological impedance baseline in MII-pH study may predict the better response to the treatment with PPIs in patients with chronic cough[34]. Therefore, MII-pH has been posited as a future standard for the reflux detection and monitoring[35].

MII-pH has the similar inherent limitations to ambulatory 24-h esophageal pH monitoring. For the estabishment of a temporal association between cough and reflux, the calculation of the symptom association probability (one of significant criteria in the diagnosis of GERC) still depends upon the counts and timing of cough reported by patients and the reflux recorded by MII-pH. Since patients usually underestimate the frequency of cough events or misreport their timing during MII-pH, the symptom association probability determined with above method is not adequately accurate. Several studies have demonstrated that only 40% of cough bursts are indicated by the patients, with a delay of around 30 s, and a positive symptom association probability is found in only 35% of GERC patients[21]. Even though with synchronized intraesophageal manometric monitoring or 24-h ambulatory cough sound recording for precise recognition of cough, the positive rate of the symptom association probability is only improved to 45%-48%[21,36]. Therefore, the sensitivity of MII-pH in the diagnosis of refractory GERC is not high enough to meet the need in clinical practice.

The diagnostic criteria for the refractory GERC can be defined as follows[4,7,8]: (1) chronic cough, with or without the classical reflux-related symptoms such as regurgitation and heartburn; (2) MII-pH confirms the abnormal acid or non-acid reflux, defined as DeMeester score of $\geqslant$ 14.72 and/or the symptom association probability for acid or non-acid reflux of $\geqslant$ 95%. However, our study has shown that $\geqslant$ 80% may be an optimal cut-off value for the symptom association probability and can maintain the better balance between sensitivity and specificity in the diagnosis of GERC[37]; (3) cough fails to improve after 8-wk standard anti-reflux treatment with omeprazole (or equivalent PPIs) at 20 mg twice daily and domperidone at 10 mg thrice daily with life style modification, but responds to the subsequent intensified anti-reflux therapy; and (4) other causes of chronic cough are excluded. When a patient meets all the above criteria, refractory GERC can be definitely diagnosed.

THERAPEUTIC INTERVENTIONS

Refractory GERC can be treated pharmacologically and non-pharmacologically. Currently, the intensified medical anti-reflux treatment is the most common therapeutic option (Table 2).

OPTIMIZATION OF PPIS THERAPY

The modulation of brands and doses of PPIs is a useful strategy for the management of refractory GERC. When a PPI fails, switching to another PPI is possibly effective. Several clinical studies have demonstrated

Table 2 Evaluation of therapeutic options for refractory chronic cough due to gastroesophageal reflux

Therapeutic options	Evaluations
Pharmacologically	
Optimization of PPIs therapy	
Switch to another PPI	Useful for some refractory cough due to acid reflux
Doubling the current dose of PPI	Useful for refractory cough due to severe acid reflux
Add-on therapy	
Histamine H2 receptor antagonists	Useful for refractory cough due to severe acid reflux and night-time reflux
TLESRs inhibitors (baclofen)	Useful for refractory cough due to acid or non-acid reflux resistant to PPI therapy
Gabapentin	Useful for refractory cough due to acid or non-acid reflux resistant to PPI and baclofen therapy
Surgically	
Laparoscopic fundoplication	A treatment option for long-term therapy of refractory cough due to acid or non-acid reflux
Endoscopic therapy or transoral incisionless fundoplication	Not recommended for refractory cough due to reflux on the basis of lack of long-term efficacy
Radiofrequency augmentation	Not recommended for refractory cough due to reflux on the basis of lack of long-term efficacy

PPI: Proton pump inhibitor. TLESRs: transient lower esophageal sphincter relaxations.

that, when adequate symptom relief is not achieved with omeprazole, the switch to esomeprazole (40 mg once daily) for 8 wk may attenuate the symptoms and improve the health-related quality of life in 78% of the patients with gastroesophageal reflux disease[38,39]. This option is also cost-effective. When a single dose of lansoprazole (30 mg once daily) fails, switching to either omeprazole or esomeprazole (40 mg once daily) may achieve adequate symptom control in the patients with gastroesophageal reflux disease[40,41]. However, there is no study to demonstrate the efficacy of a switch to another PPI in patients with refractory GERC.

To increase the dose of PPIs may help to achieve complete acid suppression, and eliminate the residual acid reflux in patients with refractory GERC. Doubling the original dose of PPIs is a common selection. When the dose of lansoprazole is increased from 30 mg daily to 60 mg daily, adequate symptom control is achieved in approximately 20%-30% of patients who are unresponsive to the original low dose lansoprazole for 6-8 wk[40,41]. Our study has demonstrated that the cough in 38.9% of patients with refractory GERC was controlled after the treatment with a doubled-dose omeprazole (40 mg twice daily)[12]. These patients had a more severe esophageal acid exposure, as indicated by a mean 85-point DeMeester score, suggesting the standard dose PPIs were not enough to obviously reduce the acidity of refluxate. After treatment with doubled-dose omeprazole, the significant increase in the pH value of refluxate may markedly attenuate the acid-induced stimulation to the esophageal receptors, inhibit the esophageal-tracheobronchial reflex and finally resolve the cough[12].

ADD-ON THERAPY WITH HISTAMINE H_2 RECEPTOR ANTAGONISTS

Seventy-five percent of patients with refractory gastroesophageal reflux disease still present with abnormal nocturnal gastric acid secretion even after the treatment with PPIs twice daily[42]. The addition of a histamine H_2 receptor antagonist at bedtime may help to achieve complete acid suppression in these patients. Retrospective studies have shown that the combination of PPIs with histamine H_2 receptor antagonists may improve the overall symptoms in 72% of patients with refractory gastroesophageal reflux disease[43]. Since long-term use of histamine H_2 receptor antagonists may develop tachyphlaxis and decrease its therapeutic efficacy, its intermittent or on-demand use at bedtime is advocated. Our results showed the addition of ranitidine 150 mg twice daily attenuated the cough symptoms in 25% of patients with refractory GERC who were unresponsive to the therapy with high dose PPIs[12]. This combined therapy is not to eliminate the night-time reflux but to completely inhibit the day-time gastric secretion by acting on multiple targets in the parietal cells of the stomach. Surprisingly, refractory GERC responsive to the add-on therapy with ranitidine had lower severity of acid reflux (mean DeMeester score of only 36.3) than that responsive to treatment with doubled dose PPIs, suggesting that the esophageal hypersensitivity to acid is a major cause in these patients[12].

ADD-ON THERAPY WITH TLESRS INHIBITORS

Baclofen is a selective gamma-aminobutyric acid (GABA) B receptor agonist primarily used for the treatment of spasticity. It has been demonstrated baclofen can reduce the frequency of TLESRs, decrease the reflux episodes[44-46], and relieve the acid reflux related symptoms by 72% and non-acid reflux related symptoms by 21%[47]. In addition, baclofen has non-specific antitussive activity and has been used for the treatment of refractory chronic cough of unknown causes[48]. As an add-on therapy to PPIs, baclofen may significantly improve the cough symptoms and decrease the cough sensitivity to inhaled capsaicin in 56.3% of patients with refractory GERC[7]. Therefore, baclofen may be useful for treatment of refractory GERC unresponsive to other anti-reflux therapies.

However, baclofen can decrease but not completely abolish TLESRs. It has been documented that baclofen only reduces the frequency of TLESRs by 40%-60% and decrease the reflux episodes by 43%[44-46]. Therefore, the residual reflux may continue to produce cough through stimulating the receptors in the distal esophageal mucosa. This explains why baclofen is not always effective to relieve the refractory GERC. To develop more potent TLESRs inhibitors with few side effects may be a future direction. Furthermore, some refluxes may be secondary to the decreased pressure difference between stomach and esophagus because of the lower baseline pressure of lower esophageal sphincter, and thus be unrelated to TLESRs. Therefore, baclofen is not effective for these refluxes.

The main side effects of baclofen are related to the central nervous system since it can permeate the blood-brain barrier. This limits its clinical application. Nevertheless, the drug-related somnolence, drowsiness and fatigue are usually tolerable and may disappear within 3 wk in most patients. Only a few patients have to stop the baclofen treatment due to severe dizziness and drowsiness[7]. A gradual increase in the dose of baclofen from 5 mg to 20 mg per time may help to improve the patients' tolerance and avoid the severe adverse effects.

ADD-ON THERAPY WITH GABAPENTIN

Gabapentin is a lipophilic structural analogue of GABA, an important central neurotransmitter, and may prevent the synaptic release of neurotransmitters by binding selectively to the $Ca_{v}\alpha_{2}\beta$ subunit of the voltage gated calcium channels. It is primarily used to treat chronic neuropathic pain. Since patients with chronic cough have a similar central sensitization to those with chronic neuropathic pain, the possible inhibition of hypersensitized cough center with gabapentin may be a new therapy for the refractory chronic cough. Ryan *et al*[49] have demonstrated that gabapentin can improve the cough symptoms and cough-specific quality of life in the patients with refractory chronic cough with 8-wk treatment with gabapentin starting at 300 mg daily and titrating up to 1800 mg daily. Madanick *et al*[50] have reported that, after the add-on therapy with gabapentin, approximately 75% of GERC patients experienced at least 50% subjective improvement in cough, irrespective of findings from the esophageal pH monitoring. The dose of gabapentin they used was 300 mg daily in most patients and 900 mg daily or more in a few patients, obviously lower than that reported by Ryan *et al*[49]. At present, it remains unclear whether the cough attenuation after gabapentin therapy is associated with the inhibition of reflux. Further studies are needed to clarify this issue.

ANTI-REFLUX SURGERY

Anti-reflux surgery can treat GERC by artificially reestablishing the mechanical barrier between esophagus and stomach to block both acid and non-acid reflux. Currently, the most commonly used anti-reflux surgery is laparoscopic fundoplication, which can improve more in cough symptom and PPI elimination[51]. In contrast, the radiofrequency augmentation, silicone injection and endoscopic suturing of the lower esophageal sphincter as well as transoral incisionless fundoplication are not recommended due to the absence of evidence supporting their long-term efficacy[32]. The reported successful rate of laparoscopic fundoplication for refractory GERC was about 85%[52]. However, the efficacy of anti-reflux surgery reduces over time. The rate of cough resolution decreases post-operationally from 83% at 6 mo to 74% at 2 years, and 71% within 5 years[53]. Because of its invasiveness and uncertain efficacy, anti-reflux surgery is not a first-line treatment and not extensively used in clinical practice.

In conclusion, refractory GERC is a disorder difficult to manage. Its underlying mechanisms may be associated with incomplete acid suppression, non-acid reflux, TLESRs and esophageal hypersensitivity. MII-pH is a major laboratory examination and can establish the temporal association between reflux and cough, which, however, need to be confirmed by the subsequent intensified anti-reflux therapy. Refractory GERC can be treated pharmacologically and non-pharmacologically. The optimization of PPIs and add-on therapy with histamine H_2 receptor antagonists, TLESRs inhibitors baclofen and gabapentin are the selective pharmacological therapies for refractory GERC. However, the further study is needed to satisfy its management.

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Early probiotics to prevent childhood metabolic syndrome: A systematic review

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Data sharing statement: Technical appendix, statistical code, and dataset available from the corresponding author Prof. Sanjay Patole at olof.skoldenberg@ki.se.

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Abstract

AIM: To conduct a systematic review of studies on early probiotic supplementation to prevent childhood metabolic syndrome (MS).

METHODS: Using the Cochrane systematic review strategy we searched PubMed, EMBASE, CENTRAL, CINAHL, and the conference proceedings of the Pediatric American Society meetings and trial registries in December 2014. Randomised controlled trials (RCTs) and non RCTs of probiotic supplementation to the mother and/or infant for a minimum duration of 4 wk were selected. Of these, studies that reported on MS or its components (obesity, raised blood pressure, hyperglycemia, dyslipidemia) in children between 2-19 years were to be eligible for inclusion in the review. Risk of bias (ROB) in selected RCTs and quality assessment of non-RCT studies were to be assessed by the Cochrane ROB assessment table and New Castle Ottawa scale.

RESULTS: There were no studies on early probiotic administration for prevention of childhood MS (CMS). Follow up studies of two placebo controlled RCTs (*n* = 233) reported on the effects of early probiotics on one or more components of MS in children aged 2-19 years. Meta-analysis of those two studies could not be performed due to differences in the patient population, type of outcomes studied and the timing of their assessment. Assessment of childhood metabolic outcomes was not the primary objective of these studies. The first study that assessed the effects of prenatal and postnatal supplementation of *Lactobacillus rhamnosus GG* on body mass index till 10 years, did not report a significant benefit. In the second study, *Lactobacillus paracasei* 19 was supplemented to healthy term infants from 4-13 mo. No significant effect on body mass index, body composition or metabolic markers was detected.

CONCLUSION: Current evidence on early probiotic

administration to prevent CMS is inadequate. Gaps in knowledge need to be addressed before large RCTs can be planned.

Key words: Metabolic syndrome; Obesity; Probiotic; Infant; Perinatal; Children

Core tip: Metabolic syndrome (MS) is a state of dysregulated glucose and lipid metabolism. The global health burden due to increasing prevalence of MS in children and adolescents, warrants urgent preventive interventions. Altered gut microbiota has been implicated in the pathogenesis of MS. The role of maternal and/or infant probiotic supplementation in improving metabolic health of the offspring is being researched. We provide a systematic review of this evidence. Gaps in the knowledge and issues regarding selection of patient population, probiotic intervention and outcomes for future trials have been discussed.

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INTRODUCTION

Metabolic syndrome (MS) is defined to include an array of risk factors that increase the chances of Cardiovascular morbidity and type 2 diabetes in an individual. The World Health Organisation (WHO) in 1998, defined MS to comprise of insulin resistance in the presence of any two of the risk factors - obesity, hypertension, high triglyceride level, reduced high-density lipoprotein cholesterol level, or micro albuminuria[1]. Since then, various definitions for MS have been proposed. MS is increasingly being recognised in children. The diagnostic criteria for MS have been adapted for children and adolescents by the WHO, National Cholesterol Education Program, International Diabetes Federation and I Guidelines for prevention of atherosclerosis in childhood and adolescence[1-4]. There are more than ten different clinical definitions for childhood MS (CMS)[5]. Additionally, there are no unifying criteria that are representative of ethnically diverse groups. The age of onset of CMS is also unclear. A systematic review of 36 epidemiological studies analysing the prevalence of MS in children aged 2-19 years, reported a prevalence ranging from 1.2%-22.6% with rates up to 60% in overweight and obese children[6]. A recent systematic review has reported a mean overall prevalence of CMS as 3.3% (range: 0%-19.2%)[7]. The prevalence was higher in overweight [11.9% (2.8%-29.3%)] and obese [29.2% (10%-66%)] children. It was also higher among males and older children. Higher prevalence has been reported in the Middle East and the United States compared to Europe and the Far East. The variations in the definition of MS, ethnicity, age and nutritional status of the study population may explain the wide range of prevalence reported in these studies. To our knowledge, there is no data available on the health burden of CMS. Follow up of the Framingham Heart study cohort has revealed that the combination of central obesity, hypertension and hyperglycemia led to 2.36 times increase in the incidence of cardiovascular events and three-fold increase in mortality among adults[8]. MS results in a seven fold increase in the risk of type 2 diabetes[9].

Obesity is considered the most important component of CMS. Data from the National Centre of health Statistics, United States reveal that prevalence of obesity has doubled in children and quadrupled in adolescents in the past 30 years[10]. Increased prevalence of CMS is a direct result of the increasing trends of childhood obesity. Cost of illness studies from the United States, Australia, Germany have confirmed that health care utilisation by children with obesity, is significantly higher than their normal weight counterparts[11]. The annual medical expenditure due to childhood obesity in the United States is approximately 14 billion United States dollar (USD) and the projected costs for the next 30 years due to currently prevailing trends of adolescent obesity would be 45 billion USD[12,13]. Thus CMS has the potential to be a major public health concern to both the developed and developing countries[14].

Pathogenesis of MS

Pathogenesis of MS is complex and involves insulin resistance, lipid partitioning, hepatic steatosis, free radical injury and hormonal changes (leptin, adiponectin, resistin)[15-17].

Role of gut microbiota

Numerous reviews have indicated the role of altered gut microbiota in the pathogenesis of MS[18-20]. Gut microbiota (*e.g., bacteroides*) can mediate energy harvest from diet resulting in obesity and type 2 diabetes[21]. Increased level of inflammatory markers (Lipopolysaccharides, Transforming growth factor-β) by gram negative bacteria in the gut can increase gut permeability and oxidant injury and thereby affect the metabolic health[22]. The firmicutes: bacteroides ratio in the gut flora was significantly reduced in children with type 1 diabetes as compared to healthy children[23]. Obesity and excessive weight gain during pregnancy was associated with aberrations in the maternal gut microbiota[24]. Collado *et al*[25] have reported lower stool bifidobacterial counts and reduced microbial diversity in infants born to obese mothers. Follow up of that cohort revealed an increased risk of obesity at seven years of age[26]. It was suggested that early infancy gut microbial alteration could influence metabolic health of children

and adolescents. Gut microbiota is also more amenable to modulation, prior to the establishment of adult type microbiota; *i.e.,* in the first two years of life[27]. Hence, it could be hypothesised that interventions modulating gut microbiota in early infancy can potentially reduce the risk of CMS.

Role of probiotics in prevention of MS

Probiotics are "live micro-organisms which when administered in adequate amounts confer a specific health benefit on the host". Probiotics have been shown to decrease body weight gain, adipose tissue mass, leptin and cholesterol levels. Diet induced hyperglycemia and hyperinsulinemia was controlled by probiotic supplementation. However, majority of the clinical evidence is from adult and animal studies[28-30]. The potential of probiotics in improving metabolic outcomes in children has been studied by maternal and/or early infant supplementation. The pathways for the potential benefits are direct modulation of the infant gut flora through breast milk or placenta and regulation of risk factors such as maternal hyperglycemia and obesity[31-34].

Given the significance of the health issue and the potential of probiotics as an intervention, we aimed to conduct a systematic review of studies reporting on probiotic supplementation to prevent CMS.

MATERIALS AND METHODS

Study selection criteria

The study selection criteria is described as follows: (1) Studies: Randomized controlled trials (RCTs) and non-RCT studies; (2) Participants: Pregnant women and/or infants that received probiotic supplementation for at least 4 wk; (3) Intervention*:* Probiotic supplement of any strain, dose and form with or without prebiotic oligosaccharide for a duration of at least 4 wk; (4) Control*:* Standard treatment but no probiotics or placebo; and (5*)* Outcome measures*:* We broadly defined our outcome measures to account for variations in age, gender and ethnicity based cut offs for individual risk factors of CMS. To be included in this review, the studies should have assessed at least one of the following four components of MS - obesity, raised blood pressure, dyslipidemia (hypertriglyceridemia or low HDL cholesterol), hyperglycemia in children between 2-19 years. The outcome equivalents for each of the components are described as follows: (1) Obesity: body mass index, waist circumference; (2) Hyperglycemia: Fasting plasma glucose, insulin levels, insulin resistance (assessed by homeostatic model assessment for insulin resistance), insulin sensitivity, fasting plasma glucose; (3) Dyslipidemia: Plasma lipid profile (total cholesterol, LDL cholesterol, HDL cholesterol, triglycerides, total cholesterol/HDL); and (4) Hypertension: Systolic blood pressure and /or diastolic blood pressure.

We followed the standard Cochrane methodology and the preferred reporting items for systematic reviews and meta-analysis, for conducting and reporting RCTs in this systematic review[35]. We followed the Meta-analysis of observational studies in epidemiology guidelines for conducting and reporting outcomes of non-RCTs in this systematic review[36].

Literature search

We searched the Cochrane central register of controlled trials (CENTRAL) (http://www.cochrane.org/cochrane/hbook.htm), MEDLINE *via* PubMed (http://www.ncbi.nlm.nih.gov/Pubmed), EMBASE (http://www.embase.com) and annual conference proceedings of the Pediatric Academic Societies (www.pas-meeting.org)/ in December 2014. PubMed was searched using two search strategies: (1) *S*tudies of probiotics related to the components of MS using the Medical Subject Heading keywords "insulin resistance" or " insulin sensitivity" or "hyperglycemia" or "type 2 diabetes mellitus" or "obesity" or " overweight" or "adipose tissue" or "dyslipidemia" or " body composition" or "bodyweights and measures" or "hypertension" or "blood pressure" and "probiotics"; and (2) Studies of probiotic supplementation in pregnant women and infants using the MeSH keywords "infant" or "infant, newborn" or "pregnancy" and "probiotics".

For both searches, the MeSH word "probiotics" was replaced by lactobacillus, bifidobacterium and saccharomyces and citations were retrieved. We combined both the search strategies to retrieve studies of probiotics in pregnant women and infants that assessed one or more components of MS as defined for this review. The results of the database search are shown in a flow diagram (Figure 1).

No restrictions were applied on study design or language. Animal studies and studies involving patients > 19 years were excluded. References of the obtained studies were also reviewed to identify additional studies. The international trial registry (www.clinicaltrials.gov) and Australian Clinical Trials Registry (www.anzctr.org.au)were checked for ongoing/registered trials in this area.

Data collection and analysis

Selection of studies: Balasubramanian H and Patole S independently assessed for inclusion all the potential studies identified as a result of the search strategy. Any disagreements about study selection were resolved by discussion.

Data extraction and management: Both the authors independently completed a pre specified data extraction form for all included studies. Any disagreements in the extracted data were resolved through discussion.

Assessment of risk of bias in included studies: Risk of bias (ROB) in selected RCTs and quality assessment of non-RCT studies were assessed by the Cochrane ROB assessment table and the New Castle Ottawa scale[37,38]. Both the authors separately assessed each

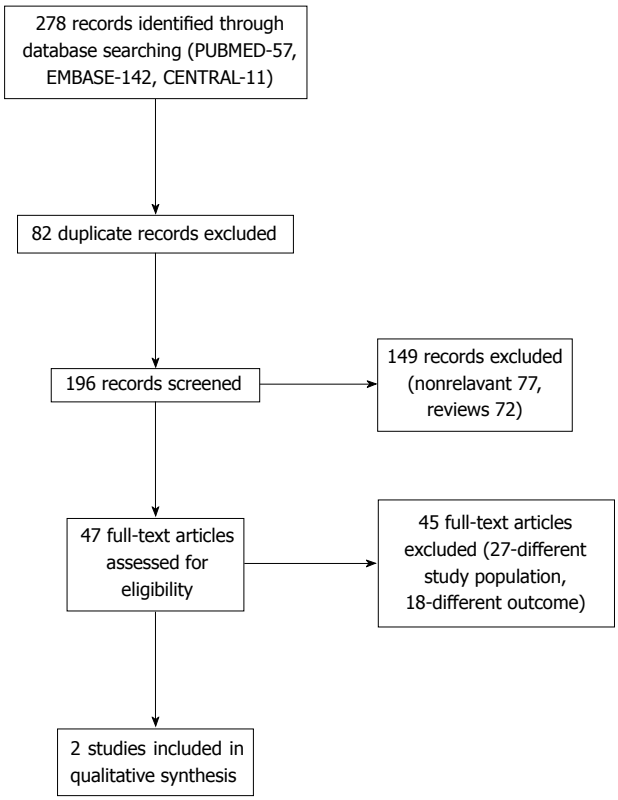

Figure 1 Flow diagram of study selection process.

study. Additional information from the trial authors was requested to clarify methodology as necessary. Any disagreement was resolved by discussion.

RESULTS

Initial broad search yielded 278 citations. We could not find any study on the effects of probiotic administration on CMS. However, we retrieved two RCTs (n = 233) reporting on the effects of early probiotics on one or more components of MS in children aged 2-19 years[39,40] (Figure 1). Meta-analysis of these 2 studies could not be performed due to differences in the patient population, type of outcomes and the timing of their assessment. Hence we decided to conduct a narrative synthesis.

Luoto 2010

This was a follow up study of a double blinded RCT involving 159 mothers with family history of atopic eczema, allergic rhinitis or asthma. They were randomized to receive probiotics (n = 77) or placebo (n = 82). The intervention group received 1 x 10^{10} cfu/d of *Lactobacillus rhamnosus GG* for 4 wk before expected delivery and extending for 6 mo postnatally to the mother/infant.

Frequency of atopic eczema in their children till 2 years of age was the primary end point of the study. The BMI and frequency of overweight and obesity was assessed in 113 children (Probiotic: 59, Placebo: 54) at 2, 4, 7, and 10 years of age. Obesity and overweight was assessed in both groups using the international obesity task force criteria. There was no significant difference in the adjusted mean BMI at any age between the 2 groups. Among the children that were overweight at 10 years, (Probiotic: 13, Placebo: 12), there was tendency towards lower mean BMI at 4 years in the probiotic group (P = 0.063, Analysis of variance for repeated measures).

Videhult 2014

This was the follow up study of a RCT involving 179 vaginally delivered term infants with birth weight > 2500 g. These infants were fed cereals with or without probiotic (*Lactobacillus paracasei ssp F19* - 1 x 10^8 CFU) between 4-13 mo. The outcomes of interest were the number of days with infections and antibiotic prescriptions before and after the second and third doses (5.5 and 12 mo) of DTaP vaccine. A total of 120/179 children were assessed at 8-9 years for the following outcomes-BMI Z score, sagittal abdominal diameter, body composition (fat free mass, fat mass index, truncal fat %, android or gynoid fat %), plasma lipids, insulin, glucose and transaminases. No significant differences in body composition, growth and metabolic markers were noted in the two groups at 8-9 years of age.

Results of the ROB assessment are reported in Table 1.

DISCUSSION

Our systematic review identified two RCTs (n = 233) studying the effects of early probiotic supplementation on metabolic health in children. Meta-analysis of these 2 studies could not be performed due to differences in the patient population, type of outcomes studied and the timing of their assessment. The current evidence on the administration of probiotics to the mother or infant to prevent CMS is thus inadequate.

To our knowledge this is the first systematic review assessing the role of early probiotic supplementation in the prevention of CMS. Small number and sample size of the included studies was the main limitation of this systematic review. Included studies were not designed to study metabolic outcomes in children and had follow up losses of up to 30%. Considering the global burden of CMS and the metabolic benefits of probiotics in adults and animal models it is important to assess this intervention in large RCTs. Few issues need to be discussed with regards to the patient population, probiotic intervention and outcome assessment in such trials.

Selection of the infant population for such trials is crucial as the current evidence on benefits of probiotics with regards to CMS related outcomes is based on healthy term infants. Preterm infants and those with intrauterine growth restriction are at high risk for MS due to catch up growth and reduction in insulin sensi-

Table 1 Assessment of risk of bias in the included studies

Study ID	Random sequence generation	Allocation concealment	Blinding of participants and personnel	Blinding of outcome assessment	Incomplete outcome data	Selective reporting	Other bias
Luoto *et al*[40]	Low	Low	Low	Low	High	Low	Low
Videhult *et al*[39]	Low	Low	Low	Low	High	Low	Low

tivity[41,42]. Infants of diabetic mothers are also at higher risk of MS[43]. Factors that put these infants at high risk of early infancy gut microbial aberrations include increased risk of caesarean delivery, prolonged hospital stay, decreased maternal contact, perinatal and/or postnatal antibiotic exposure, delayed enteral feeding, need for tube feeds, formula feeding and suboptimal nutrition[44]. Hence research should focus on these high risk infant groups, and maternal population, especially obese and diabetic mothers. Comprehensive assessment of gut flora and immunological profile would also be essential as they relate to the mechanisms/pathways of benefit of probiotic supplementation. Considering that the effects of probiotics are strain specific and host specific, selection of probiotics is an important issue. A comparative meta-analysis by Million *et al*[45] has shown that Lactobacillus acidophilus administration resulted in significant weight gain in humans and in animals and Lactobacillus gasseri was associated with weight loss both in obese humans and in animals. The same authors have also reported that obesity-associated gut microbiota is rich in Lactobacillus reuteri and depleted in Bifidobacterium animalis and Methanobrevibacter smithii[46]. Assessment of the effects of probiotics on body composition is helpful considering the nutritional benefits of probiotics[47,48]. Assessment of optimal timing and duration of intervention are also important issues in the RCTs of early probiotic supplementation for preventing CMS. Rinne *et al*[49] have demonstrated that probiotic administration during the last 6 mo of pregnancy and first 6 mo postpartum did not influence long term (2 years) composition of the infant gut flora. Perinatal metabolic programming and immune mediated effects on the infant gut flora by the administration of probiotic could explain the pathway of benefit[50]. Controlling for confounders (*e.g.*, dietary and lifestyle changes), assuring compliance during the prolonged period of supplementation, and monitoring for complications will be necessary[51-53]. Currently there are no universally accepted criteria for defining CMS or its components[54]. Selection of primary outcomes representative of some or all components of MS would be essential. Since the minimum age cut off described for CMS is unclear, standardisation of surrogate end points will be essential. Currently there are no studies showing a causal relation between MS and gut microbiota. Moreover, the risk factors for MS differ with age. Prematurity, low and high birth weight, rapid catch up growth, maternal undernutrition, maternal obesity and diabetes are potential risk factors for the components of CMS. This highlights the necessity to test early interventions (perinatal, early postnatal) for preventing CMS.

In summary, current evidence is insufficient to assess the effects of probiotics in reducing the risk of MS in children and adolescents. Considering the global health burden of CMS and the potential role of a low cost intervention such as probiotic supplementation, clinical and epidemiological studies are urgently required in this field. Better understanding of the pathogenesis and population specific cut offs of the various components of CMS is required before high quality randomised trials can be undertaken to address this important issue.

COMMENTS

Background

Metabolic syndrome (MS) in children and adolescents is defined to include central obesity, hyperglycemia, high blood pressure and dyslipidemia. Probiotics have shown to reduce adipose tissue, glucose and triglyceride levels in animal models, but evidence in children and adolescents is insufficient.

Research frontiers

Observational studies have shown that altered gut flora in infancy is associated with obesity in childhood. Altered gut flora has also been noted in children with type 1 diabetes. Whether modulation of gut flora in early infancy by probiotic supplementation would decrease the risk of childhood obesity and glucose intolerance is unclear.

Innovations and breakthroughs

Evidence from a clinical trial suggests that perinatal probiotic interventions may decrease the risk of gestational diabetes and central obesity. A prospective study of perinatal probiotic supplementation in early infancy has shown to reduce the risk of excessive weight gain in obese children. However, data on childhood metabolic outcomes is limited. Currently there is insufficient evidence to support the role of early probiotics in childhood MS (CMS).

Applications

To date, there is no systematic review on early interventions to prevent CMS. Given the magnitude of the problem, they analysed the potential role of probiotic exposure in early life for prevention of CMS or its components. The cause - effect relationship of altered gut flora *vs* CMS needs to be studied. High quality RCTs analysing all components of CMS are required.

Peer-review

Definitely this is an interesting topic and is properly argued by the authors. Unfortunately they could not present a meta-analysis because of the data available in the literature. However, it is important to publish this work to emphasize the urgent need for this kind of research.

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SYSTEMATIC REVIEWS

Prevalence of antibiotic resistance in *Helicobacter pylori*: A recent literature review

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Author contributions: Ghotaslou R conceptualized, designed the review and carried out the analysis; Ebrahimzadeh Leylabadlo H and Mohammadzadeh Asl Y contributed equally to the work; all authors reviewed and approved the final manuscript as submitted.

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Abstract

AIM: To review previous studies (the last 6 years) about the *Helicobacter pylori* (*H. pylori*) antibiotic resistance in order to evaluate the trend in antibiotic resistance.

METHODS: In this study, the PubMed, MEDLINE, Science Direct, Google Scholar and Scielo manuscripts were reviewed from 2009 to 2014.

RESULTS: On the whole rates of *H. pylori* antibiotic resistance were 47.22% (30.5%-75.02%) for metronidazole, 19.74% (5.46%-30.8%) for clarithromycin, 18.94% (14.19%-25.28%) for levofloxacin, and 14.67% (2%-40.87%) for amoxicillin, 11.70% (0%-50%) for tetracycline, 11.5% (0%-23%) for furazolidon and 6.75% (1%-12.45%) for rifabutin. The frequency of tetracycline, metronidazole and amoxicillin resistance was higher in Africa, while clarithromycin and levofloxacin resistance was higher in North America and Asian, respectively.

CONCLUSION: The most sensitive drug is rifabutin and the lowest sensitive drug is metronidazole in the world. The worldwide *H. pylori* antibiotic resistance to clarithromycin and levofloxacin has increased during the last 6 years. The present systematic review show alarming results and a novel plan is needed for eradication therapy of *H. pylori* infections.

Key words: Antibiotic resistance; *Helicobacter pylori*; Worldwide

Core tip: Because of the rising frequency of antimicrobial resistance, management of *Helicobacter pylori* (*H. pylori*) infections is a challenge for physicians. We found global frequency rate of resistance is high in Africa. The

most sensitive drug is rifabutin and the lowest sensitive drug is metronidazole in the world. The worldwide *H. pylori* antibiotic resistance to clarithromycin and levofloxacin has increased during the last 6 years. Resistances to antimicrobial agent's reports describe dramatic decrease of antibiotics efficacy.

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INTRODUCTION

Helicobacter pylori (H. pylori) is a motile, curved and Gram negative bacillus[1]. *H. pylori* certainly is the most prevalent human infection, the frequency of infection due to *H. pylori* is nearly 50% in the world and in developing country is as high as 80%-90%[2]. This bacterium colonizes the stomach of human and its infection is correlated with gastritis, peptic ulcer disease and extra-digestive diseases[3,4]. *H. pylori* is also considered as a human carcinogen[5]. Since, *H. pylori* eradication therapy represents a key clinical essential. Unfortunately, therapy against *H. pylori* has turned out to be more difficult over the years, principally due to the great decrease of standard eradication therapies efficacy.

Although *H. pylori* is sensitive to many antibiotics *in vitro*, just a few antibiotics can be used *in vivo* to treat infected patients. Management of *H. pylori* infections are recommended in all suggestive individuals[6]. According to the latest Maastricht Guidelines, in regions of low clarithromycin resistance, clarithromycin-containing treatments are recommended for first-line empirical treatment[7]. In regions of high resistance to clarithromycin, the quadruple treatment including bismuth has been proposed as first-line treatment. In case of unavailability of this therapy, non-bismuth (three antibiotics plus Proton pump inhibitors) quadruple therapy and the so-called "sequential therapy" (that includes five days of PPIs plus amoxicillin followed by five more days of PPIs plus metronidazole and clarithromycin) have been recommended as an alternative[7]. Table 1 is shown mode of actions and resistance mechanisms of antibiotics used for treatment of *H. pylori* infection.

Failure of treatment in *H. pylori* infections has become an actual subject for physicians. The cause of treatment failure is many that can be grouped into microorganism-related factors, host-related factors and treatment-related factors. *H. pylori* resistance to antibiotic is widely recognized as the chief reason for treatment failure[1,8]. Furthermore, antibiotic resistance should be considered as a lively idea, since its prevalence can change not only among diverse countries, but also between two different periods in the same area[1,9-11]. The rate of antibiotic resistance in *H. pylori* has been evaluated worldwide. However, most researches originated from single center, included only a small number of bacteria, were often restricted to selected patients, and used different techniques to evaluate antibiotic susceptibility. Though, the investigation platform is luxurious; and only performed in few countries as: United Kingdom, German, Finland[12-18]. Antibiotic use for infections other than *H. pylori* is accounting for the extensive raise antibiotic resistance rate in *H. pylori*[19]. Because of the value of *H. pylori* therapy, antimicrobial susceptibility testing has been widely done. Since, *H. pylori* antibiotic resistance is fast growing worldwide, an eradication policy based on pre-treatment susceptibility testing is going to get more attractive than in the past[1,7].

The objective of this paper was to review previous studies about the rates of antimicrobial resistance in *H. pylori* isolates obtained from worldwide during last 6 years in order to evaluate the trend of antibiotic resistance.

MATERIALS AND METHODS

In the present study, different computer-assisted searches were achieved using PubMed, MEDLINE, Science Direct, Google Scholar and Scielo. Separately searches were carried out on all English language literatures published through 2009 to 2014, by the key words: *Helicobacter pylori*, *H. pylori,* resistance, metronidazole, levofloxacin, amoxicillin, clarithromycin, tetracycline, and rifabutin. Full articles related searches were saved, and articles written in foreign languages were translated when essential. When more than one publication from the same author was obtainable, only new version, counting the whole population was enrolled. Two investigators (Ebrahimzadeh Leylabadlo H and Mohammadzadeh Asl Y) independently and in a blinded manner assessed the articles using pre-designed data extraction.

The following information was collected: (1) sum of bacteria incorporated; (2) rate of antibiotic resistant; and (3) the geographic area involved. The data were summarized in extraction table and analyzed manually. Finally, Excel 2007 software was used to draw charts.

RESULTS

During 6 years a total of 52008 *H. pylori* isolates meeting the inclusion criteria were identified. Eighty-seven studies from 2009 to 2014 on *H. pylori* antimicrobial resistance in the different countries were included; there were 43 Asian[20-62], 10 American[63-72], 5 African[73-77], and 29 European studies[78-106]. On the whole rates of *H. pylori* antibiotic resistance were 47.22% (30.5%-75.02%) for metronidazole, 19.74% (5.46%-30.8%) for clarithromycin, 18.94% (14.19%-25.28%) for levofloxacin, and 14.67% (2%-40.87%) for amoxicillin, 11.70% (0%-50%) for tetracycline, 11.5% (0%-23%) for

Table 1 Mode of action, resistance mechanisms of antimicrobial agents used for treatment of *Helicobacter pylori* infection

Antibiotic	Mode of action	Resistance mechanisms
Metronidazole	Electron reduction processes, leads to the formation of nitro-anion radicals and subsequent DNA damage	(1) Poor drug uptake and/or increased drug efflux; (2) enhanced activity of DNA repair enzymes; (3) increased oxygen scavenging abilities; and (4) decreased antibiotic activation arising from changes in metronidazole-reducing enzymes[16]
Clarithromycin	The inhibition of protein synthesis by binding and slowing down the activity of the bacterial ribosomal unit[17]	rRNA-point mutations
Amoxicillin	The inhibition cell wall synthesis	*pbp* gene mutations, membrane permeability alterations and efflux pumps[17]
Tetracycline	Reversible inhibition protein synthesis	Three contiguous nucleotides mutation in the 16S rRNA gene[17]
Fluoroquinolones	Inhibiting DNA gyrase, type Ⅱ topoisomerase, and topoisomerase Ⅳ[17]	Point mutations in the quinolones resistance determining regions
Rifabutin	Inhibits the b-subunit of *H. pylori* DNA-dependent RNA polymerase encoded by the *rpo*B gene[18]	Mutation of the *rpo*B gene[18]

H. pylori: *Helicobacter pylori*.

Table 2 Antibiotic resistance rates in different continental areas

Region (*n*)	Cla %	Amo %	Met %	Tet %	Lev %	Rif %	Fur %
Asia (23748)	27.46	23.61	46.57	7.38	25.28	12.45	23
South America (587)	12.88	6.56	52.85	0	21.23	NR	0
North America (818)	30.8	2	30.5	0	19	NR	NR
Europe (26024)	22.11	0.35	31.19	1.15	14.19	1	NR
Africa (831)	5.46	40.87	75.02	50	15	NR	NR
Total (52008)	19.74	14.67	47.22	11.70	18.94	6.75	11.5

Amo: Amoxicillin; Cla: Clarithromycin; Met: Metronidazole; Tet: Tetracycline; Lev: Levofloxacin; Rif: Rifabutin; Fur: Furazolidon; *n*: Number; NR: Not reported.

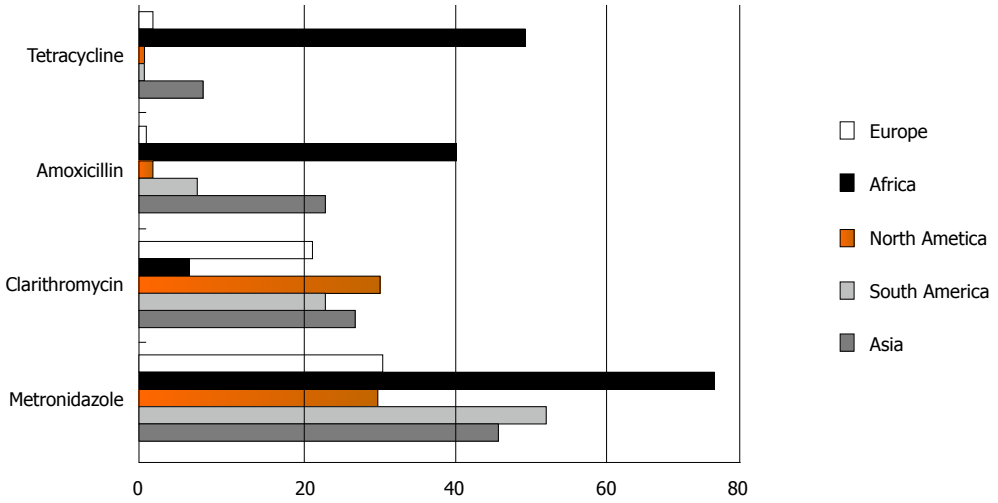

Figure 1 Antibiotic resistance rates to 4 most common used antibiotics in different continental areas.

furazolidon and 6.75% (1%-12.45%) for rifabutin. The frequency of resistance to antibiotics in various continents and countries are demonstrated in Tables 2 and 3, Figures 1 and 2.

DISCUSSION

Monitoring of resistance to antimicrobial agents is important for *H. pylori* infections therapy in medical practice[17]. Resistance to antimicrobial agents creates at risk *H. pylori* eradication in the world[10,98]. The most recent recommendations on *H. pylori* therapy suggested that initially management had better be personalized based on clarithromycin and metronidazole resistance. In fact, fourteen days triple-therapy is recommended in area where resistance to clarithromycin is more than 15% to 20%, if resistance to metronidazole is more than 40%, the association with amoxicillin is

Table 3 Quantitative data of the articles

Countries	Year	Isolates (*N*)	Cla (%)	Amo (%)	Met (%)	Tet (%)	Lev (%)	Rif (%)	Fur (%)	Method	Ref.
	2014	95	33.7							E-Test	[20]
	2013	82	17.1	9.8	64.4	0				DDM	[21]
	2013	78	15.3	6.4	55.1					DDM	[22]
Iran	2012	150	34	10	78.6	9.3	5.3			E-T,ADM	[23]
	2012	112	14.3	28.6	76.8	18.7		28.6		DDM	[24]
	2011	197	45.2	23.9	65.5	37.1			61.4	DDM	[25]
	2011	42	14.3	2.4	40.5	4.8				ADM	[26]
	2010	121	5	20	44	3				E-Test	[27]
	2010	132	30	6.8	73.4	9				E-Test	[28]
	2014	73	80.8	0	58.9		12.3			E-Test	[29]
	2013	17731	21.5	0.1	95.4		20.6		0.1	ADM	[30]
China	2011	73	84.9	0	61.6	0	13.7	6.8		PCR	[31]
	2010	374	37.2	0.3	63.9	1.2	50.3			E-Test	[32]
	2009	36	8.3	33.3	94.4		0		16.7	DDM	[33]
	2014	124	36.2	0	2.1					E-Test	[34]
Japan	2014	135	25.9		20.7					E-Test	[35]
	2014	1073	31.1		40.2					ADM	[36]
	2013	204	86.4	8.2	71.3		57			ADM	[37]
	2011	153	55.6							PCR	[38]
	2010	61	36.1	0	14.8					ADM	[39]
	2014	212	8.5	9	36.3	0				ADM	[40]
South Korea	2013	165	11.5	2.45	50.7	0	24.55			ADM	[41]
	2013	150		6						ADM	[42]
	2012	185	10.8	2.2	30.3	0.05				ADM	[43]
	2014	161	1.2		36.6					E-Test	[44]
Malaysia	2014	102	6.8	0	32.3	0	6.8	0		E-Test	[45]
	2011	90	0	0	75.5		0	14.4		E-Test	[46]
	2011	187	2.1	0	37.4	0	1			E-Test	[47]
	2009	187	2.1							E-Test	[48]
	2014	46	47.8	54.3	73.9	4.3				E-Test	[49]
Pakistan	2012	178	36	37	89	12				ADM	[50]
	2010	92	33	2	48					E-Test	[51]
	2014	98	23.5	3.9	11.7					DDM	[52]
	2012	149	18.2	0	45.5		18.2			E-Test	[53]
Turkey	2012	61	21.3	0	42.6	9.1	3.3			DDM	[54]
	2009	31	41.9	3.2	41.9	3.2				E-Test	[55]
	2009	38	13.5							ADM	[56]
Taiwan	2014	61	35.3	0	17.6	0	23.5			E-Test	[57]
	2009	180	10.6	0	26.7		9.4			E-Test	[58]
Thailand	2009	120	29.2							PCR	[59]
UAE	2010	26	19.2							E-Test	[60]
India	2014	80	58.8	72.5	83.8	53.8	13.8		13.8	DDM	[61]
Vietnam	2013	103	33	0	69.9	5.8	18.4			E-Test	[62]
South American											
	2014	54	11.1	1.9						E-Test	[63]
Brazil	2013	77	19.5	10.4	40	0			0	ADM	[64]
	2011	39	8	0	51	0	23		0	ADM	[65]
Colombia	2012	203	19.8	20.5						ADM	[66]
Cuba	2010	40	10		85					E-Test PCR	[67]
Peru	2011	95					36.9			ADM DDM	[68]
Uruguay	2009	79	8.9	0	35.4	0	3.8			E-Test	[69]
North America											
Mexico	2011	90	5.5		19					E-Test	[70]
Canada	2009	42	57							E-Test	[71]
United States	2011	686	30	2	42	0	19			E-Test ADM	[72]
Senegal	2013	108	1	0	85	0	15			E-test DDM	[73]
Nigeria	2009	186		66	95	100				E-test	[74]
Gambia	2012	64	0		68.8					ADM	[75]
Tunisia	2010	273	15.4	0	51.3					E-test	[76]
South Africa	2010	200		97.5						ADM DDM	[77]
	2014	1651	6.7		29.4					E-test	[78]

Germany	2013	5296	67.1	0	67.1		24.9		E-test	[79]
	2013	436	7.5	0	32.7		11.7		E-test	[80]
Italy	2012	111	35.2		59.3		22.1		E-test	[81]
	2011	253	9.9						PCR	[82]
England	2009	255						1	E-test DDM	[83]
	2013	343	23.5		33				E-test	[84]
Spain	2011	71	14.7	1.4	45.1	0	14.5		E-test	[85]
	2010	118	35.6						E-test	[86]
	2009	101	54.6		35.7				E-test	[87]
Norway	2012	102	5.9	0	22.5	0			E-test	[88]
Finland	2010	505	8	0	41		7		E-test	[89]
	2013	588	20.1		34.5	2.6			ADM	[90]
Bulgaria	2011	519	17.9		29.5	4			ADM	[91]
	2009	1057	18.7	0.5	21.35	3.15			ADM	[92]
Croatia	2012	382	11.9	0.6	10.1				E-test	[93]
	2014	210					8.1		E-test	[94]
Poland	2013	165	10.9		32.7		1.2		E-test	[95]
	2012	51	22				16		E-test	[96]
	2011	115	34	0	44		5		E-test	[97]
Portugal	2014	180	50	0.6	34.4	0.6	33.9		E-test	[98]
	2011	1115	34.7	0	13.9	0			E-test	[99]
Belgium	2013	189	13.3	0.8	26.1					[100]
	2011	10670	20.3	0	27				ADM	[101]
Netherlands	2014	417	6.14		10.1				E-test	[102]
	2013	746	20.5	0.68	19.9				E-test	[103]
Ireland	2013	85				0	11.7	0	E-test	[104]
	2010	219	13.2		31.5				E-test	[105]
Southern Europe	2014	74	34.7		16.7				E-test	[106]

Amo: Amoxicillin; Cla: Clarithromycin; Met: Metronidazole; Tet: Tetracycline; Lev: Levofloxacin; Rif: Rifabutin; Fur: Furazolidon; DDM: Disk Diffusion Agar; ADM: Agar Dilution Agar.

preferred[17]. At the present, due to *H. pylori* antibiotics resistance, eradication therapy appears was not carried out as simple as and we are now founded many failures which make the use of standard therapy unacceptable in many parts of the world[107]. This article systematically studied the latest data on *H. pylori* resistance to antibiotic.

Clarithromycin resistance

Because clarithromycin is the most potent antibiotic involved in the management of *H. pylori* infections, resistance to clarithromycin is important[8,17,105]. As presented in Table 2, the rate of clarithromycin resistance was 19.74%, and occurrence of clarithromycin resistance is increasing worldwide (Figure 2). The rate of clarithromycin resistance has been broadly studied, and information are on hand from nearly all areas in the world: it ranges from 5.46% to 30.8% (Figure 1).

In European regions, the lowest clarithromycin resistance was reported from Norway (5.9%), whilst the highest in Spain (32.01%) and Portugal (42.35%). European studies performed at the past 6 years intervals reported that *H. pylori* resistance decrease from 36.65% in 2009 to 24.38% in 2014. In Asian regions, a surprising clarithromycin resistance frequency was reported from India (58.8%) and China (46.54%), whereas the lowest rate was discovered in Malaysia (2.4%). An increase in clarithromycin resistance has been faced in the Asia, from 15.28% in 2009 to 32.46% in 2014, probably in the Asian countries macrolid drugs used more. In recent years due to widespread use of clarithromycin for respiratory infections in the public especially in children, clarithromycin resistance has augmented in diverse regions, and there is an association between outpatient use of long-acting macrolide and clarithromycin resistance[10,17,108].

In conclusion, the highest clarithromycin resistant area was North America, and this study showed a slight increasing tendency of clarithromycin resistance of *H. pylori* in the world. Since clarithromycin is the most potent antimicrobial agent involved in the standard treatment protocol as well as the resistance rates were still at the low level, where clarithromycin-containing triple therapies could be used empirically.

Metronidazole resistance

Metronidazole is used against *H. pylori* infections and is one of the few antibacterial agents as drug of choice that is effective in eradicated the microorganism. Some researcher reported that the rate of treatment failure is more than 20% with triple therapy in which metronidazole is the drug of choice, also *H. pylori* resistance to metronidazole is the chief solitary reason responsible for management failure[109,110].

Metronidazole resistance is the most common antibiotic resistance in *H. pylori* and overall metronidazole resistance found in 47.22% in descending order in Africa 75.02%, South America 52.85%, Asia 46.57%, Europe 31.19%, to 30.5% in North America. In developed countries about 30% of the *H. pylori* strains

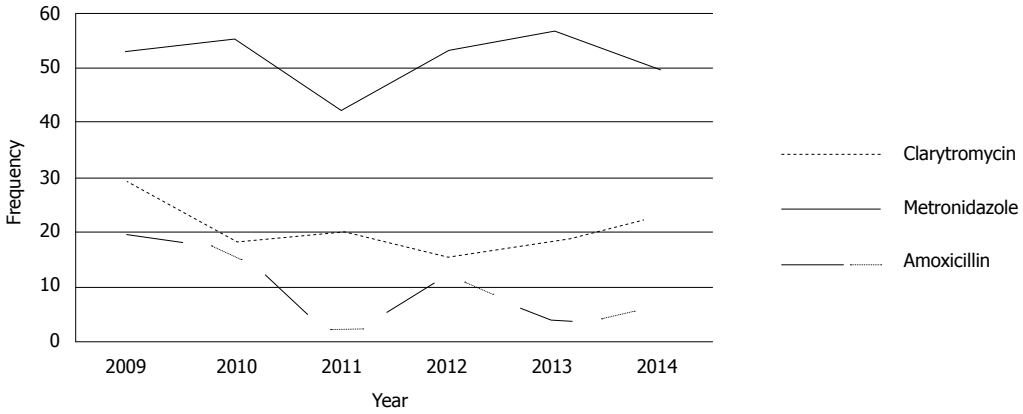

Figure 2 Trend of *Helicobacter pylori* resistance to metronidazole, clarithromycin, and amoxicillin during 6-years.

are metronidazole resistant, whereas in developing countries, the occurrence of resistance is very high. This association between metronidazole resistance and socioeconomic state level is maybe due to use of metronidazole and related drugs for gynecological, dental and parasitic related infectious diseases[13,111]. The comparison of results indicated that resistance to metronidazole have remained significantly unchanging in Asian, European and North American countries but is increasing in African countries (51.3% in 2010 to 85% in 2013). Furthermore metronidazole resistance in 2014 has stayed approximately at the similar level as in early 2009 in Europe. So, in accordance with latest guidelines, metronidazole is favored to amoxicillin in first-line therapy in Asian, Europe and North American but not in African patients.

Amoxicillin resistance

Amoxicillin is suggested for anti-*H. pylori* triple therapy in region where metronidazole resistance is high. Universal resistance to amoxicillin is uncommon; it was detected in 14.67%. The frequency of amoxicillin resistance extensively differs in Asian regions, ranging from zero in Malaysia, Taiwan and Vietnam to 72.5% in India. The rate of amoxicillin resistance in Africa was 40.87%.

The prevalence of amoxicillin resistance in Europe countries and North American is low from zero in certain area as Finland, Germany, Norway and Poland, 1.4% in Spain to 2% in United States. It seems the government policy possibly to limit the use of antibiotic for infectious diseases in European and North American countries. The incidence of amoxicillin resistance in *H. pylori* seems to increase specially in Asia and South America, where these antibiotics can be obtained without prescription. *H. pylori* resistance rates of 97.5%, 72.5%, 66% and 20.5% for amoxicillin have recently been reported in South Africa, India, Nigeria and Colombia, respectively.

Tetracycline resistance

Among the 4 most common used antimicrobial agents, tetracycline resistance was the lowest (Table 3). In general *H. pylori* resistance to tetracycline was detected 11.7% in the world. The total rate of tetracycline resistance did not vary in South America and North America (the resistance was absent), whilst it was relatively high in Africa (50%). In Asia, the resistance was absent in Thailand, and very low in China (0.6%) and South Korea (0.01%). In contrast, increased values were found in India (53.8%), and Iran (11.7%). The prevalence of tetracycline resistance stays very low (less than 7.4%) in almost most parts of the world except for Africa. The comparison of data showed that tetracycline resistance is decreasing in the world, 26.85% in 2009 to 6.11% in 2014.

Tetracycline is a bacteriostatic and broad spectrum antimicrobial agent that is active against *H. pylori* and tetracycline is the most generally used antibiotic for treatment of *H. pylori* and other infectious diseases[109]. Tetracycline is extensively used in many countries, but resistance to this antibiotic has not become a great problem yet. Management failure owing to the tetracycline resistant has been reported[112,113], though there is not enough data obtainable until now to determine the impact of this resistance on management success.

Rifabutin resistance

However, the study on *H. pylori* rifabutin resistance is inadequate and in South America, North America and Africa has not been done during previous 6 years. The rate of rifabutin resistance was higher in Asia (12.45%) as compared to Europe (1%). The frequency of rifabutin resistance differs in Asian countries, ranging from 28.6% in Iran to about 7% in China and Malaysia. Rifabutin is structurally related to rifampin group, and it has potential efficacy against *H. pylori*[114]. Rifabutin is usually used to treat mycobacterium diseases, so the secondary resistance of *H. pylori* to rifabutin is not currently expected in the healthy people.

Levofloxacin resistance

Generally, resistance to levofloxacin is low (< 19%) worldwide. The prevalence rate was higher in Asia

(25.28%) and South America (21.23%) as compared to Africa and Europe (less than 15%). The frequency of levofloxacin resistance widely differs in Asian regions, about 57% in Japan, 24.55% in South Korea, 5.3% in Iran and 2.6% in Malaysia. In addition the levofloxacin resistance rate differs between European countries, ranging from 7% to 33.9%. The rate of levofloxacin resistance seems to be increasing universal from 4.25% in 2009 to 17.55% in 2014. Furthermore, during the past 3 years levofloxacin resistance rates have even been more increasing.

Due to the dramatic increase in clarithromycin resistance, levofloxacin, a wide spectrum quinolone, has been used as an option of clarithromycin in some regimens. But the frequent use of quinolones for urinary tract infections has increased the incidence of *H. pylori* resistance in the world[17]. Failure of therapy due to levofloxacin resistance and the emerging development of quinolones resistance, use of levofloxacin as first-line therapy is generally discouraged, and its utilize should be reserved as a second-line or save regimens after failure of a clarithromycin and/or a metronidazole based regimen[7,80].

Furazolidon resistance

The study on furazolidon resistance was not widely performed in the world, and in Europe, North America and Africa has not been achieved during past 6 years. The rate of furazolidon resistance was higher in Asia (13.8%) as compared to South America (0%). The rate of furazolidon resistance broadly differs in Asia, from 61.4% in Iran to 16.8% in China and 13.8% in India. Furazolidon is a cheap and synthetic nitrofuran with a wide spectrum activities usually used in the treatment of bacterial and protozoa infections. Since high *H. pylori* resistance to metronidazole in some region as China and South America, furazolidon sometimes has been used as an option for *H. pylori* infections[65]. However some researchers were reported that the rate of cure with furazolidon-based regimens is low and a large amount of furazolidon increases the therapy rate but it significantly raises complications[81].

The prevalence of *H. pylori* metronidazole resistance is at a high level, and resistance to clarithromycin and levofloxacin is increasing worldwide. The most effective drug is rifabutin and the lowest sensitive drug is metronidazole. Resistance to levofloxacin does not show any region difference. There are no studies regarding rifabutin and furazolidon resistance of *H. pylori* in America and Africa. According to the present findings, the mean resistance rate in *H. pylori* isolated from European and North American patients is lower than other countries. The rate of tetracycline, metronidazole and amoxicillin resistance is higher in African patients, while clarithromycin and levofloxacin resistance is higher in North America and Asian patients. In conclusion, antibiotic resistance is increasing, so empirical therapy must be based on information of antimicrobial drug resistance, and this paper highlight a steady worldwide surveillance of *H. pylori* antibiotic resistance.

COMMENTS

Background

Helicobacter pylori (*H. pylori*) is a most important human pathogen associated with significant disease and fatality.

Research frontiers

Due to the rising frequency of antimicrobial resistance, management of *H. pylori* remains a challenge for physicians in most parts of the world.

Innovations and breakthroughs

Search was carried out about *H. pylori* antimicrobial resistance literatures published through 2009 to 2014.

Applications

The frequency of antibiotic resistance is increasing, and this article highlight a steady worldwide surveillance of *H. pylori* antibiotic resistance.

Peer-review

This is a systematic review article on *H. pylori* resistance to antibiotics. The manuscript is well written and the topic of interest.

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