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EDITORIAL

Where is hidden the ghost in phantom sensations?

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Abstract

The term phantom sensations (PS) refers to sensations in a missing body part. They are almost universal in amputees and can be both painful and not painful. Although PS have been frequently described in limb amputees, they can also occur in other clinical conditions and several pathophysiological interpretations have been proposed, with a predominance of theories based on a central origin. Actually, different mechanisms are able to create a phantom sensation. After an amputation, PS are frequently generated by the genesis of ectopic action potentials in the interrupted nerve fibers but the PS generator can also be more proximal. Sometimes PS are not created by the stimulation of somatosensory fibers with a missing territory, but they can be the result of central sensitization or neuroplastic changes that allow for the convergence of impulses coming from different body parts (referred sensations), one of which is missing. In conclusion, PS can be generated by both neuropathic and non-neuropathic mechanisms developed in the amputated body part or in other parts of the nervous system. Since these mechanisms are not pathognomonic of amputation there are no hidden ghosts to look for in phantom sensations. The only interpretative rule is just to follow the pathophysiological principles.

Key words: Phantom sensations; Phantom pain; Neuropathic pain; Referred pain; Pain pathophysiology

Core tip: The term phantom sensations (PS) refers to sensations in a missing body part. They are almost universal in amputees and can be both painful and not painful. Several pathophysiological interpretations have been proposed, with a predominance of theories based on a central origin. Actually, PS can be generated by both neuropathic (ectopic) and non-neuropathic (referred) mechanisms developed in the amputated body part or in other parts of the nervous system. Since these mechanisms are not pathognomonic of amputation there are no hidden ghosts to look for in phantom sensations. The only interpretative rule is just to follow the pathophysiological principles.

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The term phantom sensations (PS) refers to sensations in a missing body part, phenomena that obviously appear paradoxical (but also intriguing) for the most part of the people. A literary example is in the very famous book "Moby Dick, or the whale" (1851) by Herman Melville, who describes in a short sentence the PS of the Captain Achab who had a leg amputated by the whale: "here is only one distinct leg to the eye, yet two to the soul".

PS are almost universal in amputees and can be both painful and not painful[1-3]. More precisely, patients can describe their PS in several ways, according to the anatomical and pathophysiological characteristics of the amputation: burning[4], tingling[3] or painful[3] sensations, illusory limb movement[5], visual hallucinations[6], and so forth.

Although PS have been frequently described in limb amputees, they can also occur in other clinical conditions such as after orchiectomy[7], mastectomy[8], tooth' s root canal treatment[9], penis amputation[10], ocular evisceration or enucleation[6].

Several pathophysiological interpretations have been proposed for PS, with a predominance of theories based on a central origin, including psychiatric explanations[11]. Actually, different mechanisms (neuropathic or non-neuropathic) are able to create a phantom sensation in a missing body part.

It is largely accepted that any neuropathic mechanism is characterized by the ectopic generation of action potentials in somatosensory afferent fibers[12]. In amputees, neuropathic pain mechanisms of PS can be localized at the level of amputation or more proximally. Sometimes they are strictly linked to the amputation, sometimes not.

After an amputation, PS are frequently generated by the genesis of ectopic action potentials in the interrupted nerve fibers, as demonstrated by human microneurographic recordings[13].

Nevertheless, several studies suggested that the PS generator can be proximal to the amputation site. On a pathophysiological point of view, this is not at all strange. In physiology, it is well known that the direct (ectopic) stimulation of a sensory nerve fiber induces a sensation localized in the territory of the stimulated fiber, *i.e.*, in the body part where the receptors are located. When Penfield and Rasmussen described for the first time the sensory homunculus, they reported patients' sensations evoked in different body parts during the electrical stimulation of the somatosensory cortex[14]. All that considered, when the territory of the stimulated nerve fibers is missing, the adequate ectopic stimulation of somatosensory nerve fibers always creates a phantom sensation, wherever the stimulation is applied.

Several examples can be given. For instance, in a recent paper, selective peripheral nerve blockades suggested a major role played by dorsal root ganglia in the generation of PS in a group of amputees[15].

Very interesting is also the recently described case of a patient with an old hip disarticulation amputation due to a malignant sarcoma[16]. After 1.5 years from amputation, this patient started to complain a severe phantom limb pain, mainly localized at the right phantom thigh. Computed tomography and magnetic resonance imaging showed the presence of a metastatic spinal mass involving the L3 vertebra with stenosis of the right lateral recess. Importantly, the resection of the vertebral mass completely resolved the phantom limb pain, demonstrating that the pain generator was in the sensory nerve fibers compressed at the lateral recess of the lumbar spine and not at the site of amputation.

Moving proximally in the central nervous system, the electrical stimulation of the thalamus during functional stereotactic mapping constantly evoked various PS, including pain, in a group of amputees[17].

Sometimes PS are not created by the stimulation of somatosensory fibers with a missing territory, but they can be the result of central sensitization or neuroplastic changes that allow for the convergence of impulses coming from different body parts (referred sensations), one of which is missing. All that happens because the conscious representation of the body lies in the activation of one or more parts of the sensory cortex, independently from what really occurs in the periphery. This seems to be clearly confirmed in patients with arm amputation where the stimulation of face and trunk can evoke a phantom sensation. Since face and trunk are close to the hand in the cortical representation of the human body, the interpretation for this referred sensation was again the change of cortical representation after the amputation[18].

On a neurobiological point of view, great importance has been attributed to the rearrangement of the central nervous system in response to the loss of inputs coming from the periphery[19], but it is important to underline that any injury can induce a change in the cortical body representation, independently from PS occurrence[20].

It is also worth highlighting that referred sensations are not neuropathic per se and can also be observed in healthy subjects, although only in special situations[21]. Since the beginnings of the 20th century it was clear that a painful sensation can be complained in a part of the body as a consequence of a disease in another[22]. This is confirmed by several studies on experimental pain demonstrating how the intense stimulation of some tissues is able to evoke a painful sensation not only in the stimulation site, but also at a distance from it[23,24]. Nowadays referred pain is truly considered a rather common complaint in several clinical conditions.

Moreover, the (traumatic) amputation of a body part is not necessary for the development of PS as demonstrated by the evidence that patients with congenital limb absence can experience PS after minor trauma or minor surgery[25,26].

Interestingly, cortical representation changes can also explain other quasi-phantom phenomena. For instance, in patients with complete spinal cord injury the stimulation of body parts above the lesion can evoke a

sensation below the injury level[27].

All that considered, referred sensations can thus represent an additional pathophysiological basis of PS in amputees.

In conclusion, PS can be generated by both neuropathic and non-neuropathic mechanisms developed in the amputated body part or in other parts of the nervous system.

Since these mechanisms are not pathognomonic of amputation there are no hidden ghosts to look for in phantom sensations. The only interpretative rule is just to follow the pathophysiological principles. In this respect, since PS are generally very stressful for patients, according to Sherman[4], physicians have an important role in easing the patients' suffering by educating them about the PS pathophysiology in order to explain that their sensations are not so strange as they appear.

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REVIEW

Treatment strategies for multiple sclerosis: When to start, when to change, when to stop?

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Abstract

Multiple sclerosis (MS) is a chronic inflammatory condition of the central nervous system determined by a presumed autoimmune process mainly directed against myelin components but also involving axons and neurons. Acute demyelination shows as clinical relapses that may fully or partially resolve, while chronic demyelination and neuroaxonal injury lead to persistent and irreversible neurological symptoms, often progressing over time. Currently approved disease-modifying therapies are immunomodulatory or immunosuppressive drugs that significantly although variably reduce the frequency of attacks of the relapsing forms of the disease. However, they have limited efficacy in preventing the transition to the progressive phase of MS and are of no benefit after it has started. It is therefore likely that the potential advantage of a given treatment is condensed in a relatively limited window of opportunity for each patient, depending on individual characteristics and disease stage, most frequently but not necessarily in the early phase of the disease. In addition, a sizable proportion of patients with MS may have a very mild clinical course not requiring a disease-modifying therapy. Finally, individual response to existing therapies for MS varies significantly across subjects and the risk of serious adverse events remains an issue, particularly for the newest agents. The present review is aimed at critically describing current treatment strategies for MS with a particular focus on the decision of starting, switching and stopping commercially available immunomodulatory and immunosuppressive therapies.

Key words: Multiple sclerosis; Disease-modifying therapy; Treatment start; Treatment switch; Treatment stop; Interferon beta; Glatiramer acetate; Azathioprine; Natalizumab; Fingolimod

Core tip: Disease-modifying therapies for multiple sclerosis (MS) modulate or suppress with different mechanisms the autoimmune process that underlies the

disease. Patients with relapsing MS may benefit from treatment but individual response to a given therapy and adverse events occurrence are largely unpredictable and many cases need to change several drugs to stabilize their disease. Nevertheless, a high proportion of patients evolve to a progressive phase, which is not responsive to any existing therapy. As opposed, some cases have a benign course without treatment. A critical review of strategies for starting, switching and stopping disease-modifying therapies for MS is here presented.

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INTRODUCTION

Multiple sclerosis (MS) is a chronic neurological disease of unknown cause sustained by a widespread inflammatory process within the central nervous system (CNS) leading to multifocal demyelination and axonal loss mostly in the white matter but importantly also in the grey matter of both brain and spinal cord[1]. Clinical manifestations are heterogeneous depending on the anatomical location of inflammatory lesions, and are expression of acute demyelination which can fully or partially resolve, of chronic demyelination and neuroaxonal injury, that are generally irreversible, or both. Based on the predominance of episodic acute demyelinating events or of the chronic neurodegenerative process, the clinical course is defined either relapsing-remitting, which represents around 60% of prevalent cases, or progressive (primary if progression starts from onset or secondary if it begins after a preceding relapsing-remitting phase). About 10% of MS cases have a primary progressive (PP) course, while transition to the secondary progressive (SP) phase occurs in around half of RR MS patients, generally decades after clinical onset. An initial acute episode of neurological disturbance that is suggestive of MS but does not fulfill diagnostic criteria is defined clinically isolated syndrome (CIS), which is the typical presentation of relapsing forms of MS, although many patients may remain asymptomatic and free of disease-defining brain/spinal cord MRI activity for several years after a CIS has occurred[2,3].

MS predominantly affects young adults of female sex (female to male ratio 2.5:1 or greater), although the disease may begin in children and subjects over the age of 60. Caucasians are more frequently affected and the prevalence of the condition varies profoundly across different areas of the world, roughly following an increasing gradient from the equatorial zone - where it is below 5 cases per 100000 inhabitants - to the poles, reaching rates over 130 cases/100000 in several regions of Northern America, Europe and Australia[4-6]. Epidemiological studies indicate that genetic susceptibility, infections (particularly Epstein-Barr virus), reduced sun light exposure/blood levels of vitamin D, cigarette smoking, obesity, and increased dietary salt intake are risk factors for developing the disease but have not yet a completely established causative role[7]. Although the etiology of MS remains unknown, there is strong biological evidence of an autoimmune pathogenesis sustained by migration of peripheral T and B cells - reactive against one or more unidentified myelin or neuronal antigens - into the CNS, in which lymphocytes induce and maintain inflammation also through persistent microglia activation among other mechanisms that cause demyelination, axonal loss, and ultimately neuronal death[8].

Currently disease-modifying therapies (DMTs) for MS approved by the European Medicine Agency (EMA) and Food and Drug Administration (FDA) include interferon beta (IFNB) 1-a and 1-b, glatiramer acetate (GA), mitoxantrone, natalizumab, fingolimod, teriflunomide, dimethyl fumarate, and alemtuzumab. In addition, azathioprine and cyclophosphamide are used off-label or approved in some countries for MS treatment as a consolidated indication not initially registered (Table 1). Also methotrexate and rituximab are used as an off-label option in some cases. All mentioned agents act by modulating and/or suppressing the immune system at various levels and with different mechanisms of action, the description of which is beyond the scope of this review[9]. As a general rule, available DMTs have a favorable impact on relapsing-remitting MS, while they have no significant benefit in progressive MS in which neurological disability continues to worsen over time[10]. Even in relapsing-remitting MS, the efficacy, tolerability and safety profile vary greatly across treatments, ranging from combinations of modest effect and excellent safety to options that are highly effective but at increased risk of serious adverse events, which may be fatal in rare cases[11]. These include but are not limited to: cardiomyopathy and acute leukemia after long-term treatment with mitoxantrone; natalizumab-associated progressive multifocal leukoencephalopathy (PML); bradyarrhythmias, macular edema, and varicella-zoster virus infections occurring with fingolimod therapy; autoimmune thyroiditis, thrombocytopenia, and glomerulonephritis induced by alemtuzumab. Ideally, optimal treatment responders should be free from relapses, disability worsening and adverse events, outcomes that are difficult to assess experimentally in the long term given the relatively short duration of clinical trials for a lifelong condition such as MS. As a consequence, surrogate outcomes - mainly represented by brain MRI measures - have been increasingly used in trials for the last 20 years to demonstrate the biological activity of MS therapies[12,13]. However, the precise correlation between short-term effect on MRI measures and long-term clinical changes remains to be fully elucidated[14-16]. In addition, MS may have an extremely

Table 1 Main characteristics of available disease-modifying therapies for multiple sclerosis

Agent	Indication and line of therapy	Dosage, route and frequency	Clinical efficacy in placebo-controlled phase III trials	Tolerability issues	Safety issues
Interferon beta 1b	RR MS; SP MS with relapses; CIS First line	250 mcg *s.c.* every other day	34% reduction of ARR over two years (RR MS) 50% risk reduction of conversion to CD MS at two years (CIS) No statistically significant effect on disability progression	Flu-like syndrome; injection site reactions	Hepatotoxicity; myelotoxicity; autoimmune thyroiditis; microangiopathy; epileptic seizures (rare)
Interferon beta 1a	RR MS; CIS First line	30 mcg *i.m.* once a week	18% reduction of ARR over two years (RR MS) 44% risk reduction of conversion to CD MS at two years (CIS) No statistically significant effect on disability progression	Same as above	Same as above
Interferon beta 1a	RR MS; CIS First line	44 mcg *s.c.* three times a week	32% reduction of ARR over two years (RR MS) 45% risk reduction of conversion to CD MS at two years (CIS) 30% reduction of progression of disability at two years (RR MS)	Same as above	Same as above
Peginterferon beta 1a	RR MS First line	125 mcg *s.c.* every two weeks	36% reduction of ARR over one year	Same as above	Same as above
Glatiramer acetate	RR MS; CIS First line	20 mg *s.c.* every day	29% reduction of ARR over two years (RRMS) 45% risk reduction of conversion to CDMS at three years (CIS) No statistically significant effect on disability progression	Injection site reactions; post-injection reaction (chest pain, flushing and dyspnea)	Cutaneous necrosis; anaphylaxis (rare)
Mitoxantrone	RR MS; SP MS; PR MS Second or third line	12 mg/m^2 *i.v.* every three months or 8 mg/m^2 *i.v.* every month	65% reduction of relapse risk over two years (mostly in RR MS)[98] 66% reduction of risk of disability progression at two years (mostly in RR MS)[98]	Nausea/vomiting; amenorrhea/infertility; alopecia; blue discoloration of sclera and urine	Infusion site tissue necrosis; myelotoxicity; infections; cardiotoxicity; acute leukemia
Natalizumab	RR MS Second line	300 mg *i.v.* every four weeks	68% reduction of ARR over two years 42% reduction of progression of disability at two years	Headache	Infusion associated reactions; anaphylaxis; infections; hepatotoxicity; progressive multifocal leukoencephalopathy
Fingolimod	RR MS Second line (first line in the United States)	0.5 mg per *os* every day	48%-54% reduction of ARR over two years 30% reduction of progression of disability at two years	Fatigue; headache	Bradyarrhythmias after first dose; lymphopenia; viral infections (VZV); macular edema; hepatotoxicity; hypertension
Teriflunomide	RR MS First line	14 mg per *os* every day	31%-36% reduction of ARR over one year or more 26%-32% reduction of progression of disability at one year or more	Nausea; diarrhea; alopecia	Myelotoxicity; hepatotoxicity; infections; peripheral neuropathy; pancreatic fibrosis; teratogenicity (requires accelerated elimination procedure)
Dimethyl fumarate	RR MS First line	240 mg per *os* twice a day	44%-53% reduction of ARR over two years 38% reduction of progression of disability at two years	Flushing; gastrointestinal symptoms; pruritus	Lymphopenia; progressive multifocal leukoencephalopathy
Alemtuzumab	RR MS Second or third line	12 mg/d *i.v.* for five days followed by 12 mg/d *i.v.* for three days one year after the first course	49%-55% reduction of ARR over two years compared to *s.c.* interferon beta 1a 42% reduction of progression of disability at two years compared to *s.c.* interferon beta 1a	Infusion associated reactions; myalgia; arthralgia; irregular menstruation	Infusion associated reactions; cytokine release syndrome; lymphopenia; infections; autoimmune thyroiditis; thrombocytopenic purpura; glomerulonephritis
Azathioprine[1]	MS of all types First or second line	2.5 mg/kg per *os* every day	23% relative risk reduction of the frequency of relapses over two years No statistically significant effect on disability progression at two and three years[98]	Gastrointestinal symptoms; photosensitivity; irregular menstruation/reduced fertility	Myelotoxicity; hepatotoxicity; lymphopenia; infections; acute pancreatitis; increased toxicity in subjects with thiopurine methyltransferase deficiency; malignancies (cumulative dose > 600 g)
Cyclophosphamide[1]	SP MS; PP MS Third line	1 g *i.v.* over three days or 500 mg *i.v.* over five days	No statistically significant effect on disability progression at two and three years[98]	Nausea/vomiting; amenorrhea/infertility; alopecia	Myelotoxicity; hepatotoxicity; infections; hemorrhagic cystitis; bladder cancer

[1]The use of these drugs for the treatment of multiple sclerosis is off-label in most countries. ARR: Annualized relapse rate; CD: Clinically definite; CIS: Clinically isolated syndrome; PP: Primary progressive; PR: Progressive-relapsing; RR: Relapsing-remitting; SP: Secondary progressive.

Table 2 Critical factors affecting the decision of starting disease-modifying therapies for multiple sclerosis

Factors suggesting not to start a DMT	CIS with favourable prognostic factors
	RR MS with no relapses in previous two years, no disability, and no evidence of MRI activity (potential "benign" case)
	Progressive forms of MS with no relapses or evidence of MRI activity
	Pregnancy planning
	High risk of low adherence to treatment
Factors suggesting to start a first line DMT	CIS with unfavourable prognostic factors
	RR MS with at least one relapse in previous two years but less than two relapses in the last year, low residual disability, and/or active MRI
Factors suggesting to start a second line DMT	RR MS with at least 2 disabling relapses in the last year
	Progressive forms of MS with relapses and/or active MRI

DMT: Disease-modifying therapy; CIS: Clinically isolated syndrome; MS: Multiple sclerosis.

variable clinical course both within and between subjects, who may show extremely active and break-through disease despite treatment or, on the contrary, very mild forms or phases not necessarily requiring a potentially harmful and costly pharmacological therapy[17].

Here we will discuss current and potential strategies to start, change and stop disease-modifying MS therapies in the clinical practice.

WHEN TO START TREATMENT FOR MS?

Primum non nocere

To avoid overtreatment, it is important to start on a DMT MS patients who carry the highest probability of optimal therapy response, making decisions based on multiple factors, including evidence of efficacy and safety profile of drugs, disease course and activity, expected adherence and preferences of the individual case (Table 2)[18-20]. Placebo-controlled randomized trials of IFNB and GA in patients with CIS have shown that active treatment significantly delays conversion to definite MS and prevent accumulation of new brain lesions on MRI[21-25]. However, there is little or no significant benefit of early *vs* delayed therapy on worsening of neurological disability in the open-label extension phase of these trials up to 10 years after study initiation[26-28].

Randomized trials of DMTs for relapsing-remitting MS included patients who had experienced at least one or two relapses in the previous one or two years prior to randomization and showed that all therapies significantly reduce relapse rate over 2-3 years of treatment with largely different effect size depending on the specific drug considered (Table 1)[29-45]. Comparisons between old and new drugs or between pivotal and recent trials are limited by the changed profiles of MS subjects enrolled in clinical trials who are now generally in earlier phases of disease and with much lower clinical and MRI activity compared to patients included in studies between 1988 and 2000[46].

When taking the decision of treating a patient with MS for the first time, clinicians choose either an escalation or an induction approach[10]. The first consists of starting with a first-line medication - intended as a moderate-efficacy high-safety drug - and switching to a second-line treatment (more effective but also with more safety risks) in case of unsatisfactory response to the first line: this is reasonable in most patients seen in the clinical practice who present with mildly or moderately active disease. The induction approach is the initial use of a highly effective second-line treatment in order to obtain the rapid remission of a very active disease, which justifies the risk of serious adverse events. This strategy is intended for MS cases with frequent (*i.e.*, two or more per year) and severe relapses who are at increased risk of rapid accumulation of disability.

IFNBs, GA, teriflunomide, and dimethyl fumarate are considered first-line therapies, while natalizumab, alemtuzumab, are mitoxantrone are second-line or third-line drugs. Fingolimod is approved as a second-line treatment in the EU and as first-line in the United States, Canada and other countries[47]. Azathioprine and cyclophosphamide, which are not registered for MS treatment, are used by clinicians as first-line and second-line medications, respectively. Among first-line drugs, differences exist in terms of efficacy and tolerability, although direct comparison data are limited. Existing evidence indicates that high dose IFNB (particularly IFNB 1-a 44 mcg subcutaneously three times a week) is more effective than low dose IFNB, *i.e.,* IFNB 1-a 30 mcg intramuscular once a week[48,49]. However, high dose IFNB and GA have similar efficacy on clinical parameters, while they slightly differ in terms of impact on MRI measures, that is greater for IFNB than GA, and tolerability profile[50-53]. There is less experience worldwide with dimethyl fumarate given its recent introduction to the market. One of the pivotal studies included a group of GA-treated patients as reference arm: MS subjects receiving the experimental drug or GA had similar statistically significant reductions of relapse rate, while differences in disability progression at 2 years were not significant, compared to placebo[42]. Teriflunomide has shown a similar efficacy to high dose IFNB and, as dimethyl fumarate, has the advantage of being an oral medication[54]. Recently, an independent comparative study has shown that azathioprine is not inferior to IFNBs in relapsing-remitting MS in terms of relapse rate and disability progression reduction, confirming the utility of an old and safe drug as a low cost and oral administration treatment option for this

Table 3 Critical factors affecting the decision of changing current disease-modifying therapy for multiple sclerosis

Factors suggesting to switch from a first line DMT to another	Tolerability/safety issues Suboptimal efficacy with disease activity not suitable for escalation to a second line DMT Persistent high-titre neutralizing antibodies in patients treated with interferon beta
Factors suggesting to switch from a first line to a second line DMT	RR MS patients experiencing at least one relapse and with an active MRI during the previous year on treatment RR MS patients transitioning to the secondary progressive phase with evidence of relapses or MRI activity
Factors suggesting to switch from a second line DMT to another or to a third line DMT	RR MS patients continuing to experience relapses Progressive forms of MS with relapses and/or active MRI despite treatment Safety issues (*e.g.*, patients on natalizumab at high risk of developing progressive multifocal leukoencephalopathy)
Factors suggesting to switch from a second line to a first line DMT	Tolerability/safety issues Risk perception of patient

DMT: Disease-modifying therapy; RR: Relapsing-remitting; MS: Multiple sclerosis.

condition[55].

Natalizumab, fingolimod, and mitoxantrone are consolidated second-line DMTs, which can be used as initial treatment in patients with aggressive MS requiring an induction approach. In addition, EMA and FDA recently approved alemtuzumab with the indication for "active" MS. In patients not previously treated with other medications, all the mentioned drugs strongly reduce the frequency of attacks compared to standard first-line therapy (around 50% relapse rate decrease *vs* IFNB) and have a profound effect on MRI activity measures[44,56-58]. However, the benefit on disability progression appears less robust and consistent across studies.

There are no approved DMTs for the PP form of MS[59-61], which carries the worst prognosis. For this reason, some patients - particularly in presence of rapid neurological worsening, superimposed relapses and evidence of inflammatory activity on brain/spine MRI - are treated off-label with immunosuppressants such as cyclophosphamide or mitoxantrone, based on the possible efficacy on disability progression suggested by some randomized trials[36,62].

WHEN TO CHANGE TREATMENT FOR MS?

Evidence-based data and guidelines on criteria and timing for DMT change in MS are limited and choices of clinicians on this matter are often based on observational reports and guided by good clinical practice (Table 3). In fact, MS patients who start a DMT discontinue it in a proportion ranging from 30% to 80% for various possible reasons[63]. One of the biggest challenges is the definition of treatment response/failure. An easy-to-apply and fairly validated tool is the Rio score, which combines clinical and MRI parameters to predict disability progression over five years[64,65]. In any case, MS patients receiving a first-line DMT who continue to have a similar relapse rate compared to the pre-treatment phase, have persistent MRI activity, and/or show irreversible neurological disability worsening, have a sub-optimal response and a therapy switch needs to be considered[66]. Second-line options for these cases are natalizumab, fingolimod and alemtuzumab, considering potential differences across drugs in efficacy and safety profiles[37-39,56,57,67,68].

For patients on first-line DMT with evidence of partial response but not fulfilling requirements for escalation to a second-line treatment (*e.g.*, isolated persistent MRI activity) or with adverse reactions/tolerability issues that affect patient safety or quality of life, a so called "lateral" switch to another first-line DMT is justified, *e.g.*, shifting from low-dose to high-dose IFNB (or the reverse in case of side effects), from GA to IFNB or *vice versa*[69,70]. In the near future switching from IFNB or GA to one of the newest oral agents such as teriflunomide and dimethyl fumarate will likely become very common. An additional option is switching from IFNB or GA to azathioprine.

Some authors suggest that patients treated with IFNB should be monitored for the serological status of neutralizing antibodies (NABs) both in cases in which suboptimal efficacy is suspected and with stable disease: persistent high-titer NABs positivity reflects IFNB biological activity loss, is associated with a higher risk of disease activity, and indicates the need of switching to a non-IFNB therapy[71]. Although NABs assay is not routinely performed in all IFNB-treated patients in all Centers, positivity is currently reported in less than 10% of cases on IFNB 1-a and over 30% of subjects receiving IFNB 1-b[72].

Finally, one has to consider the possibility or necessity of changing a second-line or third-line treatment in a patient with MS. If a patient continues to experience relapses and - more importantly - shows disability progression, a DMT change is needed as well as in case safety concerns arise during treatment. MS patients on fingolimod with break through disease will typically switch to natalizumab if this is safe, or to "rescue-therapy" with cyclophosphamide, which is also a possible option for cases not responsive to natalizumab, although this rarely occurs and should raise the suspicion of NABs presence[73]. Anyway, this scenario will likely change in the next future as the use of alemtuzumab catches on as a third-line or earlier therapeutic strategy. A debated issue in the community of MS neurologists is changing therapy in patients treated with natalizumab and at risk of developing PML,

since treatment discontinuation is associated with a high risk of disease reactivation[74]. However, also switching to another DMT, including fingolimod, does not prevent relapse occurrence and MRI worsening in many cases, particularly if new therapy start is delayed[75-77]. Other strategies, such as continuing natalizumab with a strict surveillance of early PML signs[78], or shifting to a third-line option such as cyclophosphamide or alemtuzumab are being adopted in some Centers, although it is not excluded that PML risk could be carried over by prolonging immunosuppression after natalizumab[79].

WHEN TO STOP TREATMENT FOR MS?

Effective DMTs are essential to guarantee the highest possible well-being to people with MS. For the same reason there are circumstances in which ongoing DMT should or must be stopped to avoid that risks or costs overcome benefit. Given the nature of MS, DMT discontinuation is usually temporary but in some cases it can be permanent[19,80].

First, DMT must be stopped when a serious adverse event potentially correlated to treatment occurs or is suspected, in particular if it is life threatening since MS itself does not lead to a meaningful increase of mortality. Several MS therapies, especially among the newest, expose patients to the risk of infectious, hematologic, cardiac, and neoplastic complications that are potentially lethal and must be monitored carefully[81]. If a DMT is discontinued for this reason, a treatment change has to be considered with caution since other drugs with similar mechanism of action may interfere with recovery of the adverse event or even aggravate it. In some cases a precautionary interruption of treatment, which may be temporary or prolonged, is dictated by factors that are known to increase the risk of certain adverse events. This is the case of PML risk during natalizumab in patients with anti-JCV antibodies positivity, previous immunosuppressive exposure, and treatment duration of 2 years or more[68]. Other examples include: risk of opportunistic infections in patients treated with fingolimod or dimethyl fumarate and persistently low lymphocyte count in the peripheral blood[82,83]; risk of cardiotoxicity and leukemia for patients treated with mitoxantrone[84]; increased risk of cancer with immunosuppressive cytotoxic therapies prolonged for more than 3 years in the case of cyclophosphamide or more than 10 years for azathioprine[85,86]. Beside serious adverse events, DMTs may cause "minor" side effects and tolerability issues that disrupt patient quality of life[87]. Cases not obtaining a satisfactory management of such symptoms or not perceiving treatment benefit that justifies undesired effects generally have low adherence to the prescribed medication. This is known to be a risk factor for poor control of disease activity and progression: if lack of adherence to treatment cannot be improved DMT has to be discontinued[88].

Pregnancy is another event that requires immediate DMT interruption in women with MS who, however, must be carefully informed of the need of adequate contraception prior to and during treatment, of the possibility that some DMTs may reduce fertility, and of the importance of becoming pregnant when the disease is as stable as possible[89]. Treatment cannot be resumed during breast-feeding meaning that nursing mothers should be advised of stopping breast-feeding and (re)starting therapy only in presence of disease activity or in case of aggressive course prior to treatment interruption. Pregnancy planning requires DMT discontinuation with the appropriate timing according to the pharmacokinetic of the specific drug[90]. IFNB and GA may be continued until few weeks in advance or even up to conception; natalizumab, fingolimod and dimethyl fumarate should be stopped at least two months prior to planned conception; cytotoxic agents, such as mitoxantrone and azathioprine, need to be discontinued at least three months in advance. In addition to therapy interruption, patients on teriflunomide are required to undergo an accelerated elimination procedure with colestyramine or activated charcoal at least two months before conception (in case of unexpected pregnancy the procedure must be started immediately)[91]. For patients on alemtuzumab pregnancy program appears more complex as the effects of a single five-days course of the drug may last up to four years; however, based on pharmacokinetic data, maintaining contraception for at least four months after last alemtuzumab administration is currently recommended[92]. Data and guidelines regarding paternity planning for men with MS receiving DMT are lacking. Treatment interruption is generally not recommended for IFNB and GA, since the outcome of pregnancies fathered by patients receiving those drugs does not differ from general population[93]. However, male patients receiving therapies with mutagen potential that could lead to an increased risk of fetal malformations should be encouraged to avoid conception while on treatment.

Although it might be difficult to establish, MS patients who gradually accumulate irreversible disability without experiencing relapses and MRI inflammatory activity - *i.e.,* have transitioned to the SP phase of the disease - do not benefit significantly from any of currently available DMT, which should be therefore discontinued in this group of subjects[94]. On the other hand, for treated patients with prolonged stable disease and no apparent side effects DMT discontinuation is not recommended because the disease could reactivate. However, available data have been obtained from few patients treated for less than three years who had high pre-treatment MS activity and were not selected according to an a priori definition of stable disease[95]. In this context, patients treated with natalizumab represent an exception because it has been consistently reported that treatment interruption even in cases with no sign of MS activity for several years, frequently leads to disease reactivation - with a very severe clinical

picture in some cases - soon after stopping therapy[96].

CONCLUSION

General consensus and detailed guidelines on starting, changing and stopping DMTs for MS are lacking. Recently, an effort to guide the use of DMTs based on evidence from the literature with the aim of improving access to therapies for MS patients, led to a consensus paper by the MS coalition[97].

Based on current evidence and good clinical practice principles, we suggest the following.

When to start treatment for MS?

First-line DMT should be started in patients with a diagnosis of relapsing MS (according to 2010 McDonald's criteria) and at least one documented attack in the previous two years; as for the choice of the specific drug, high dose IFNB 1-a and GA are the preferred options among established injectable therapies, although oral therapies such as azathioprine, teriflunomide and dimethyl fumarate have at least comparable efficacy.

First-line DMT may be initiated in patients with a CIS or MS with a single attack and dissemination in space and time according to 2010 McDonald's criteria in presence of factors known to be associated with poor prognosis, such as male sex, incomplete recovery from attack, prominent neurological efferent systems involvement, and more than nine lesions on brain MRI (good clinical practice point - there is no evidence that subgroups of patients with such features are significantly protected by DMTs against long-term disability progression).

DMT-naïve MS patients experiencing at least two disabling relapses in the last year and with an active MRI scan should be treated with a second-line regimen, such as fingolimod or natalizumab; also alemtuzumab may be considered for patients with aggressive disease from onset.

Available DMTs are of no utility in PP MS, although cases with rapid progression, superimposed relapses and active MRI might benefit from immunosuppressants such as mitoxantrone, cyclophosphamide, or methotrexate.

When to change treatment for MS?

Given the current availability of multiple options, a DMT change needs to be considered in any MS patient with suboptimal response: in case of one or more relapses during the previous year on a first-line DMT, particularly in case of incomplete recovery, switching to a second-line medication is appropriate, while isolated MRI activity and/or increased relapse frequency not qualifying for second-line escalation are conditions for switching to another first-line DMT; patients relapsing while on fingolimod may be switched to natalizumab, or the reverse (although natalizumab is expected to reduce relapse rate more than fingolimod based on indirect comparison); alternatively, these cases may be shifted to a third line of treatment such as alemtuzumab or intravenous cytotoxic immunosuppressants.

Patients on IFNB who develop persistent high-titer NABs need to change treatment even if disease is stable.

Subjects with intolerable side effects from their current medication need to be switched to another DMT within the same line of treatment.

Patients receiving natalizumab for more than two years who are anti-JCV antibody positive and previously received cytotoxic immunosuppressants should be switched to another DMT due to the significantly increased risk of PML; possible options include fingolimod, alemtuzumab, cyclophosphamide, and less convincingly first-line DMTs; to minimize the risk of disease reactivation the wash-out interval should be shortened as much as possible.

When to stop treatment for MS?

DMT must be stopped in case a serious adverse event potentially related to the drug occur or is likely to occur, in patients becoming pregnant, and in subjects who are not adherent to treatment.

DMT should be also discontinued in patients with confirmed disability progression over one year in the absence of relapses and new/enhancing lesions on MRI; these subjects have progressive MS, which does not respond to any DMTs, and priority should be given to symptomatic treatment, physical therapy, and management of disability.

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REVIEW

From variome to phenome: Pathogenesis, diagnosis and management of ectopic mineralization disorders

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Abstract

Ectopic mineralization - inappropriate biomineralization in soft tissues - is a frequent finding in physiological aging processes and several common disorders, which can be associated with significant morbidity and mortality. Further, pathologic mineralization is seen in several rare genetic disorders, which often present life-threatening phenotypes. These disorders are classified based on the mechanisms through which the mineralization occurs: metastatic or dystrophic calcification or ectopic ossification. Underlying mechanisms have been extensively studied, which resulted in several hypotheses regarding the etiology of mineralization in the extracellular matrix of soft tissue. These hypotheses include intracellular and extracellular mechanisms, such as the formation of matrix vesicles, aberrant osteogenic and chondrogenic signaling, apoptosis and oxidative stress. Though coherence between the different findings is not always clear, current insights have led to improvement of the diagnosis and management of ectopic mineralization patients, thus translating pathogenetic knowledge (variome) to the phenotype (phenome). In this review, we will focus on the clinical presentation, pathogenesis and management of primary genetic soft tissue mineralization disorders. As examples of dystrophic calcification disorders Pseudoxanthoma elasticum, Generalized arterial calcification of infancy, Keutel syndrome, Idiopathic basal ganglia calcification and Arterial calcification due to CD73 (NT5E) deficiency will be discussed. Hyperphosphatemic familial tumoral calcinosis will be reviewed as an example of mineralization disorders caused by metastatic calcification.

Key words: Ectopic mineralization; Pseudoxanthoma elasticum; Pseudoxanthoma elasticum-like syndrome; Generalized arterial calcification of infancy; Keutel syndrome; Idiopathic basal ganglia calcification; Arterial calcification due to CD73 deficiency; Hyperphosphatemic familial tumoral calcinosis; Etiology; Phenotype

Core tip: Ectopic mineralization disorders represent a broad range of phenotypically heterogenous diseases, often leading to significant morbidity and mortality. Involving a complex interplay between different pro-osteogenic mediators and inhibitors of calcification, the mechanisms of ectopic mineralization are progressively being unveiled. Though current knowledge is beyond any doubt the tip of the proverbial iceberg, insights already have significant implications in the diagnosis and daily management of these patients. As such, ectopic mineralization diseases are a fine example of translating variome data to the clinic. Here, we will discuss prototype hereditary ectopic calcification diseases with respect to their presentation, diagnosis and management.

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Table 1 Causes of metastatic/dystrophic calcification and ectopic ossification

	Metastatic calcification	Dystrophic calcification	Ectopic ossification
Primary	Primary hyperparathyroidism Pseudo(pseudo)hypoparathyroidism HFTC	PXE PXE-like syndrome GACI Keutel syndrome IBGC ACDC AI	Fibrodysplasia ossificans progressiva
Secondary	Sarcoidosis Vitamin D intoxication Milk-Alkali syndrome Secondary hyperparathyroidism Renal failure Hemodialysis Tumor lysis Therapy with vitamin D and phosphate	Scleroderma Dermatomyositis SLE	Nonhereditary myositis ossificans

ACDC: Arterial calcification due to CD73 deficiency; AI: Amelogenesis imperfecta; GACI: Generalized arterial calcification of infancy; HFTC: Hyperphosphatemic familial tumoral calcinosis; IBGC: Idiopathic basal ganglia calcification; PXE: Pseudoxanthoma elasticum; SLE: Systemic lupus erythematosus.

INTRODUCTION

Physiological biomineralization is a complex multifactorial metabolic process, which in normal conditions is restricted to the extracellular matrix (ECM) of specific body structures, namely the bones, teeth, hypertrophic growth plate cartilage and calcified articular cartilage[1,2]. The intracellular and extracellular mechanisms, underlying physiological biomineralization, rely on a balanced interplay between mineralization inhibitors and propagators (Figure 1)[2,3]. Although in physiological circumstances calcium and inorganic phosphate (Pi) concentrations exceed their solubility in most human tissues, this does not result in mineralization of soft tissues, suggesting that these tissues possess regulatory mechanisms preventing mineral deposition. Mineralizing tissues must be able to modulate these mechanisms to enable calcification[2], but should also contain anti-mineralizing factors to prevent escalation of the calcification process leading to excessive and uncontrolled mineral deposits[1,2]. When these regulatory mechanisms are inadequate, ectopic mineralization, *i.e.,* inappropriate biomineralization in soft tissues, occurs and causes a spectrum of ectopic calcification disorders (Table 1)[2,4].

Uncontrolled mineralization occurs frequently in response to tissue injury or a systemic mineral imbalance. This leads to the development of a calcified lesion, which can occur throughout the body, though tissues as articular cartilage, the cardiovascular (CV) tissues and kidneys seem particularly prone[3,5,6]. Unlike physiological mineralization deposits, which only contain calcium phosphate crystals such as hydroxyapatite, ectopic mineralization depositions may also contain other calcium salts, including calcium oxalates or octacalcium[4].

Regarding the initiation of and pathogenetic mechanisms underlying ectopic mineralization several hypotheses have been proposed (Figure 1): (1) increasing evidence is found that soft tissue calcification can be initiated in matrix vesicles (MVs), extracellular membrane particles (approximately 20-200 nm in diameter), which have a key role in the normal physiological mineralization process[3]. MVs contain calcium-binding non-collagenous matrix proteins, such as secreted phosphoprotein 1 (SPP1; OMIM*166490), which can boost mineralization *in vitro*[7]. MVs initiate mineralization in 2 phases: (1) initial formation of hydroxyapatite in the MV itself: after budding from the plasma membrane, tissue-nonspecific alkaline phosphatase (TNAP; OMIM*171760) activity induces an increase of extracellular Pi concentration, which then enters the vesicles *via* sodium-dependent inorganic phosphate transporters (PiTs). This is followed by calcium influx into the MVs, which is enabled by annexin A5 (ANXA5; OMIM*131230) and phosphatidyl serine (PS), located at the MV inner membrane leaflet[1,3]; and (2) propagation of the calcium salts in the ECM: in the MVs hydroxyapatite crystals continue to grow, eventually rupturing the MV membrane. As a result, the crystals are exposed to the ECM, inducing their further expansion[3,8]; pathological calcification can also be influenced by ectopic osteogenic and chondrogenic signaling, leading to the activation of multiple pro-mineralization proteins[9]. This conversion of tissue-specific cells to bone-like cells has been mainly

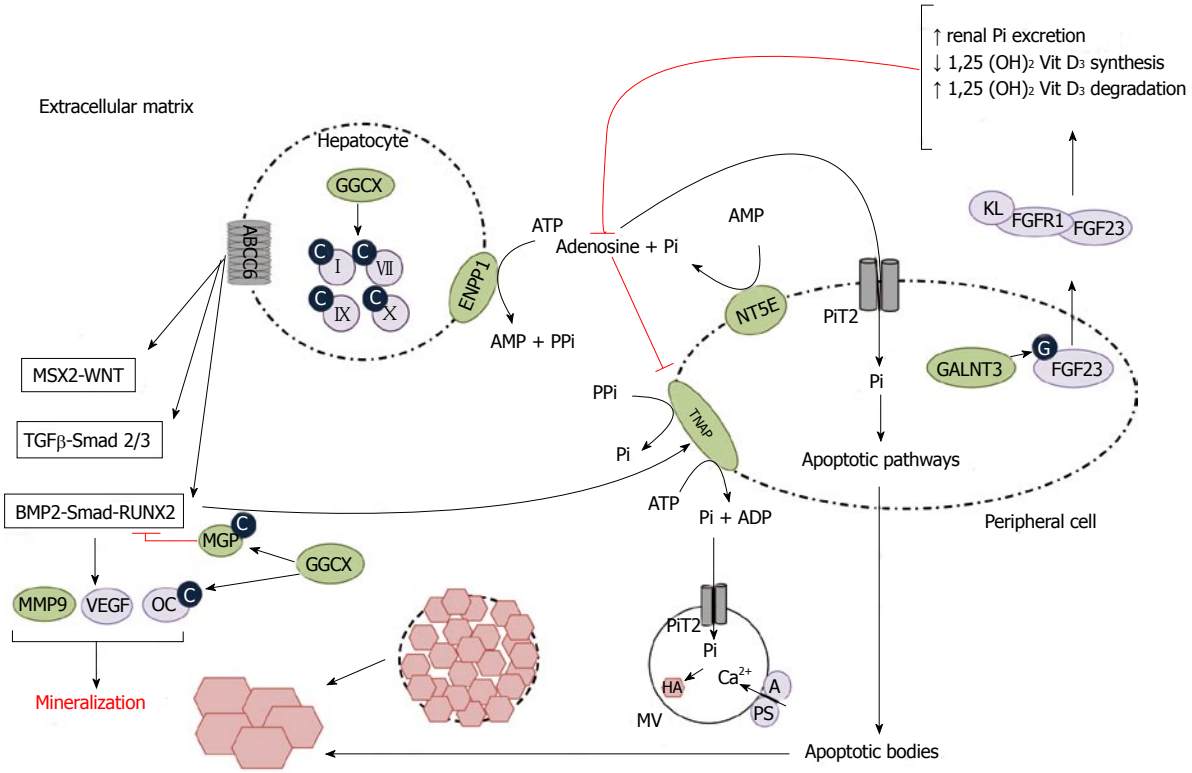

Figure 1 Schematic representation of the pathophysiological mechanisms leading to ectopic mineralization. Hepatocyte: Impairment of ABCC6 function leads to upregulation of pro-osteogenic pathways (MSX2-WNT, TGFβ-Smad 2/3, BMP2-Smad-RUNX2), upregulation of their downstream targets and eventually to ectopic mineralization. GGCX carboxylates and hence activates multiple targets, such as coagulation factors and MGP, the latter being a potent BMP2-inhibitor and hence mineralization inhibitor. When GGCX function is impaired, these targets stay inactive, leading to increased mineralization. ENPP1 converts ATP to AMP and PPi, the latter being a mineralization inhibitor. Impairment of this conversion and hence a decrease in the PPi level leads to increase in ectopic mineralization. Peripheral cell: After glycosylation by GALNT3, FGF23 forms a complex with FGFR1 and KL (coreceptor) which leads to increased renal excretion of Pi, a pro-mineralizing agent and decreased 1,25 dihydroxyvitamin D3, causing a decrease in intestinal Pi absorption. NT5E converts AMP to Pi and adenosine, which inhibits the pro-mineralizing TNAP. Impairment of NT5E function leads to increased TNAP activity and decreased PPi concentration, hence leading to ectopic mineralization. Pi is internalized into the peripheral cell by PiT2 and leaves the cell through apoptotic bodies, which cause ectopic mineralization through apoptotic pathways (not shown). In MVs an influx occurs of Pi *via* PiT2 and of Ca^{2+}, which is facilitated by A and PS. This leads to an accumulation of growing hydroxyapatite crystals, eventually causing the MVs to burst and the crystals to grow in the extracellular matrix. A: Annexin A5; ABCC6: Adenosine triphosphate-binding cassette, subfamily C, member 6; ADP: Adenosine diphosphate; AMP: Adenosine monophosphate; ATP: Adenosine triphosphate; BMP2: Bone morphogenetic protein 2; C: Carboxyl; Ca^{2+}: Calcium 2+; ENPP1: Ectonucleotide pyrophosphatase/phosphodiesterase 1; FGF23: Fibroblast growth factor 23; FGFR1: Fibroblast growth factor receptor 1; G: Glycosyl-; GALNT3: UDP-N-acetyl-alpha-D-galactosamine: Polypeptide N-acetylgalactosaminyltransferase 3; GGCX: Gamma-glutamyl carboxylase; HA: Hydroxyapatite; KL: Klotho; MGP: Matrix gla protein; MMP9: Matrix metalloproteinase; MSX2: Muscle segment homeobox, drosophila, homolog of, 2; MV: Matrix vesicle; NT5E: Ecto-5-prime nucleotidase or CD73; OC: Osteocalcine; Pi: Inorganic phosphate; SLC20A2: Solute carrier family 20 (phosphate transporter), member 2; PPi: Inorganic pyrophosphate; PS: Phosphatidyl serine; RUNX2: Runt-related transcription factor; Smad: Mothers against decapentaplegic, drosophila, homolog of; TGFβ: Transforming growth factor β; TNAP: Tissue-non-specific alkaline phosphatase; VEGF: Vascular endothelial growth factor; WNT: Wingless-type MMTV integration site family; II, VII, IX, X: Vitamin K-dependent coagulation factors; 1,25 $(OH)_2$ Vit D3: 1,25-dihydroxyvitamine D3 (calcitriol).

described in vascular calcification, and is probably due to the common mesenchymal origin of vascular smooth muscle cells (VSMCs) and bone cells[1]; (3) apoptosis or programmed cell death is accompanied by the release of apoptotic bodies, which exteriorize PS to the outer membrane of the apoptotic body and therefore face the ECM. There, PS may bind calcium, resulting in an accumulation of calcium and phosphate, as is also seen in MVs, thus contributing to physiological and pathological mineralization[1,10]. Another potential apoptosis pathway includes elevated phosphate levels to induce VSMC apoptosis, a process that is possibly caused by downregulation of growth arrest-specific 6 (Gas6; OMIM*600441) and B-cell CLL/Lymphoma (BCL2; OMIM + 151430), with subsequent caspase 3 activation[11,12]; and (4) reactive oxygen species (ROS), highly reactive oxygen-containing molecules, are formed as byproducts of normal oxygen metabolism and has important roles in cell signaling and metabolism. Nonetheless, if ROS concentration surpasses a critical threshold, oxidative stress, accompanied by important cell damage, can occur[13]. Potential sources of ROS in soft tissues are nicotinamide adenine dinucleotide (phosphate) (NAD(P)H) oxidase, nitric oxide synthase (NOS), xanthine oxidase, cytochrome P450 and cyclo-oxygenase; in addition, mitochondrial dysfunction may also lead to the formation of ROS. ROS possibly causes soft tissue mineralization through either the IκB-NF-κB pathway (inhibitor of κB - nuclear factor kappa-light-chain-enhancer of activated B cells), upregulation of the pro-osteogenic bone morphogenetic protein 2 (BMP2; OMIM*112261) pathway and/or osteogenic conversion

of soft tissue cells[1].

These pathophysiological mechanisms are however not mutually exclusive and display significant crosstalk[1].

Ectopic soft tissue mineralization is a common finding in aging and several common disorders, including atherosclerosis, hypertension, diabetes, chronic kidney disease and autoimmune diseases, and can be related to significant morbidity and mortality in each of these. It has been shown that vascular calcification correlates with an increased risk of myocardial infarction and that it is an independent risk factor for death in patients with coronary artery calcification[14,15]. However, in these complex, multifactorial disorders, multiple genes are likely to contribute, with each gene having only a small effect[16]. Contrary, in primary genetic mineralization disorders mutations in a single gene or few genes can cause an often extreme and life-threatening phenotype. Though individually rare, as a group they affect a considerable number of patients with important impact on quality of life and high morbidity and mortality rates.

Ectopic mineralization disorders are conventionally classified based on the mechanism through which the mineralization takes place: *i.e.,* metastatic or dystrophic calcification or ectopic ossification (Table 1)[14]: (1) metastatic calcification, due to hyperphosphatemia and/or hypercalcemia; (2) dystrophic calcification, which occurs in diseased (metabolically impaired or dead) tissue under normal calcium and phosphate homeostasis[1]; and (3) ectopic or heterotopic ossification, leading to true bone formation[1,4,17,18].

For many of these disorders, important advances have been made in defining their clinical presentation (phenome), their (molecular) etiology (variome) and the correlation between both. This has led to novel insights and perspectives for the management and treatment of the patients, but also supports the complexity of the pathophysiology of soft tissue mineralization.

This review will focus on the clinical presentation, pathogenesis and management of primary genetic soft tissue mineralization disorders due to dystrophic (Pseudoxanthoma elasticum, Generalized arterial calcification of infancy, Keutel syndrome, Idiopathic basal ganglia calcification, Arterial calcification due to CD73 deficiency) or metastatic calcification (Hyperphosphatemic familial tumoral calcinosis).

PSEUDOXANTHOMA ELASTICUM

Pseudoxanthoma elasticum (PXE; OMIM#264800) is a rare, autosomal recessive connective tissue disorder, resulting from ectopic mineralization and fragmentation of elastic fibers. The prevalence of PXE is estimated between 1/25000 and 1/100000 with a carrier frequency of 1/80, although this may be an underestimation due to the variability of the phenotype, which in some cases may hinder the diagnosis[19-21].

Clinical characteristics

PXE primarily affects 3 organ systems, *i.e.,* the skin, the eyes and the CV system, albeit with important inter- and intrafamilial variability in severity[19,21,22]. Usually the skin symptoms are the first to arise, though they are not present in all patients, presenting as soft yellowish papules in flexural body areas (*i.e.,* neck, axilla, elbow, groin and knees) (Figure 2A-E)[19]. These solitary papular lesions can coalesce into larger plaques. Loss of resilience may give the skin a wrinkled aspect and can cause an esthetic burden[1,19]. Less frequently, mucosal lesions (usually at the inner lower lip) are present (Figure 2F)[19]. The emergence of additional inelastic skin folds[1], especially in neck (and thigh) area(s) can also cause functional problems, *e.g.,* when sleeping or riding a bicycle[1,19].

The most common ocular features in PXE patients are peau d'orange and angioid streaks (AS), which themselves cause no functional impairment (Figure 2G). In later stages choroidal (subretinal) neovascularization (CNV) occurs and these neovessels may rupture, causing retinal hemorrhage (Figure 2I). Symptoms will include metamorphopsia and vision loss, which can be permanent if left untreated. More recently, chorioretinal atrophy, subretinal fluid independent from CNV, pattern dystrophy-like changes, debris accumulation under the retinal pigment epithelium, reticular drusen and a decreased fluorescence on late phase indocyanine green angiography were described[23].

CV symptoms, usually arising when patients are 30-40 years old, include accelerated coronary and peripheral artery disease (hypertension, myocardial infarction, intermittent claudication), diastolic cardiac dysfunction and gastrointestinal hemorrhage[24]. In 15% of PXE patients ischemic stroke may occur, at an average age of 49[1,24]. Heterozygous carriers usually develop neither skin nor eye symptoms but can suffer from accelerated atherosclerosis and (mild) diastolic dysfunction of the heart[24].

To date, no correlation has been established between the PXE phenotype and mutations in the main causal gene adenosine triphosphate (ATP)-binding cassette, subfamily C, member 6 (*ABCC6*; OMIM*603234), which complicates the prediction of the evolution and severity of the symptoms in an individual patient[19,25]. The absence of a reliable genotype-phenotype correlation suggests that other genes, so-called modifier genes, may play an important role in influencing the disease course, apart from other factors such as lifestyle, environmental factors and - although less probable - dietary habits[19]. Thus far, only one promising modifier gene, vascular endothelial growth factor A (*VEGFA*; OMIM + 192240) has been identified for the PXE retinopathy[26].

Pathogenesis

PXE is caused by mutations in the *ABCC6* gene, enco-

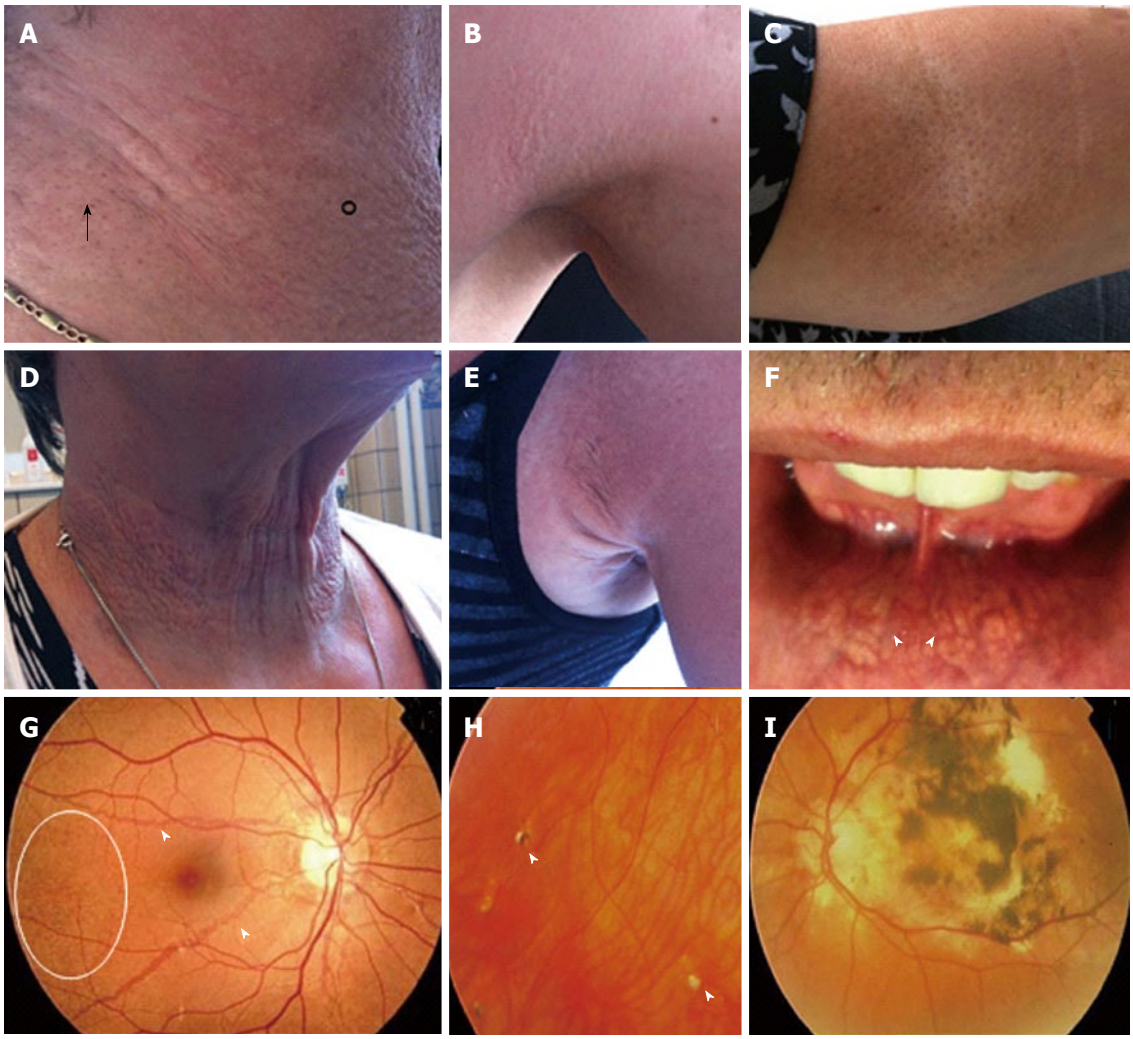

Figure 2 Dermatological (A-F) and ophthalmological (G-I) manifestations of pseudoxanthoma elasticum. A, B: Flexural areas can show papular lesions (°) and coalesced plaques of papules (arrow); C: Cutaneous peau d'orange; D, E: Additional skin folds; F: Yellowish, reticular pattern on the mucosae of the lip (arrowed); G: Ocular fundi show peau d'orange (circle) and angioid streaks (arrowed); H: Comets and comet tails (arrowhead); I: Choroidal and subretinal hemorrhage.

ding an ATP-binding efflux transporter, the substrate and (patho)physiological role of which are yet to be elucidated[27]. The ABCC6 transporter is mainly expressed in the liver and kidneys while only minimally present in the organs affected by PXE[28,29]. This led to the hypothesis that PXE is a metabolic disorder in which a defective transporter causes inefficient transport of one or multiple substrates into the bloodstream[28,29]. As a result, a deficiency of vitamin K (VK) -dependent and -independent mineralization inhibitors occurs, favoring ectopic soft tissue mineralization[23,30,31]. The metabolic hypothesis was reinforced several times, until very recently Ziegler *et al*[32] reported that a conditional, liver-specific *Abcc6*$^{-/-}$ mouse model does not develop ectopic mineralization and concluded that mineralization in PXE occurs through a liver-independent mechanism. This would correspond with a second, so-called cellular, hypothesis which states that the local environment in the affected organ systems is altered; in this respect it was shown that PXE fibroblasts suffer mild chronic oxidative stress because of overexpression of oxidative stress-favoring mediators[31,33].

More recently, 3 pro-osteogenic pathways, *i.e.*, BMP2-Smad (mothers against decapentaplegic, drosophila, homolog of; OMIM*601366)- runt-related transcription factor 2 (RUNX2; OMIM*60021) and transforming growth factor β2 (TGFβ2; OMIM*190220)-Smad2/3 pathways and the MSX2 (muscle segment homeobox, drosophila, homolog of, 2; OMIM*123101)-canonical WNT (wingless-type MMTV integration site family; OMIM*164820) pathway which are associated with vascular mineralization, were found to be upregulated in the skin and eyes of PXE knock-out mice and in PXE patients (Figure 1)[34]. The relevance of BMP2-Smad-RUNX2 signaling was alluded on by previous observations in PXE, including the low levels of carboxylated (active) matrix gla protein (MGP; OMIM*154870)/gamma-carboxyglutamic acid, a potent inhibitor of BMP2, the upregulation of several target genes of RUNX2 such as SPP1, osteocalcin, matrix metalloproteinase (MMP9; OMIM*120361), TNAP and VEGFA, the influence of oxidative stress on BMP2

Table 2 Differential diagnosis of pseudoxanthoma elasticum manifestations[1,19,47,49,53-59]

Disease	Distinct differences with PXE
Beta-thalassemia (PXE phenocopy)	Severe anemia Reduced production of hemoglobin
PXE-like syndrome (*AR*; *GGCX* gene)	More severe cutaneous phenotype not restricted to flexural areas Vitamin K-dependent coagulation factor deficiency
GACI (*AR*; *ENPP1* gene)	Onset in infancy or early childhood Arterial stenosis Early-onset severe myocardial ischemia High mortality rate in early childhood
Fibroelastolytic papulosis, Treatment with D-penicillamine	No ophthalmological or CV phenotype
Buschke-Ollendorf syndrome (*AD*; *LEMD3* gene)	Skeletal manifestations (osteopoikilosis, stiff joints, osteosclerosis) No ophthalmological or CV phenotype No mineralization
Solar elastosis	Dermatological features (lentigines, mottled pigmentation, actinic keratoses, telangiectasias, xerotic texture) No ophthalmological or CV phenotype No mineralization
Late-onset focal dermal elastosis	Onset in 7th to 9th life decade No ophthalmological or CV phenotype
Cutis laxa	No ophthalmological or CV phenotype Histopathology: scarce and mottled elastic fibers, no mineralization
A(R)MD (age-related macular degeneration)	No AS No CV or dermatological phenotype Less unique lesions (outer retinal tabulation or Bruch's membrane undulation)
Presumed ocular histoplasmosis	No AS No CV or dermatological phenotype

AD: Autosomal dominant; AR: Autosomal recessive; A(R)MD: Age-related macular degeneration; AS: Angioid streaks; CV: Cardiovascular; ENPP1: Ectonucleotide pyrophosphatase/phosphodiesterase 1; GACI: Generalized arterial calcification of infancy; GGCX: Gamma-glutamyl carboxylase; LEMD3: Lem domain-containing protein 3; PXE: Pseudoxanthoma elasticum.

expression and the overexpression of RUNX2 in calcified cardiac tissue of the *Abcc6*-related dystrophic cardiac calcification mouse[34-36]. Furthermore, apoptosis was identified as an important process in PXE contributing to mineralization, by activation of BCL2 and multiple caspases[34].

Some insights in the dysfunction of pro- and anti-mineralizing factors in the PXE pathogenesis, have been described in the PXE murine model and/or PXE patients. Several local pro-mineralizing factors seem to be upregulated *in vitro* and/or *in vivo* (TNAP, BMP2) while mineralization inhibitors, such as ecto-5-prime-nucleotidase or CD73 (NT5E; OMIM*129190), SPP1, ankyrin (mouse, homolog of) (ANKH; OMIM*605145) and VK-dependent calcification inhibitors, were found to be less expressed[37-39].

Besides local factors, systemic inhibitors of mineralization such as Fetuin A and more recently inorganic pyrophosphate (PPi), were shown to be less abundant in PXE. PPi is a potent endogenous inhibitor of vascular calcification, both *in vitro* and *in vivo*, which was already shown to be downregulated in PXE fibroblasts, thus promoting pro-calcifying stimuli leading to tissue mineralization[40,41]. Jansen *et al*[42] found low PPi serum levels in both *Abcc6*$^{-/-}$ mice and PXE patients, and concluded that an impaired ABCC6 transporter negatively influences PPi efflux from hepatocytes to the hepatic circulation, though the exact mechanism is poorly understood.

Diagnosis

The diagnosis of PXE is a clinical one to begin with, based on the presence of typical skin and/ or fundus changes. While the skin lesions of PXE can be mimicked macroscopically by other disorders (Table 2), the presence of peau d'orange and/or AS in the ocular fundus can be considered pathognomonic. Diagnostic confirmation can be obtained by skin biopsy, showing shortened, fragmented elastic fibers as well as mineral deposits in the mid-dermis using H&E (hematoxylin and eosin) and von Kossa staining. Molecular analysis of the *ABCC6* gene detects both causal mutations in approximately 95% of patients[1,33,43,44]. Sanger sequencing is still the gold standard, but should be complemented with multiplex ligation-dependent probe amplification, to detect larger deletions and insertions[45]. If no or only one *ABCC6* mutation can be identified, it is worthwhile to screen for gamma-glutamyl carboxylase (*GGCX*; OMIM*137167) mutations as digenic inheritance has been described[46]. Furthermore, the initial presentation of the *GGCX*-related PXE-like disorder with coagulation factor deficiency (see below) can be identical to PXE[47,48]. Sequencing of the ectonucleotide pyrophosphatase/phosphodiesterase 1 (*ENPP1*; OMIM*173335) gene is useful when no *ABCC6* mutations can be detected in a patient with a histologically confirmed diagnosis of PXE; digenic inheritance of an *ABCC6* and *ENPP1* mutation has so far not been reported[49]. This increasing number of genes which may cause PXE as well as the potential importance of modifier genes, brought about a gradual shift from Sanger sequencing towards the more recently introduced next generation sequencing[26,47,50-52].

Differential diagnosis

The differential diagnosis of PXE manifestations is summarized in Table 2.

Management

To date, PXE management is mainly symptomatic, focusing on prevention and treatment of complications[19]. For ophthalmological complications, preventive measures include wearing glasses and avoiding sports and activities with a (relative) high risk of (head) trauma or increased pressure[19,21,60]. Once fundus changes have appeared, an annual control by an ophthalmologist is important, as well as weekly self-examination using the Amsler Grid. If distortion or metamorphopsia occurs,

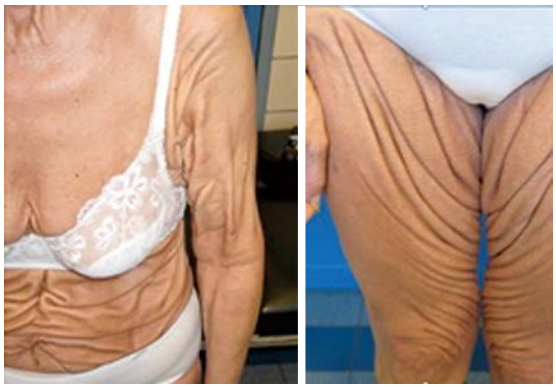

Figure 3 Cutaneous features of a pseudoxanthoma elasticum-like patient with increased amount of generalized thick leathery skin folds.

the patient should contact his/her ophthalmologist immediately[20,23]. Timely treatment with anti-VEGF antibodies, such as bevacizumab or ranibizumab, were shown to be successful in forcing back neovessels and preserving visual acuity[1,20,30]. Prophylactic anti-VEGF therapy has however not been proven to be advantageous[19].

The prevention of CV complications consists of controlling traditional CV risk factors (*e.g.,* smoking, obesity, hypercholesterolemia and diabetes)[1]. Upon diagnosis, a baseline screening should be performed with measurement of blood pressure, assessment of biochemical CV risk factors, echocardiography, determination of the ankle-brachial index and duplex ultrasound of the arteries of the neck and lower extremities. If hypertension is found, further assessment with 24-h blood pressure monitoring and an exercise test should be done. Further CV management is tailored based on the results of this screening, usually comprising an annual checkup by a cardiologist and if necessary initiation of secondary prevention[24]. Since heterozygous carriers suffer CV complications more frequently compared to the general population, they should also undergo a baseline CV screening and regular checkups by a cardiologist[24]. Furthermore, the use of anticoagulants, aspirin and nonsteroidal anti-inflammatory drugs should be avoided as they may elevate the risk of gastrointestinal bleeding[1]. When complications do occur, standard interventional or surgical procedures can usually be applied[24,61].

For the skin problems no prevention is possible and therapeutic options are scarce. When functional problems arise, mainly due to excessive skin folds, plastic surgery can be attempted[61]. Possible post-surgery complications include slower wound healing and apparition of skin lesions in the scars[61-63]. Recently, Salles *et al*[64] described a PXE patient in which skin lesions in the neck were successfully treated with fractional carbon dioxide laser therapy. The post-laser reaction - redness, pain, swelling and crusting - was the same as seen in normal skin. After a follow-up of 2 years, the treatment showed an overall satisfactory esthetic result, showing improvement of the skin texture, irregularity, volume and distensibility. Moreover, lipofilling to reduce esthetically disturbing skin folds, especially in the neck region, is being evaluated in an experimental setting[65].

PXE-LIKE SYNDROME WITH MULTIPLE COAGULATION FACTOR DEFICIENCY

In 2007, Vanakker *et al*[1,47] described a new autosomal recessive disorder which was closely related to PXE and coined it the PXE-like syndrome with multiple coagulation factor deficiency (OMIM#610842). To date, the disorder has been described in 12 patients, 8 of which had molecular confirmation of the clinical suspicion[47,48,66-68].

Clinical characteristics

The initial presentation of the PXE-like syndrome is nearly identical to that of PXE, making it often difficult to distinguish the two diseases in young adults. The natural evolution of the PXE-like disorder is however completely different and characterized by severe cutaneous symptoms with the development of thick and redundant skin folds, not restricted to flexural areas but variably expanding toward limbs and abdomen (Figure 3), a mild retinopathy and a deficiency of the VK-dependent coagulation factors (coagulation factors Ⅱ, Ⅶ, Ⅸ, Ⅹ)[1,47,48]. Furthermore, subclinical atherosclerosis and cerebral aneurysms have been described[47].

Pathogenesis

Biallelic mutations have been described in the *GGCX* gene, encoding a gamma-carboxylase enzyme which performs an essential post-translational modification step of a number of so-called VK-dependent proteins, including clotting factors and mineralization inhibitors (Figure 1). It was shown that the PXE-like mutations result in a reduced activity of the enzyme, thus leading to inadequate carboxylation (or activation) of these VK-dependent proteins. This causes a deficiency of coagulation factors and creates an environment which favors ectopic mineralization[1].

Diagnosis

The diagnosis of PXE-like syndrome is relatively straightforward when typical skin lesions are seen in combination with a deficiency in the VK-dependent clotting factors. Biochemically, a prolonged prothrombin time can be found, though the coagulation factor deficiency can be very mild[47]. In young individuals, the diagnosis should be considered in every patient suspected of having PXE in whom no *ABCC6* mutations are found. Histopathology shows fragmentation and calcification of the mid-dermal elastic fibers, being located in the periphery of the fiber[69]. Light microscopy will not allow differentiating with PXE, but on electron microscopy the elastic fibers are more ragged and the calcification is located in the periphery of the fibers

Table 3 Differential diagnosis of the pseudoxanthoma elasticum-like syndrome[19,47]

Disease	Distinct differences with PXE-like syndrome
PXE (*AR*; *ABCC6* gene)	More severe CV and ophthalmological manifestations Skin lesions are less severe and restricted to flexural areas No coagulation factor deficiency associated EM: mineralization in the core of the EF
Cutis laxa	No retinopathy No deficiency of coagulation factors Atherosclerosis and cerebral aneurysm are infrequent Histopathology: scarce and mottled elastic fibers, no mineralization

ABCC6: Adenosine triphosphate-binding cassette, subfamily C, member 6; AR: Autosomal recessive; CV: Cardiovascular; EF: Elastic fiber; EM: Electron microscopy; PXE: Pseudoxanthoma elasticum.

(compared to fiber core mineralization in PXE). The diagnosis can be confirmed by *GGCX* sequencing[47].

Differential diagnosis

The differential diagnosis of PXE-syndrome is summarized in Table 3.

Management

The management of PXE-like patients is similar to that of patients with classic PXE. In most, treatment of the coagulation deficiency is not necessary, though the use of anticoagulants is not advised. In rare cases, supplementation with VK may be useful[47].

GENERALIZED ARTERIAL CALCIFICATION OF INFANCY

Generalized arterial calcification of infancy (GACI; OMIM#20800) is an early-onset, autosomal recessive disorder, which has only been described in approximately 100 mostly Caucasian patients[1,70]. The disease typically affects infants of less than 6 mo of age[71,72].

Clinical characteristics

GACI is characterized by arterial stenosis, resulting from myointimal proliferation of muscular arteries, and early-onset severe myocardial ischemia due to extensive deposition of hydroxyapatite in the inner elastic lamina of medium- and large-sized arteries[1,70,73]. Complications include myocardial infarction, hypertension and congestive heart failure, leading to early demise[1]. Other possible manifestations include dermatological and ophthalmological findings typical of PXE, extravascular (mostly periarticular) calcifications, hearing loss and development of hypophosphatemic rickets after infancy[49,70,71,74-77]. The majority of patients die before the age of 1, with the highest fatality rate in the first six months of life, most commonly due to myocardial infarction, congestive heart failure, multiple organ failure or persistent arterial hypertension[71,72]. Recently, Rutsch *et al*[71] reported a mortality rate of 55%, with a marked decrease in the mortality of patients, which survived the first 6 mo. In some of these, spontaneous resolution of the mineralization was seen[78,79].

Pathogenesis

GACI is caused by inactivating mutations in the *ENPP1* gene, which encodes ectonucleotide pyrophosphatase/phosphodiesterase 1. Under normal conditions, ENPP1 is associated with the outer plasma membrane of VSMCs in arteries and generates extracellular PPi through hydrolysis of ATP to adenosine monophosphate (AMP)[2]. PPi is a potent calcification inhibitor, which was already shown to hinder mineral crystal growth by binding to the crystal surface in osteoblast cultures[1,2,17].

Diagnosis

Neonates with GACI can present with rather aspecific symptoms, such as poor feeding and respiratory distress. Consequently the diagnosis is often only established by detecting arterial calcification using plain radiography, ultrasound or computed tomography. Typically, diffuse vascular and periarticular ectopic mineralization is found. GACI should be considered antenatally when ultrasonographic anomalies include arterial calcifications, hydrops, abnormal cardiac contractility and/or hyperechoic kidneys[80]. Confirmation of the diagnosis is possible through molecular analysis of the *ENPP1* gene which detects mutations in approximately 70% of cases[49,71,81,82]. When no mutations can be found in *ENPP1*, *ABCC6* sequencing should be performed, due to an overlap in the phenotypes of both diseases[70,73]. An arterial biopsy shows fragmentation in the integral elastic lamina with calcium deposition and fibrointimal hyperplasia causing luminal narrowing, which can occur in places devoid of mineralization[72,83]. Conversely, mineralization can occur without luminal narrowing[84]. Apart from calcium, the depositions also contain iron and mucopolysaccharides[84,85]. The lesions are surrounded by a giant cell reaction[86].

Differential diagnosis

The differential diagnosis of GACI is summarized in Table 4.

Management

The treatment options in GACI are limited and rely mostly on the use of bisphosphonates, such as etidronate and pamidronate, which are analogs of PPi. These bisphosphonates possibly act through decreasing bone turnover, inhibiting further growth of mineralized crystals and/or providing an alternative form of PPi that may influence the regulation of mineralization[91]. Vascular calcifications have been reported to disappear under bisphosphonate therapy within a variable time period (2, 5 wk to 2 years). Calcifications do not tend to reappear after cessation of the therapy, although arterial stenosis persists[92,93]. Since prolonged etidronate use in GACI

Table 4 Differential diagnosis of generalized arterial calcification of infancy[73,78,87-90]

Disease	Distinct differences with GACI
PXE (AR; *ABCC6*)	GACI-like phenotype possible, however infrequent CV phenotype usually less severe No onset in infancy Dermatological and ophthalmological phenotypes more prominent
Singleton-Merten Calcification (AD; unknown causal gene)	Dental anomalies (delayed eruption and early loss of permanent teeth, alveolar bone erosion) Osteopenia Acroosteolysis
Metastatic calcification due to hypervitaminosis D, hyperparathyroidism or end-stage renal disease	Different distribution of extravascular calcification (renal tubules, bronchial walls and basal mucosa and muscularis mucosae of the stomach) Microscopic vascular changes in media instead of intima
Congenital syphilis	Only calcification of the (ascending) aorta Diagnosed mainly in adults Hutchinson teeth, interstitial keratitis, saber tibiae, saddle-shaped nose Histopathology: endarteritis obliterans of vasa vasorum with perivascular plasma cells, lymphocytic cuffing and adventitial fibrosis
Iliac artery calcification in healthy infants	Only calcification in the common and internal iliac arteries

ABCC6: Adenosine triphosphate-binding cassette, subfamily C, member 6; AD: Autosomal dominant; AR: Autosomal recessive; CV: Cardiovascular; GACI: Generalized arterial calcification of infancy; PXE: Pseudoxanthoma elasticum.

patients has been linked to severe skeletal toxicity, bisphosphonate therapy should be closely monitored and according to some, should be stopped as soon as the calcifications have disappeared[94]. Nevertheless, the prognosis of patients remains poor with only few long-term survivors, the oldest GACI patient being 25[1,71,79,95]. Recently, Towler *et al*[96] suggested that restoring PPi levels by inhibition of alkaline phosphatase (ALP) and/or upregulation of vascular ENPP1 or ANKH-mediated secretion of intracellular PPi may serve as possibilities to limit vascular calcification.

KEUTEL SYNDROME

Since its first identification by Keutel *et al*[97] in 1971, approximately 30 cases have been described of Keutel syndrome (OMIM#245150), which is an autosomal recessive multisystem disease with an age of onset in childhood (5-15 years)[97,98].

Clinical characteristics

Keutel syndrome is mainly characterized by peripheral pulmonary stenosis, abnormal cartilage ossification or calcification of typically (para)tracheal, bronchial and rib cartilages as well as auricular and nose cartilage[99]. Less frequently soft tissue calcification, *i.e.*, of blood vessels, brain and kidneys, occurs[1]. Other clinical features include CV (ventricular septal defect, pulmonary artery hypoplasia, hypertension) respiratory (recurrent respiratory infections), skeletal (brachytelephalangism, typically sparing the 5th distal phalanx, height below the 25th percentile), neurological symptoms (subnormal intelligence quotient (IQ) in multiple cases) and recurrent otitis media causing inner ear or mixed deafness. Patients have a typical facial gestalt with mild midface hypoplasia, a depressed nasal bridge, small alae nasi and a deep philtrum[100-104]. A long-term follow-up of 4 sisters with Keutel syndrome showed that all clinical manifestations were progressive. Further, these patients developed skin lesions, *i.e.*, multiple erythematous, irregularly bordered macular lesions without induration, typically after the age of 30. Skin biopsy of these lesions failed to show calcification or ossification and loss of elastic fibers was only seen in the papillary dermis[99]. Nevertheless, the prognosis of Keutel syndrome is good in the majority of patients, with life expectancy mainly depending on the severity of the pulmonary complications[99].

Pathogenesis

Keutel syndrome is caused by loss-of-function mutations in the *MGP* gene, encoding matrix gla protein[1]. MGP is an inhibitor of the pro-osteogenic BMP2-Smad-RUNX2 pathway, by inhibiting BMP2 to bind to its receptor. Consequently, MGP, expressed in chondrocytes, functions as a local mineralization inhibitor under physiological conditions (Figure 1)[105,106]. Impairment of its inhibitory function favors pro-mineralizing signaling, leading to ectopic mineralization[98]. Moreover, Cranenburg *et al*[107] reported a patient in whom the levels of carboxylated/uncarboxlated MGP were very low, unresponsive to VK supplementation, but in whom high levels of phosphorylated MGP were found. Phosphorylation is a VK-independent posttranslational modification of MGP which may allow binding of calcium crystals in the absence of optimal carboxylation. It was hypothesized that this phosphorylation-dependent residual MGP activity might be sufficient to prevent development of arterial calcification[108].

Diagnosis

The majority of Keutel syndrome patients are diagnosed during childhood based on clinical presentation and radiographic examinations, with abnormal cartilage calcification and brachytelephalangism as major signs. The clinical diagnosis can be confirmed by sequencing of the *MGP* gene, in which to date 7 loss-of-function mutations have been reported[98,104,109].

Differential diagnosis

The differential diagnosis of Keutel syndrome is summarized in Table 5.

Management

No etiologic treatment exists for Keutel syndrome, hence management is merely symptomatic, including

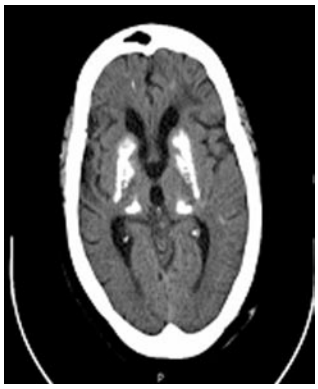

Figure 4 Transverse computed tomography of the brain displaying symmetrical bilateral ganglia calcification in an idiopathic basal ganglia calcification patient.

(angiographic) dilatation of peripheral artery stenosis and bronchodilating agents for respiratory symptoms (dyspnea and wheezing); the latter however can be inefficient in certain patients[99,108]. Most patients develop hypertension before the age of 20, which can be treated with antihypertensive medication such as perindopril, amlodipine or nifedipine[99].

IDIOPATHIC BASAL GANGLIA CALCIFICATION

Idiopathic basal ganglia calcification (IBGC) is a rare neurodegenerative disorder with unknown prevalence. The disease is sometimes referred to as Fahr's disease, although the the patient Fahr described primarily had mineralization in blood vessels of the white matter of the brain[113]. IBGC affects young to middle aged adults, with an average onset in the 3rd or 4th life decade; however the disease has also been described in childhood[114-116].

Clinical characteristics

IBGC is characterized by bilateral and (almost) symmetrical basal ganglia calcifications (Figure 4)[116]. Ectopic mineralization may also occur in other brain regions, including the nucleus dentatus, thalamus, cerebral cortex and centrum semiovale[116,117]. Neurological symptoms include neuropsychiatric (cognitive impairment, depression, hallucinations, delusions, manic symptoms, anxiety, schizophrenia-like psychosis, personality changes) and movement disorders (*a.o.* parkinsonism, ataxia due to cerebellar involvement, tremor and paresis), as well as headache, vertigo, stroke-like events, orthostatic hypotension, dysarthria, seizures and papilledema due to raised intracranial pressure[116,118]. Both sporadic and familial IBGC cases have been reported, the latter predominantly with autosomal dominant inheritance[116].

Pathogenesis

To date, mutations in 3 genes have been associated with IBGC, *i.e.,* solute carrier family 20 (phosphate transporter), member 2 (*SLC20A2*; OMIM*158378), the beta polypeptide of platelet-derived growth factor (*PDGFB*; OMIM*190040) and platelet-derived growth factor receptor, beta (*PDGFRB*; OMIM*173410). So far, no genotype-phenotype correlation has been found[119]. The *SLC20A2* gene, encoding a Pi transporter (also known as PiT2 which belongs to the type III sodium-dependent phosphate transporter family), is expressed abundantly in a variety of tissues and likely plays a housekeeping role in cellular phosphate uptake (Figure 1)[119,120]. Mutations in the gene have been described in more than 40 IBGC families worldwide and *in vitro* resulted in impaired Pi transport, leading to accumulation of this pro-mineralizing factor[119,121,122].

More recently, a few IBGC patients were reported harboring mutations in *PDGFB* or *PDGFRB*[123-128]. In animal models, *Pdgfrb* has been identified as an essential mediator in the development of pericytes in brain vessels, which have a key role in the maintenance of the blood-brain barrier (BBB). The BBB is hypothesized to be defective in IBGC[123]. Moreover, Villa-Bellosta *et al*[129] found that the PDGFB-PDGFRB pathway seems to be involved in phosphate-induced calcifications in VSMCs by downregulating SLC20A2. All these data suggest that cerebral phosphate homeostasis plays a role in the development of vascular mineralization[129]. The mineralization generally develops within the vessel wall and in the perivascular space, ultimately extending to the neuron. Upon progression, the calcifications start to compress the vessel lumen, which causes impaired blood flow, starting off a vicious circle with further neural tissue damage and mineral deposition. The mineral depositions tend to vary in composition according to

Table 5 Differential diagnosis of Keutel syndrome[98,110-112]

Disease	Distinct differences with Keutel Syndrome
X-linked chondrodysplasia punctata (XL; *ARSE* gene)	Ichtyosis Cataracts Microcephaly, intellectual disability ASD, VSD, PDA Failure to thrive in infancy Age at diagnosis: usually infancy
Warfarin embryopathy	Pectus carinatum Congenital heart defects different from those seen in Keutel syndrome (ASD, PDA, ventriculomegaly)
Combined Vitamin K-dependent coagulation factor deficiency	Easy bruising, mucocutaneous bleeding Osteoporosis with normal serum markers
Relapsing polychondritis	Age at diagnosis: 40-60 yr Cartilage inflammation, possibly progressing to destruction Aortic or mitral valvular disease Facies: saddle nose deformity, multifocal, tender chondritis, including variably floppy or calcified auricles Cranial neuropathies, hemiplegia

ARSE: Arylsulfatase E; ASD: Atrial septal defect; PDA: Patent ductus arteriosus; VSD: Ventricular septal defect; XL: X-linked.

Table 6 Differential diagnosis of idiopathic basal ganglia calcification[116,140-143]

Disease	Distinct differences with IBGC
Basal ganglia calcification as incidental finding on CT scans/ aging	In 1% of CT scans Usually benign No clear etiology, especially when in older patients Asymptomatic
Hypoparathyroidism	Early onset: childhood/adolescence Hypoparathyroidism, hypocalcemia, hyperphosphatemia Alopecia, dry hair Dental dysplasia, caries Moniliasis Albright osteodystrophy symptoms (short stature, round facies, obesity, short metacarpals/metatarsals)
Pseudohypoparathyroidism (AD/maternal imprinting; *GNAS*, *GNASAS1* and *STX1A* gene)	Early onset: childhood/adolescence Hyperparathyroidism, hypocalcemia, hyperphosphatemia Baseline cAMP in urine low; after Ellsworth Howard test subnormal Intellectual disability Albright osteodystrophy symptoms
Pseudo-pseudohypoparathyroidism (AD/paternal imprinting; *GNAS* gene)	Similar phenotype as pseudohypoparathyroidism Normal serum PTH, calcium and phosphorus Intellectual disability (more obvious than in PHP)
Kenny-Caffey syndrome, type 1 (AR; *TBCE* gene)	Growth delay Cortical thickening of long bones Hypocalcemia, hypoparathyroidism
PKAN (AR; *PANK2* gene)	Early onset (10% > 10 yr) Pigmentary retinopathy
DRPLA (AD; CAG expansion in *DRPLA* gene)	Phenotype similar to IBGC
Neuroferritinopathy (AD; *FTL* gene)	Dysphagia
PLOSL (AR; *TYROBP* and TREM2 gene)	Radiography: polycystic osseous lesions Frontal lobe syndrome
Cockayne syndrome; Aicardi-Goutières syndrome	Onset in infancy/early childhood

AD: Autosomal dominant; AR: Autosomal recessive; CAG: Cytosine, adenine, guanine; cAMP: 3'-5'-cyclic adenosine monophosphate; CT: Computed tomography; DRPLA: Dentatorubropallidoluysian atrophy; *FTL*: Ferritin light chain; *GNAS*: GNAS complex locus; *GNASAS1*: GNAS complex locus, antisense transcript 1; IBGC: Idiopathic basal ganglia calcification; PANK2: Panthothenate kinase 2; PHP: Pseudohypoparathyroidism; *PKAN*: Panthothenate kinase-associated neurodegeneration; PLOSL: Polycystic lipomembranous osteodysplasia with sclerosing leukoencephalopathy); PTH: Parathyroid hormone; STX1A: syntaxin 1A; *TBCE*: Tubulin-specific chaperone E; TREM2: Triggering receptor expressed on myeloid cells 2; *TYROBP*: Tyro protein tyrosine kinase-binding protein.

their anatomical site and the proximity to vasculature calcifications, containing components such as calcium phosphate and carbonate; other compounds including glyconate, mucopolysaccharide and metals (iron, copper, magnesium, zinc, aluminum, silver and cobalt) may also be found[116]. Abnormal iron metabolism in IBGC has been described in a single case, showing elevated serum ferritin, reduced levels of serum iron and iron-binding capacity. At autopsy iron depositions were found in the liver, the spleen, the bone marrow and the brain[130]. More recent reports confirm abnormalities in metal metabolism (iron, copper, zinc), although there is no consensus whether the metal levels are elevated (cerebrospinal fluid) or reduced (hair) in IBGC patients[131,132].

Diagnosis

IBGC diagnosis is supported by the following criteria: (1) bilateral calcification of basal ganglia; (2) progressive neurologic dysfunction; (3) absence of biochemical abnormalities; (4) absence of infectious, traumatic or toxic cause; and (5) a significant family history (although sporadic IBGC cases have also been described)[116].

However, the diagnosis can only be established by obtaining a computed tomography (CT) or magnetic resonance imaging (MRI) scan of the brain, showing bilateral, almost symmetric calcifications of one or more of the affected brain regions, and ruling out other abnormalities (showing bilateral basal ganglia calcifications, and developmental defects)[116,133-136]. Other possible investigations, which are typically normal in IBGC patients, include blood and urine testing for hematologic and biochemical (ALP, serum creatinine, osteocalcin, serum lactic acid at rest and after exercise, 1,25-dihydroxyvitamin D_3, serum calcium, phosphorus, magnesium, calcitonin, heavy metals and parathyroid hormone (PTH)) parameters and an Ellsworth Howard test (showing a 10-20 fold increase of urinary 3'-5'-cyclic AMP (cAMP) after stimulation with 200 U of PTH)[116,137-139]. Neurological tests are usually normal (electroencephalography, nerve conduction studies, pattern shift visual-evoked potential studies) or show mild abnormalities (brainstem auditory-evoked potentials)[116].

Genetic testing can confirm the IBGC diagnosis. Sequencing of *SLC20A2* is the first choice, as well as deletion/duplication analysis if no mutation is found, with a mutation detection rate of 40%. If no mutations are found, *PDGFRB* and *PDGFB* sequencing can be performed; the precise mutation detection rate is currently unknown. If no molecular confirmation can be obtained, other (genetic) causes of brain calcification should be considered (Table 6), before establishing a clinical diagnosis of IBGC[116].

Differential diagnosis

Symmetrical calcifications of the basal ganglia are not specific to IBGC and a variety of familial and non-familial conditions should be considered. It should be noted that these calcifications can also be incidental findings on CT scan, especially in older individuals (Table 6)[116,140].

Treatment

Since no etiologic treatment is available, management and treatment options focus on symptomatic relief[116,117,120]. Pharmacologic treatment (*e.g.*, anxiolytics

and antidepressants) for the psychiatric and movement symptoms can be attempted[117,120]. Possibly, an early causative treatment may reverse the calcification process, causing complete recovery of mental functions, which was already described in hypoparathyroidism, another basal ganglia causing disorder, provided that an intervention target can be identified[116].

ARTERIAL CALCIFICATION DUE TO CD73 DEFICIENCY

Arterial calcification due to CD73 deficiency (ACDC), also referred to as calcifications of joints and arteries, is an autosomal recessive disease, which usually takes an onset in young adulthood[16,144].

Clinical presentation

ACDC is mainly characterized by prominent and often symptomatic calcification of the large arteries of the lower extremities (iliac, femoropopliteal and tibial arteries), typically sparing the coronary circulation[16,144]. Furthermore, periarticular calcifications of (large and smaller) joints of the lower extremities have been described[144]. Typical symptoms include claudication, hemodynamically significant peripheral obstructive artery disease of the lower limbs, joint swelling and pain[144]. The disease seems relatively rare, being only reported in 3 Caucasian families[144,145].

Pathogenesis

To elucidate the molecular etiology of ACDC, genome-wide homozygosity mapping was performed in three families, revealing homozygous and compound heterozygous loss-of-function mutations in the *NT5E* gene[144,145]. *NT5E* encodes the glycosyl phosphatidylinositol (GPI)-linked plasma membrane CD73 ecto-enzyme, which has 5' ectonucleotidase activity and thus converts AMP to extracellular adenosine and Pi[146]. The enzyme is located on the plasma membrane of vascular cells, supplying adenosine to cell surface receptors[145]. Adenosine is produced immediately downstream of ENPP1 in the extracellular ATP-degradation pathway on the surface of vascular cells, and a lower adenosine level leads to impaired inhibition of TNAP[16,144]. St Hilaire *et al*[144] hypothesized that increased TNAP activity reduces PPi levels, allowing calcification to occur (Figure 1). Since the vascular calcification in ACDC seems to be limited to the lower extremities, it is likely that members of other ectonucleotidase families, such as ectonucleoside triphosphate diphosphohydrolase 1 or CD39 (*ENTPD1*; OMIM*601752) and its isoforms or cardiac ectonucleoside triphosphate diphosphohydrolase 6 or CD39L2 (*ENTPD6*; OMIM*603160) (members of the ectonucleoside triphosphate diphosphohydrolase (E-NTPDase) family), may compensate NT5E activity in other vascular beds[16,147]. An alternative explanation for this predilection may be the particular distribution of adenosine receptors in these vascular beds[148].

Diagnosis

An ACDC diagnosis can be established based on clinical presentation and a full radiographic workup of the patients, as well as determination of the ankle-brachial index, which should be reduced. Plain radiography can visualize the vessel calcifications; magnetic resonance angiography and especially CT angiography can show diffuse and gross calcification of obstructing lesions. Biochemical indices, including serum electrolytes, cholesterol and vitamin D-levels, PTH, C-reactive protein, rheumatoid factor and erythrocyte sedimentation rate should all be normal. The clinical suspicion can be confirmed by *NT5E* sequencing[144].

Differential diagnosis

Other - often not - hereditary causes of vascular calcification have to be excluded, *e.g.,* diabetes mellitus type 2 and impaired renal function[144]. Since joint swelling and pain was present in all three described families, rheumatologic diseases should also be excluded[144].

Management

Because of the rarity of the disease, no treatment guidelines are available. Bisphosphonates, which were proven to be successful in GACI, possibly by restoring PPi levels, may also be a good treatment option in ACDC[91,144]. Adenosine deficiency could be addressed using dipyridamole, which inhibits its cellular reuptake *in vitro* and *in vivo*. Other possible therapeutic options include adenosine receptor agonists or direct TNAP inhibitors (*e.g.,* lansoprazole), all of which need to be further investigated[144].

HYPERPHOSPHATEMIC FAMILIAL TUMORAL CALCINOSIS

Contrary to the disorders described above, autosomal recessive hyperphosphatemic familial tumoral calcinosis (HFTC; OMIM#211900), is characterized by metastatic mineralization[1,149]. Patients usually show first signs in the first or second decade of life[150].

Clinical characteristics

The most prominent clinical manifestation of HFTC is periarticular mineralization of the skin and subcutaneous tissue, mainly affecting the upper limbs and hip regions, although involvement of other localizations (spine, temporomandibular joints, metacarpals/metatarsals and popliteal space) have also been reported[151]. The calcium salt depositions usually present as firm painful tumorlike masses, which may gradually enlarge over a period of years, causing functional problems including restricted joint mobility[149,151]. Complications of the overlying skin, including pain, infection and ulceration, can cause scarring and deformity[1,149,151]. Other possible manifestations of the disorder are dental abnormalities and retinal AS[152].

Pathogenesis

HFTC can be caused by mutations in UDP-N-acetyl-alpha-D-galactosamine: polypeptide N-acetylgalactosaminyltransferase 3 (*GALNT3*; OMIM*601756), fibroblast growth factor 23 (*FGF23*; OMIM*605380) or klotho (*KL*; OMIM + 604824), all of which are key regulators of the phosphate metabolism[1]. GALNT3 protects intact FGF23, a phosphaturia-causing protein, from proteolytic processing by O-glycosylation of Threonine residue 178 in a subtilisin-like proprotein convertase (SPC) recognition sequence motif. In this way, FGF23 is activated and enabled to secrete from the cell, while this glycosilation also competitively inhibits proteolytic FGF23 cleavage by proteases. Hence, this glycosylation step is proposed to be a post-translational regulatory model. In the presence of (nonsense/missense/splice-site) *GALNT3* mutations, intact FGF23 is cleaved prior to secretion which leads to an accumulation of fragmented FGF23 and a reduced amount of active FGF23, causing hyperphosphatemia[153,154]. In physiological conditions FGF23 binds to the FGF receptor 1, of which KL, a β-glucuronidase, is an important co-receptor, inducing high affinity interaction between FGF23 and its receptor. This activates the further downstream effects of this pathway, including the maintenance of serum phosphate levels within the normal range by increasing renal phosphate excretion and both a reduction of synthesis rate and acceleration of the degradation of 1,25-dihydroxyvitamin D_3 to reduce intestinal phosphate absorption (Figure 1)[155,156]. Moreover, KL works independently from FGF23 as an enzymatic inhibitor of renal NaPi-2a (sodium/phosphate cotransporter) transporter activity - which requires glucuronidase activity, subsequent proteolytic degradation and possibly internalization of the transporter - eventually leading to reduced renal expression of the transporter[157]. FGF23 fulfills its biological functions in a tissue-specific way, which is likely to be regulated by the limited local distribution of KL[155]. Inactivating mutations in FGF23 as well as missense mutations in KL cause FGF23 deficiency. Consequently renal phosphate reabsorption and 1,25-dihydroxyvitamin D_3 synthesis is increased, leading to elevated serum concentrations of phosphate, 1,25-dihydroxyvitamin D_3 and calcium and ectopic mineralization[155].

Diagnosis

Next to clinical examination and family history, the diagnosis of HFTC is mainly based on a full radiographic workup: (1) Plain radiographs show the typical appearance of periarticular amorphous, multilobulated and cystic calcifications[158]; (2) CT, showing cystic loculi with fluid-fluid levels caused by calcium layering; (3) MRI imaging, showing lesions of inhomogeneous intensity, help to document the extent and interconnectivity of individual lesions and can help to determine possible surgical approaches; (4) scintigraphy, using a phosphate compound radiolabel (technetium-99m methylene diphosphonate) is helpful in determining the activity level of the disease; and (5) ultrasonography can help to localize fluid collections[149]. A typical feature of HFTC is the absence of erosion/bone destruction by adjacent soft-tissue masses[149]. Biochemically, hyperphosphatemia with normocalcemia, normal or slightly elevated 1,25-dihydroxyvitamin D_3, hypoparathyroidism and low intact FGF23 proteins levels can be found[152]. A biopsy should be avoided, because of the risk of an infection and should only be done as a last resort. Histopathology shows that mineralization depositions fill up the cystic loculi (active stage), which causes them to become encapsulated by fibrous tissue, eventually ending the mineralization process (relative latent stage)[150].

Differential diagnosis

The differential diagnosis of HFTC is summarized in Table 7.

Management

HFTC should be treated according to the location, size of the lesion and its relations to its environment. A first treatment option is medically reducing hyperphosphatemia through phosphate depletion, by dietary phosphorus restriction and/or the administration of phosphate binding chelating agents such as aluminum hydroxide. This method has a variable success rate, both in normo- and hyperphosphatemic cases. When combined with acetazolamide, which induces phosphaturia, a synergistic effect occurs, especially in the hyperphosphatemic form of familial tumoral calcinosis[149].

A second treatment option is early surgical resection of the lesions; however a considerable recurrence rate of the lesions, which have the tendency of growing faster, poses a major problem. Therefore it is very important that resections contain the lesion, and preferably should contain a wider perilesional area/band, including the hypervascular region beyond the periphery of the lesion. Broad resections can cause problems in case of voluminous lesions, which may require extensive reconstructive surgery[149]. Surgery is indicated when lesions are painful, recurrently infected, ulcerated or when functional impairment occurs[149]. Surgical complications include: (1) prolonged drainage of the wound, possibly leading to delayed wound healing and sinus tract formation; (2) secondary infections due to chronic wound problems, especially when the disease is extensive or when resection is incomplete; and (3) recurrence, which is more frequently seen after incomplete resection[167].

During the active stage of the disease, primary medical treatment of HFTC is justified and may even be recommended, because the postresection recurrence rate of lesions is even higher in this stage. In the relative latent stage, encapsulation occurs which hinders ion exchange, thus making surgery more advantageous[149]. Alternative treatment options, including steroids,

Table 7 Differential diagnosis of hyperphosphatemic familial tumoral calcinosis[149,151,158-166]

Disease	Distinct differences with HFTC
Calcinosis universalis	Calcium depositions in tendons and muscle tissues Normophosphatemia High hemosedimentation Microcytic and hypochromic anemia
Calcinosis circumscripta	Adult onset Local calcinosis Fingers symmetrically affected
Calcific tendinitis	Adult onset Calcification limited to tendons
Synovial chondromatosis	Lesions arising from synovial tissue Widespread throughout the body Not all lesions are calcified
Osteosarcoma	Long bone malignant tumor 2nd life decade or late adulthood No subcutaneous/skin lesions
Fibrodysplasia ossificans progressiva (AD; *ACVR1* gene)	Hallux valgus, monophalangism and/or malformed first metatarsal Sporadic episodes of painful soft tissue swellings (flare-ups) in 1st life decade
Tophaceous gout	Severe form of gout Severe joint deformity, chronic pain and functional decline More prominent in Asian population (slow metabolizers) and in young men with strong genetic predisposition
Calcific myonecrosis	Post-traumatic (time interval of several years possible) Lower limbs only
NFTC (AR; *SAMD9* gene)	Reddish-to-hyperpigmented skin lesions during the first year of life (preceding calcified nodules) Severe conjunctivitis and gingivitis Normophosphatemia
Secondary tumoral calcinosis	Renal insufficiency, hyperparathyroidism, or hypervitaminosis D
Rheumatological diseases	Usually normophosphatemia and - calcemia Possibly positive results in antinuclear, anti-Smith, anti-centromere and anti-scleroderma antibodies, which should all be negative

ACVR1: Activin A receptor, type 1; AD: Autosomal dominant; AR: autosomal recessive; HFTC: Hyperphosphatemic familial tumoral calcinosis; NFTC: Normophosphatemic familial tumoral calcinosis; SAMD9: Sterile alpha motif domain-containing protein 9.

bisphosphonates, calcitonin and radiotherapy, have not been proven to be effective[149].

CONCLUSION

Ectopic mineralization disorders comprise a wide range of heterogeneous diseases, which can affect a variety of tissues, causing important health problems. Insights in the mechanisms that cause these diseases have led to the observation that many - if not all - are closely related to one another from a mechanistic point of view. The considerable differences in clinical presentation and natural course however suggest that our current knowledge is merely the proverbial tip of the iceberg and that the subtle mechanisms which render each disease to be unique are still largely to be uncovered. Nevertheless, the pathophysiological knowledge to date has already led to several successful treatment options and a number of promising targets for the future. As such, ectopic mineralization diseases are a fine example for the interaction between the variome and the phenome.

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REVIEW

Role of quorum sensing in bacterial infections

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Abstract

Quorum sensing (QS) is cell communication that is widely used by bacterial pathogens to coordinate the expression of several collective traits, including the production of multiple virulence factors, biofilm formation, and swarming motility once a population threshold is reached. Several lines of evidence indicate that QS enhances virulence of bacterial pathogens in animal models as well as in human infections; however, its relative importance for bacterial pathogenesis is still incomplete. In this review, we discuss the present evidence from *in vitro* and *in vivo* experiments in animal models, as well as from clinical studies, that link QS

systems with human infections. We focus on two major QS bacterial models, the opportunistic Gram negative bacteria *Pseudomonas aeruginosa* and the Gram positive *Staphylococcus aureus*, which are also two of the main agents responsible of nosocomial and wound infections. In addition, QS communication systems in other bacterial, eukaryotic pathogens, and even immune and cancer cells are also reviewed, and finally, the new approaches proposed to combat bacterial infections by the attenuation of their QS communication systems and virulence are also discussed.

Key words: Quorum sensing; Virulence; Infections; *Pseudomonas aeruginosa*; *Staphylococcus aureus*; Animal models

Core tip: In this manuscript we discuss the basics aspects of quorum sensing (QS) and its relationship with human infections, focusing in two major QS bacterial models, the opportunistic Gram negative bacteria *Pseudomonas aeruginosa* and *Staphylococcus aureus*.

Castillo-Juárez I, Maeda T, Mandujano-Tinoco EA, Tomás M, Pérez-Eretza B, García-Contreras SJ, Wood TK, García-Contreras R. Role of quorum sensing in bacterial infections. *World J Clin Cases* 2015; 3(7): 575-598 Available from: URL: http://www.wjgnet.com/2307-8960/full/v3/i7/575.htm DOI: http://dx.doi.org/10.12998/wjcc.v3.i7.575

INTRODUCTION

Several important bacterial pathogens, like *Pseudomonas aeruginosa* (*P. aeruginosa*), *Staphylococcus aureus* (*S. aureus*), and *Vibrio cholerae,* utilize quorum sensing (QS) cell communication to coordinate the expression of multiple virulence factors and associated behaviors such as swarming and biofilm formation, once a population size threshold is reached. QS systems consist of an enzyme that catalyzes the synthesis of the signal (such as acyl-homoserine lactones or cyclic peptides) and a receptor that binds the signal and reprograms the expression of several genes, including those encoding the enzyme that produces the signal, creating a positive feedback loop. In bacterial pathogens, most of the QS controlled genes codify several different virulence factors, such as proteases, toxins, and adhesins[1]. The expression of QS controlled phenotypes is energetically costly to the cells and only provides an advantage if it is expressed when cells reach high densities[2,3]; hence, in the context of bacterial infections, the expression of QS regulated virulence factors is delayed until a sufficient bacterial load is achieved and once this threshold is reached, bacteria coordinate their attack against the host, which maximizes the probability of establishing the infections and disseminating them, hence increasing the pathogen fitness. In fact, QS along with subversion of the immune system are the main factors that determine the bacterial infectious doses. Hence those bacterial pathogens that need small doses to infect, generally lack QS systems but are very effective at inactivating the immune response by killing professional phagocytes. In contrast, those bacterial pathogens that need high infectious doses rely in QS for the coordination of the expression of virulence[4]. In this review, the current knowledge about QS control of virulence factors in two main model bacterial pathogens, *P. aeruginosa* and *S. aureus* (which are also responsible for nosocomial and wound infections), will be discussed along with the relationship of their QS systems, its virulence in animal infection models, and the data available from human infections. Furthermore, the role of QS in other important infections and the role of QS in immune and cancer cells are discussed. Finally, proposed novel approaches of blocking QS/virulence as an alternative in fighting recalcitrant bacterial infections are also reviewed.

QS-CONTROLS OF THE EXPRESSION OF VIRULENCE FACTORS *IN VITRO*

P. aeruginosa

P. aeruginosa possesses at least three functional QS circuits; two of them are mediated by N-acyl homoserine lactones (HSL) signals and the other mediated by quinolones (Figure 1). The HSL-QS systems were first described and they were named after the virulence factors that were first identified under their control; hence, the Las system was discovered as a positive regulator for elastase production through the expression of the structural elastase gene lasB[5]. This system (by LasI HSL-acyl-synthase) produces the 3-oxo-C12-homoserine lactone (3-oxo-C12-HSL), that binds its receptor LasR which then dimerizes and binds promoters that contain *las* boxes, turning on the expression of several genes, including lasI, which then in a positive feedback loop increases the production of *3*-oxo-*C*12-HSL, the other HSL mediated QS system was named Rhl since it controls the expression of the biosurfactant rhamnolipids[6]. This system (RhlI) produces N-butyryl-L-homoserine lactone that is sensed by RhlR and also shows positive autoregulation[7]. The third QS system is mediated by different kinds of signals, alkyl quinolones, specifically 2-heptyl-3-hydroxy-4-quinolone(Pseudomonas quinolone signal or PQS) which is synthesized from anthranilate by the products of *pqsABCDEH* genes and sensed by PqsR (MvfR)[8,9]. The three systems are interconnected and function in a hierarchical way[10]; the Las system is the first to become activated, and it in turn it stimulates the Rhl and PQS systems[11,12], while PQS activates Rhl[13] and RhL inhibits PQS[11,14]. Moreover, 3-oxo-*C*12-HSL, the Las signal, is able to bind functional

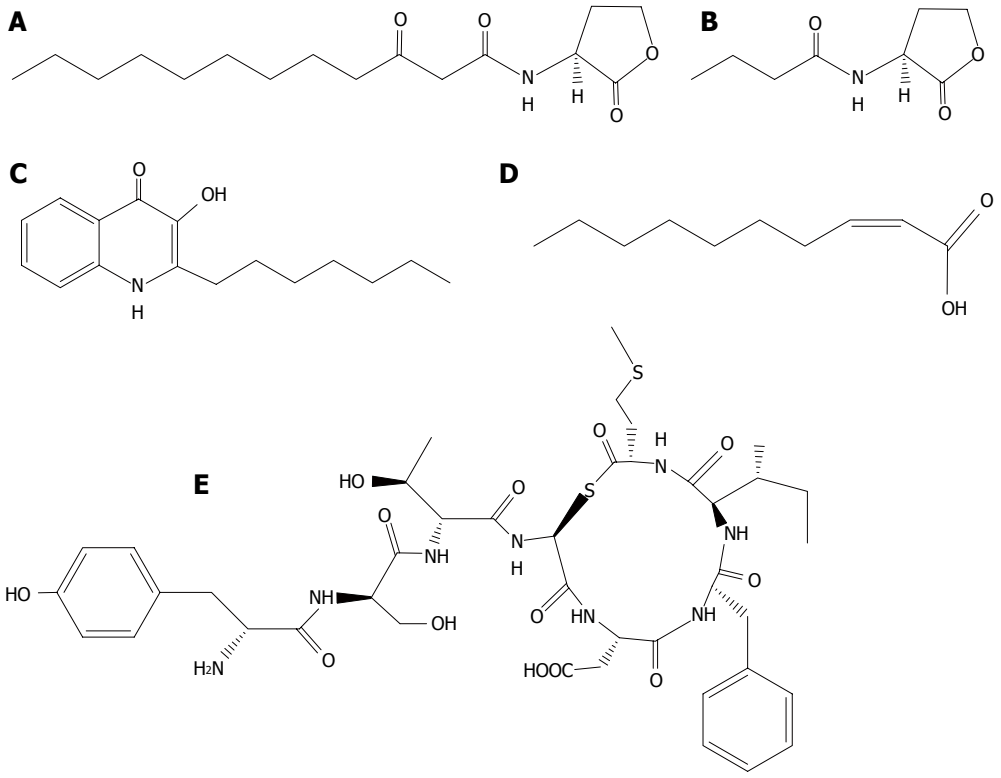


Figure 1 Structures of representative quorum sensing signal molecules of *Pseudomonas aeruginosa*. A: 3-oxo-C12-homoserine lactone; B: N-butyryl-L-homoserine lactone; C: 2-heptyl-3-hydroxy-4-quinolone; D: DSF-like fatty acids, cis-2-decenoic acid) and *S. aureus* (E: AIP group I).

RhlR dimmers, promoting their dissociation and inactivation[15]. In addition to control *lasB* elastase, the Las system also controls the expression of *lasA* elastase, exotoxin A (PA1148), and alkaline protease (PA1246)[16], and the Rhl also controls the expression of the phenazine pyocyanin a pigment able to cause oxidative damage to the eucaryotic host, promoting the production of reactive oxygen species and depleting the host antioxidant defense mechanisms[17], while the PQS system increases the expression of *lasB* elastase and pyocyanin[9]. In fact, the regulation of virulence factors by these 3 QS systems is complex and often overlaps[18]; for example, RhlR is apparently enough to compensate the absence of LasR at least in stationary phase cells in which it promotes the production of exoproteases, pyocyanin, PQS, and the 3-oxo-*C*12-HSL[18,19]. To add even more complexity, recently the role of environmental signals, such as the availability of iron and phosphate in influencing QS systems has been beginning to be explored[20]. In addition, other ions such as calcium strongly influence the production of QS modulated virulence factors such as pyocyanin, and proteases[21] and in fact there is solid evidence that indicates that the chemical composition of the sputum in cystic fibrosis patients promotes the use of the PQS system for communication, preferentially over the HSL systems[22]. Moreover the presence of metabolites like 2,3-butanediol (end product of bacterial fermentation from species that coexist with *P. aeruginosa* in the lung of cystic fibrosis patients) enhance the production of QS controlled virulence factors (phenazines and exotoxin) and improve biofilm formation *via* the Las QS system[23]; hence, the expression of QS-virulence factors *in vivo* is likely influenced by several variables, related with the state of the host as well as the presence or absence of other bacterial species. Indeed, the simultaneous utilization of several QS systems in bacteria, may serve different purposes like identifying community composition[24] or distinguish phases in population development[25], and a recent study shows that the concomitant utilization of Las and Rhl systems allows *P. aeruginosa* to simultaneously assess their population density and the presence of nutrients by combinatorial communication. Therefore, the secretion of QS controlled factors is subjected to "AND-gate" like responses to multiple signal inputs, allowing effective expression of secreted factors in high-density and low mass-transfer environments[26]. Another important role of QS systems in regulating bacterial physiology is that they are implicated in the tolerance against stress[27-29] that allow them to maximize their chances to effectively contend and survive the immune system attack[30], which may be a major determinant for the establishment and progression of *P. aeruginosa* and other pathogens infections.

S. aureus

S. aureus produces several virulence factors and many

of them are regulated by QS. In Gram positive bacteria, regulation by QS is generally mediated by autoinducing cyclic peptides. Specifically for *S. aureus,* QS controls the expression of virulence factors such as hemolysins, leukocidins, cell surface adhesins, exoenzymes, and biofilm formation *via* the Agr system, which relies on the autoinducing peptide (AIP) (Figure 1E). AIPis encoded by *agrD* and consists of 7-9 amino acids, and has a 5-membered thiolactone ring[31-33]; this peptide is secreted by the membrane protein AgrB and activated by the AgrC sensor kinase[1]. The Agr system regulates the expression of several genes by the production of two regulatory RNAs, RNAII and RNAIII[34], which are produced from promoters P2 and P3 respectively[34,35]. Transcription from the *agr* operon (*agrA*, *agrB*, *agrC* and *agrD*) is regulated by a phosphorylated AgrA homodimer from P2[36], while RNAIII is produced by AgrA from P3. RNAIII, which is the effector of the system, upregulates *α*-haemolysin, and increases the production of proteases, toxins, and the synthesis of capsule, while it repress protein A (which allows *S. aureus* to evade opzonization), and the expression of surface adhesions[1,31,34,35,37,38]. Such modulation of the expression of several virulence factors by the Agr system allows *S. aureus* to express a different repertoire of those determinants according to the kind of disease and the environmental conditions including the host status. Noteworthy is that *in vitro* the appearance of clones with diminished QS had been observed; these clones are apparently social cheaters which exploit cooperative individuals without contributing with the production of virulence factors. The presence of cheaters during infections may be very relevant for disease progression, since in controlled experiments, the ratio between cheaters and cooperating individuals strongly affects the mortality rate and extent of infection; *i.e.*, the severity of the infections are inversely proportional to the percentage of cheaters in the population[39]. Among the QS-controlled virulence factors in *S. aureus*, RNAIII is very important since it regulates biofilm formation, and resistance to antibiotics as well as the establishment of chronic infections is intimately related to the biofilm formation abilities of pathogens[40]. However, the *in vivo* biofilms in which *S. aureus* exists can be very complex environments, due the presence of several other bacterial species and their multiple interactions with each other and with the host, hence *in vitro* models for studying *S. aureus* virulence may have the disadvantage of not revealing the real expression of virulence factors. This hypothesis is supported by significant differences in the expression of several virulence factors in *S. aureus* grown in calf serum compared with those grown in defined CDM medium, since in serum the expression of hemolysins, enterotoxins, proteases, iron acquisition factors, and of RNAIII is significantly higher than in standard growth medium, and such differences are partially due the low iron concentration in serum[41,42].

QS-CONTROLS OF THE EXPRESSION OF VIRULENCE FACTORS *IN VIVO P. AERUGINOSA* QS AND VIRULENCE IN ANIMAL MODELS

A number of animal models including the nematode (*Caenorhabditis elegans*), fruit fly (*Drosophila melanogaster*), zebrafish (*Danio rerio*) and mouse (*Mus musculus*), have been used to identify and define the role of virulence determinants in the pathogenesis of *P. aeruginosa*[43,44]. The attenuation of its QS is achieved by two basic strategies: (1) the utilization of mutant strains with QS genes disrupted; and (2) the quenching of QS by treatments that interfere with it; these methods have shown that QS systems as well as QS-independent virulence determinants are required for *P. aeruginosa* infections in animals.

The main animal model that was used to discover the relationship between QS and virulence of *P. aeruginosa* is the nematode *C. elegans*. In 1999 Tan and coworkers, first described conditions to test the role of QS in virulence using this model, showing that the reference strain PA14 kills the nematode either after days (slow killing) or quickly after a few hours (fast killing)[45]. Their evidence indicate that fast and slow killing occur by distinct mechanisms; the slow killing involves an infection-like process and correlates with accumulation of PA14 within worm intestines, while the fast killing is mediated by the production of phenazines(regulated by QS); that increase active oxygen species[45,46]. A third mode by which *P. aeruginosa* can kill *C. elegans is lethal paralysis; this mechanism is mediated by QS since* Darby and coworkers, using QS-less mutant strains of *P. aeruginosa*, found that the lethal effect is associated with a rapid neuromuscular paralysis, caused by the action of diffusible unidentified factors whose production requires the *las* and *rhl* genes, since the infection with a *lasR* mutant and with a *rhlR* reduces the paralysis (by 28%-100% and 100% respectively)[47]. A potential target of these diffusible factors is the EGL-9 worm protein, which is expressed in the neuronal muscle tissues[47]. In a recent study, a reduction of 83% in the death of the nematodes by the double mutant (PA14*rhlRlasR*) was reported; however, the analysis of individual mutants, revealed that only the *rhlR* mutant reduced death 69%, implying that the RhlR system is crucial for infection under their experimental conditions[48]. In addition to lethal paralysis and slow and fast killing, a fourth kind of *C. elegans* death induced by *P. aeruginosa* is the "red death", characterized by the formation of red precipitate (PQS + Fe^{3+} complex) within the intestine of the nematodes. This mode of death is mediated by the quinolone dependant QS system Mvfr-PQS in coordination with the PhoB phosphate sensor and the pyoverdine iron acquisition system[49]. The role of QS in *P. aeruginosa* infectivity and virulence in *C.*

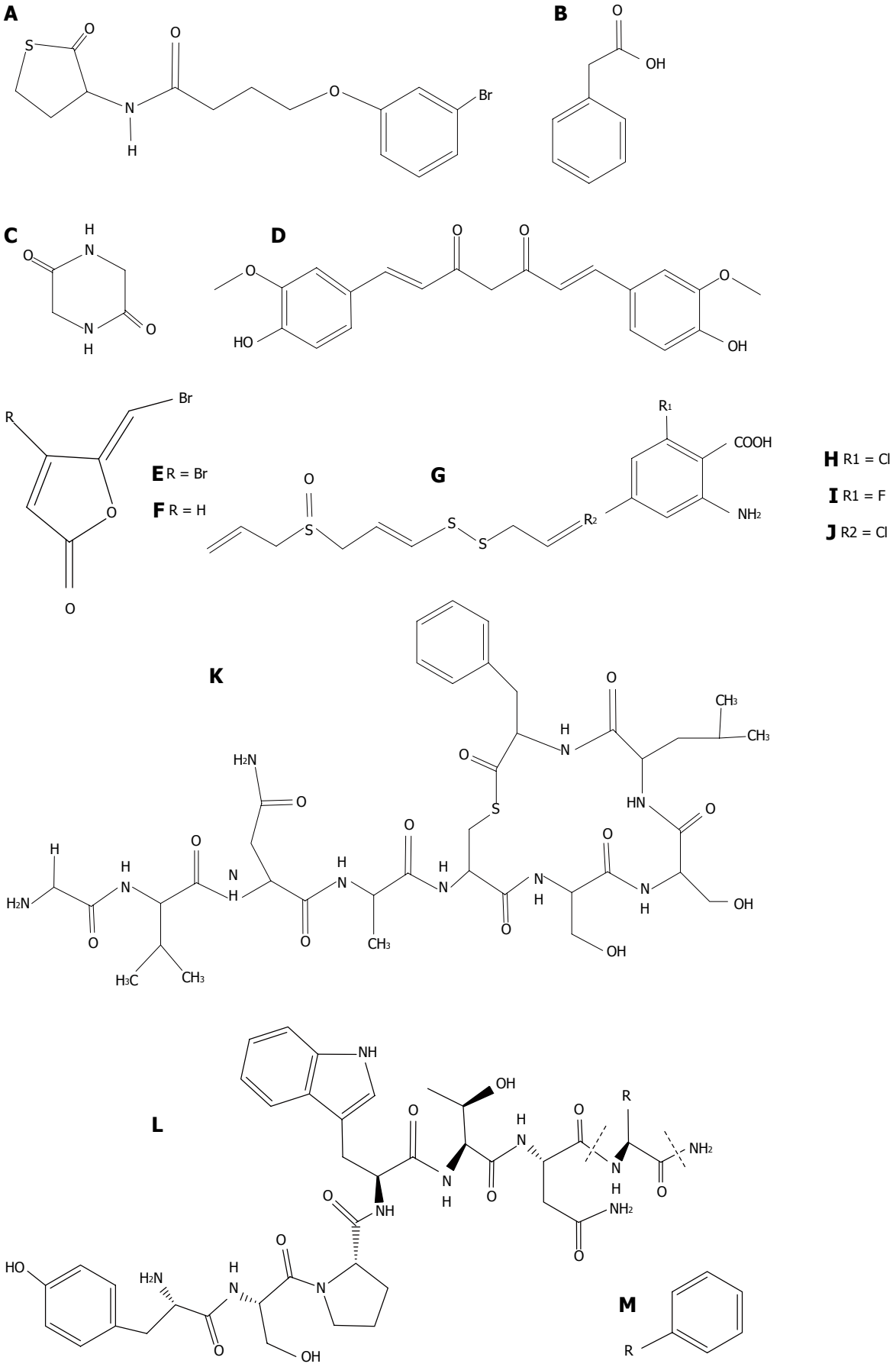


Figure 2 Structures of quorum sensing inhibitors evaluated in animal models. A: Meta-bromo-thiolactone; B: Phenylacetic acid; C: 2,5-piperazinedione; D: Cucurmin; E: Furanone C-30; F: Furanone C-56; G: Ajoene 4,5,9,-trithiadodeca-1,6,11-triene-9-oxide; H: 2-amino-6-chlorobenzoic acid; I: 2-amino-6-fluorobenzoic acid; J: 2-amino-4-chlorobenzoic acid; K: AIP-II; L: RIP (H-Tyr-Ser-Pro-Trp-Thr-Asn-Phe-NH_2); M: FS8 (H-Tyr-Ser-Pro-Trp-Thr-Asn-Ala-NH_2).

elegans is also evidenced by the effect of QS inhibitors, since a synthetic analog of HSL, meta-bromo-thiolactone (Figure 2A) that partially inhibits *in vitro* the LasR and RhrlR systems also reduces the death of worms infected with PA14, to 60% at 24 h. Interestingly, the *in vivo* action of the quencher in the worm model occurs mainly through the RhrlR system[48]. Moreover, phenylacetic acid (Figure 2B), which is a byproduct of the degradation of antibiotics such as penicillin G and cephalosporin Gby G acylase[50], increases the survival of PAO1 infected nematodes by 53%, while untreated worms die within 72 h. This protective activity is perhaps a consequence of interfering with the LasR and RhrlR systems, due to the structural similarity of phenylacetic acid with salicylic acid, a quorum quencher[51]. Another compound,2,5-piperazinedione (Figure 2C), increases the survival of worms by 66%, compared to untreated ones, and it was shown by molecular docking that it interacts with an amino acid residue (E145) in LasR, which is required for correct binding of the natural HSL ligand[52]. Similarly cucurmin (Figure 2D), a secondary metabolite from *Curcuma longa,* increases the survival of worms by 28%; this compound decreases the expression of genes involved in biofilm formation and attenuates HSL production in PAO1. Thus, it was suggested that it may act as a quorum quencher, delaying the synthesis of HSL molecules or by impairing autoinducers perception[53]. Moreover, various enzymes that degrade natural autoinducers are able to decrease the pathogenicity of *P. aeruginosa*; for example, adding the purified acylase PvdQ to *C. elegans* infected with *P. aeruginosa* PAO1, strongly reduces their pathogenicity and increases the nematodes life span[54]. Although the utilization of *C. elegans* as a model for studying *P. aeruginosa* infections has been very fruitful, recently it was proposed to use the fruit fly (*D. melanogaster*) as an animal model for the study of the *P. aeruginosa* pathogenesis, since the fly has a higher similarity to human[55-57]. The importance of both *P. aeruginosa* HSL QS pathways for infection was also demonstrated in *D. melanogaster* using the feeding assay, in which the bacteria are ingested and a local infection type is established in the intestine. In this assay the PA103 (*las*R), PDO100 (*rhl*I), PDO111 (*rhl*R), PAOR1 (*las*R) and PAOJP2 (*las*I/*rhl*I) mutant strains were avirulent with respect to wild-type PAO1 whose infected flies were killed at 14 d post-infection. Similarly, using the nicking assay (needle pricking), in which an injury is produced in the dorsum of the flies and *P. aeruginosa* is added to the wound, all mutant strains showed a lower death rate than wild-type, including the PDO100 mutant (*rhlI*) with 50% survival of the flies compared to 90% death for the PAO1 wild-type 35 d post-infection[57]. However, in contrast to the work with *C. elegans,* to date the effect of quorum quenching in *P. aeruginosa* virulence in the fly was not yet evaluated. With regard of these two infection models, Clatworthy and colleagues pointed out that a drawback to study *P. aeruginosa* infections using invertebrate hosts are the differences between their immune response and the one of vertebrates. For example, *C. elegans* and *D. melanogaster* do not have an adaptive immunity, or complex multilineage immune cells, such as those present in vertebrates[58]. Thus it is important to analyze the participation of QS in the pathogenesis of *P. aeruginosa* in vertebrate animal models, like zebrafish and mice. Specifically for zebrafish, the microinjection of PA14 QS$^-$ mutant strains (*lasR* and *mvfR*) during two different stages of fish development [28 and 50 h post-fertilization (hpf)], revealed that the participation of these two transcriptional activators during the infections is different and is influenced by the maturity of the immune system at different stages of the embryo development, since for the *lasR* mutant, only a 40% decrease in the death of the embryos at 50 hpf (a developmental stage when both macrophages and neutrophils are present) was recorded, whereas the *mvfR* mutant showed a moderate effect by decreasing death by 20% to 28 hpf (an stage in which only macrophages are present), but a higher effect of 60% decrease at 50 hpf[58].

For murine models, different protocols have been used to determine the participation of QS in *P. aeruginosa* pathogenicity[43,44]. The thermal induced injury model is frequently used and consists of producing a burn of second or third degree on the dorsal side of the mouse using water at 90 ℃ and subsequent inoculation of *P. aeruginosa.* Several experiments using this model have linked QS and virulence; for example, a PAO1-R1 (Δ*las*R) mutant has a diminished ability to spread systemically, as well as lower dispersion through the lesion at early stages[59,60]. Also, mice infected with PA14 *pqsA* show a 75% survival rate in contrast to 10% survival with wild-type PA14[61]. Similarly, virulence is reduced in PAO1 *lasR, lasI*, and *rhlI* mutants, with the greatest effect seen for the double mutant *lasI-rhlI* that decreased the mortality of animals by approximately 88%, significantly reduced the number of c.f.u in the lesion, liver and spleen, and delayed the spread of the bacteria from the lesion[59]. In agreement, similar results were found using the pneumonia model in neonatal mice, in which *lasR* mutants showed reduced virulence and are unable to replicate efficiently in the lung tissue; as a consequence, less damage occurs and the bacterial infection does not spread[62]. A third kind of experimental infection, the foreign-body infection model, consists in introducing a fragment of *P. aeruginosa* infected silicone into the peritoneal cavity of mice. This model was successfully used to determine the participation of QS in biofilm formation[43]. In this system, the mutant strain *lasR-rhlR* disappears from the silicone fragments during the first 7 d of infection, in contrast with wild-type PAO1 cells which remain in the silicon implant for at least 14 and up to 21 d. Critically, the establishment of the PAO1 infection in the implants depends in the mouse strain that is used, since for Balb/c, bacterial counts in the implants decayed constantly from day one and several of the implants were completely cleared after 21 d, while for the NMRI strain, bacterial counts initially decreased

(day 1 to 4), then remained constant and finally increased at levels similar to the initials at day 15[63].

Regarding studies testing the quorum quenching effect on virulence, by using the foreign body model, it was found that the intraperitoneal addition of furanone C-30 greatly increased the bacterial clearance rate (Figure 2E)[63]. In agreement a similar effect was observed with the lung infection model in mice in which a related compound, C-furanone 56 (Figure 2F) accelerated the bacterial clearance from the lungs, reducing the severity of the damage and significantly increasing mice survival[64]. Another quorum quencher, the synthetic molecule ajoene (ajoene 4,5,9,-trithiadodeca-1,6,11-triene-9-oxide) (Figure 2G), is able to attenuate the production of various *P. aeruginosa* QS-controlled virulence factors *in vitro*, while in the pulmonary infection model in mice infected with PAO1, its prophylactic administration from two days before the infection and during its course, reduced the bacterial c.f.u. in the lungs 500-fold relative to the non-treated mice[65]. Moreover, in the burn mice model, the intravenous administration of three related quorum quenchers, the anthranilic acid analogues: 2-amino-6-chlorobenzoic acid, 2-amino-6-fluorobenzoic acid, and 2-amino-4-chlorobenzoic acid (Figure 2H-J), that inhibit the biosynthesis of quinolone signals and disrupt the MvfR-dependant gene expression, restrict the systemic spread of the PA14 strain and decreases the animals death by 30% to 50%[61].

QS IN *P. AERUGINOSA* HUMAN INFECTIONS

The importance of the QS systems of *P. aeruginosa* in human infections is highlighted by their presence in most clinical strains that were isolated during the moment of the infection. This was demonstrated in 2004 by Schaber *et al*[66], by screening 200 isolates from patients with urinary tract, lower respiratory tract, and wound infections. Of those isolates, 97.5% (195 isolates) had robust functional HSL-QS communication systems and hence were able to produce elastase (codified by the genes *lasB* and *lasA,* which expression is QS dependent through LasR) and high levels of both HSL autoinducers while only 5 isolates failed to satisfy those criteria; however, 2 isolates were identified as being the same bacteria, but isolated at two different times from the same patient, and for one isolate there was no clinical data available to support that it was implicated in an infective process[66]. Hence only approximately 1% of the isolates that were implicated in infections appeared to be QS deficient. Critically, one of these isolates had no *lasR* and *rhlR* functional genes. In addition the authors demonstrated that those isolates deficient in HSL-QS systems produced high levels of non-QS controlled virulence factors, such as the ExoS and ExoT proteins that are components of the type three secretion system. Hence, perhaps this was an adaptive response that potentially could compensate for the decrease in virulence caused by QS deficiency., Nevertheless, the production of those proteins in the QS proficient isolates was not evaluated, and the virulence of the isolates was not tested using infection models. Other studies have reported similar results;, for example, the characterization of 442 *P. aeruginosa* isolates colonizing the respiratory tract of 13 intubated patients identified 9 genotypically different strains and of these, 6 strains produced both HSL-autoinducers and the virulence factors: elastase, exoproteases, rhamnolipid, hydrogen cyanide, and pyocyanin *in vitro*, and two of them had mutations in both *lasR* and *rhlR* genes, while the third had a mutant *lasR* gene[67]. Another study performed with 100 isolates from patients with respiratory infections that were collected from sputum, tracheal aspirate, and bronchoalveolar lavage identified 11 HSL-QS deficient isolates, six of them with absent *QS* genes (one isolate negative for *rhlR*, two isolates negative for *rhlI* and *rhlR,* and three isolates were negative for *rhlI*, *rhlR*, *lasI* and *lasR*). Interestingly, this study found a negative correlation between the expression of QS controlled virulence factors and antibiotic resistance[68]. Furthermore, the analysis of 82 *P. aeruginosa* clinical strains isolated from urinary tract infections identified 6 isolates deficient in the production of both HSL autoinducers, biofilm, rhamnolipids, and elastase, correlating with the absence of the *lasR* gene in one isolate and the absence of *lasI, lasR, rhlR* in another isolate, while the other 4 isolates harbored point mutations that probably inactivated their *lasI, lasR, rhlR*, and *rhlI* genes[69].

Taken together, these independent studies indicate that about 90% of *P. aeruginosa* isolates that cause infection generally preserve active HSL-QS systems, although clearly a small percentage of the isolates have those systems impaired by mutations or loss of the important QS regulatory genes, nevertheless, in all these studies, the third QS system of *P. aeruginosa*, the quinolone dependent system, was not evaluated; hence, it is not reliable to conclude that these isolates were indeed 100% QS deficient. In addition, the existence of cell communication systems not yet described in this organism cannot be ruled out, and indeed in the reference strain PAO1, cell communication by fatty acids was recently discovered (DSF-like fatty acids, cis-2-decenoic acid) (Figure 1D)[70]. Another possibility that may explain the isolation of QS deficient strains from infections is the presence of multiple *P. aeruginosa* strains in the infection site and that the QS deficient isolates coexist with QS proficient strains; this was demonstrated recently in 8 patients with cystic fibrosis (CF), in which a complex mixture of QS-proficient and deficient isolates were found. Interestingly, among all the patients, the deficiency of the isolates in individual QS regulated phenotypes (LasA and LasB elastase, rhamnolipids, growth in adenosine, and HSL signals) ranged from 0 to approximately 90% and no single

patient with 100% QS deficient isolates was found.

Such high diversity in isolates from the same patient likely is the result of a complex and multifactorial selective process, perhaps including social components like the advantages accrued by QS-deficient clones that use the resources made by the QS positive strains (siderophores, proteases, *etc.*), without contributing to the generation of the public goods; these bacteria are termed social cheaters[71]. Regarding the importance of QS for infections, these results indicate that at the population level, QS may be essential for CF infections; however, more studies increasing the number of CF patients and including other kinds of infections are necessary to better understand the importance of *P. aeruginosa* QS in the infective process. In addition, the elucidation of factors that shape the mosaic-like composition of isolates in patients or in animal models need to be determined in order to design better anti-QS therapies since the current ones are focused on laboratory strains with QS-proficient systems rather than clinical strains recently isolated from infections[72,73]. Although such factors are still unknown, some variables like: the severity and progression of the infection, the nutritional, health, and immunological status of the patients, the exposure of the susceptible individuals to only one, a few, or several strains and the bacterial loads during the infections could be involved. In this sense, animal models would be useful to evaluate the role of these and other valuables in the colonization diversity in the patients, for example experiments comparing the colonization of well feed animals and animals with a deficient nutrition, immune competent animals and immunosuppressed ones, or healthy animals compared to animals harboring important disorders such as the alpha-1-antitrypsin deficiency that promotes major pulmonary inflammation, degradation of lung tissue, and eventually manifestations of pulmonary emphysema, *etc*. using several bacterial strains (QS proficient and QS deficient) alone or in combination could be very valuable to determine the factors involved in the *in vivo* bacterial ecology in infections.

In addition, although *P. aeruginosa* virulence is multifactorial[74], the individual importance of QS controlled virulence factors in different kinds of infections is a current research area, and the role of molecules from the HSL autoinducers themselves, to extracellular factors like rhamnolipids, elastase, pyocyanin, *etc*. have been established. For example several independent studies have shown that the main *P. aeruginosa* autoinducer N-(3-oxododecanoyl)-HSL is readily detected in sputum samples collected from patients with cystic fibrosis[75-77], which correlates with a QS dependent gene expression during the infections[78-80]. However, besides its role as a signal, the autoinducer is also able to inhibit lymphocyte proliferation as well as secretion of tumor necrosis alpha by macrophages and interferon gamma by T-cells[27]. Moreover QS controlled secreted factors such as alkaline protease can interfere with the classical and the lectin pathway-mediated complement activation *via* cleavage of C2, blocking phagocytosis and killing of *P. aeruginosa* by neutrophils[81]. Also elastase, by cleaving the pulmonary surfactant protein-A, can contribute to phagocytosis evasion[82]. Furthermore, rhamnolipids are able to disrupt calcium-regulated pathways and protein kinase C activation, preventing the induction of human beta-defensin-2 in keratinocytes[83]. Remarkably, the production of rhamnolipids in mechanically ventilated patients is associated with the development of life-threatening ventilator-associated pneumonia (VAP), while elastase production and QS independent production of the cytotoxins ExoU and ExoS are not[84]. Another QS controlled virulence factor, the polysaccharide alginate, protects *P. aeruginosa* biofilm cells from IFN-gamma-mediated macrophage killing[85]. Surprisingly, the importance of pyocyanin, a blue redox-active compound which is one of the main *P. aeruginosa* virulence factors studied *in vitro* and one of the more notorious in infections (present in large quantities in sputum from patients with cystic fibrosis infected by *P. aeruginosa)* during clinical infection is still underexplored[86].

S. AUREUS QS AND VIRULENCE IN ANIMAL MODELS

The participation of SarA and Agr *S.aureus* QS systems in pathogenicity has been evaluated using numerous animal models, in which the bacteria induce diseases such as osteomyelitis, septic arthritis, endocarditis, endophthalmitis and soft tissue abscesses. By using the mutant strains *agr* and *sarA,* as well as QS inhibition, the participation of these systems in the infectivity of the bacterium and the damage of tissues has been proved. Intravenous inoculation of the bacteria in mice induces the development of septic arthritis. In this model, *agr* mutants showed a reduced ability to induce the pathology since it is produced in only approximately 10% of the animals while the wild-type strain produces it in approximately 60% of the inoculated mice. Furthermore, in mice infected with the mutant strain, the arthritis severity is less, and only a few developed erosive arthropathy in contrast to those infected with wild-type[87]. Similarly in the endophthalmitis-rabbit model, which is established by the intraocular injection of the bacteria, *agr* mutants produced a smaller loss of neuroretinal function during the first 3 d of the infection, with respect to the wild-type strain. In addition, those infected with the mutant strain had normal eye histology, whereas those infected with the wild-type strain showed focal retinal destruction and mild vitritis[88]. In a subsequent study employing the same animal model, no significant differences in the rabbits eyes infected with the mutant strain (*sarA*) and with the wild-type were found; however, the simultaneous deletion of genes *agr* and *sarA* resulted in a near to complete attenuation of virulence[89]. Moreover, employing the model of endocarditis in rabbits, which consists in introducing a

catheter into the ventricle and subsequently colonizing it by intravenous administration of the bacteria, it was observed that single mutations (*sarA* and *agr*) diminish the bacterial ability to induce the pathology, while a *agr*/*sarA* double mutant was incapable of inducing endocarditis in 100% of the animals inoculated with 10^3 or 10^4 c.f.u.[90]. Another infection model in mammals is the murine brain abscess model in which lesions are produced by embedding bacteria in agarose beads that are later inoculated in the cranial cavity. In this model, the *agr*/*sarA* double mutant, but not the single mutants, had reduced virulence, lower proliferation in the brain and poorly developed abscesses that were drastically smaller than those produced by the wild-type strain. Furthermore, the double mutation attenuates the expression of pro-inflammatory cytokines and chemokines[91]. Similarly invertebrate models such as the nematode *C. elegans*, which is killed by feeding on *S. aureus,* showed similar outcomes, since mutating *sarA* or *agr* increased the survival of the worms with respect to the wild-type strain[92].

Regarding the effect of quorum quenching in *S. aureus* animal infections, several inhibitory peptides have been evaluated; for example, in the murine subcutaneous abscess model, the administration of the synthetic autoinducer analog of AIP-Ⅱ peptide (Figure 2K) in a single dose was able to decrease the formation of abscesses, and although AIP-Ⅱ prevents expression of the *S. aureus agr* QS regulon for only a short time period, this transient inhibition is sufficient to achieve significant effects[93]. Other QS inhibitory peptides such as the RNAⅢ-inhibiting peptide (RIP) and its analogues (Figure 2K and L), that inhibit the phosphorylation of a target protein called "target of RNAⅢ-activating protein" (TRAP), leading to the suppression of virulence factor production *in vitro*[94,95], are also effective *in vivo.* For example, in the vascular-graft rat model, the administration of RIP (Figure 2L), both locally and systemically, is able to completely inhibit the formation of biofilms in graft and in polymethylmethacrylate beads infected with methicillin-susceptible and resistant *S. aureus*. Similarly, in the mouse sepsis model, administering RIP significantly reduced the bacterial load and mice mortality; this effect is potentiated by co-administration of antibiotics like cefazolin, imipenem, or vancomycin[96]. In addition, using the graft rat model, a RIP treatment increases its effectiveness in combination with antibiotics rifampin and temporin, and the complete elimination of infection is achieved by combining it with temporin A[97]. The same phenomenon has been documented with a derivative of RIP, termed FS3, which contains a substitution of alanine in the second position, since FS3 in combination with daptomycin has higher efficiency than single compounds, in the rat model of vascular graft staphylococcal infection[98]. Also in this model, a similar effect was obtained, by combining tigecycline and the RIP analogue called FS8 which contains a terminal alanine (Figure 2M)[99]. Taken together these extensive studies demonstrate the participation of *S. aureus* QS systems in its pathogenicity and indicate that QS inhibition in combination with antibiotics is a promising new strategy that may be effective to treat the infections produced by this important pathogen.

QS IN *S. AUREUS* HUMAN INFECTIONS

Although it is not always pathogenic to humans, the Gram (+) bacterium *S. aureus,* is frequently found in the human respiratory tract and on the skin and it is considered as a transient member of the human microbial flora[32], it is able to cause several kind of infections with a plethora of clinical manifestations. There are risk factors that complicate the infection caused by *S. aureus*, including the presence of prosthetic material and immunosuppression[100], and it is considered one of the three main causes of nosocomial bacterial infections. Among the many kinds of *S. aureus* infections, skin ones are very common; for instance, in children it is the main cause of impetigo, a superficial skin infection that according to its clinical manifestations is divided into non-bullous and bullous impetigo, the non-bullous is the most common form, the lesions begin as papules that progress to vesicles with erythema on its periphery. These become pustules that later form adherent crusts with a golden appearance and can be accompanied by regional lymphadenitis, although systemic symptoms are usually absent. Bullous impetigo is seen in young children in which the vesicles enlarge to form flaccid blisters with clear yellow liquid, which later becomes darker and turbid, leaving a thin brown crust[3,4]. Other common infections produced by *S. aureus* are hair follicle infection or folliculitis, furunculosis, and cellulitis. In addition to skin infections, *S. aureus* also causes respiratory tract infections such as nosocomial and septic pneumonia, septic pulmonary emboli and post viral empyema. It also infects the apocrine glands, causes musculoskeletal infections, produces bacteremia and its complications like sepsis, septic shock, and infective endocarditis and is able to produce toxic shock syndrome and food poisoning. Therefore, *S. aureus* is a major health concern worldwide.

Although the role of QS in regulating the expression of several virulence determinants including toxin production, and biofilm formation have been extensively studied *in vitro* and in animal models, its importance in actual human infections is yet under studied; nevertheless, it is known that as in the case of *P. aeruginosa* the great majority of *S. aureus* clinical isolates implicated in human infections possess active QS systems (agr^+). Although some agr^- strains are also commonly found in *S. aureus* infections[101-103], the presence of both kind of strains during infection indicates that agr^+ and agr^- variants may have a cooperative interaction[103] and also raises the possibility that social interactions like QS cheating may exist during the

infections[39]. In addition, the link of QS and biofilm formation in *S. aureus* strongly suggests that this QS is important for the development and establishment of its chronic infections[32]; however, further work in this area is needed to define the importance and the specifics of QS in regulating *S. aureus* virulence in human infections.

QS ANTIVIRULENCE DRUGS

P. aeruginosa

To date, there are a large number of quorum quenching (QQ) compounds reported. In general, the three types of QQ compounds are degraders of AHL autoinducers, synthase inhibitors, and receptor inhibitors. Here the QQ compounds used against *P. aeruginosa* are sorted into seven categories.

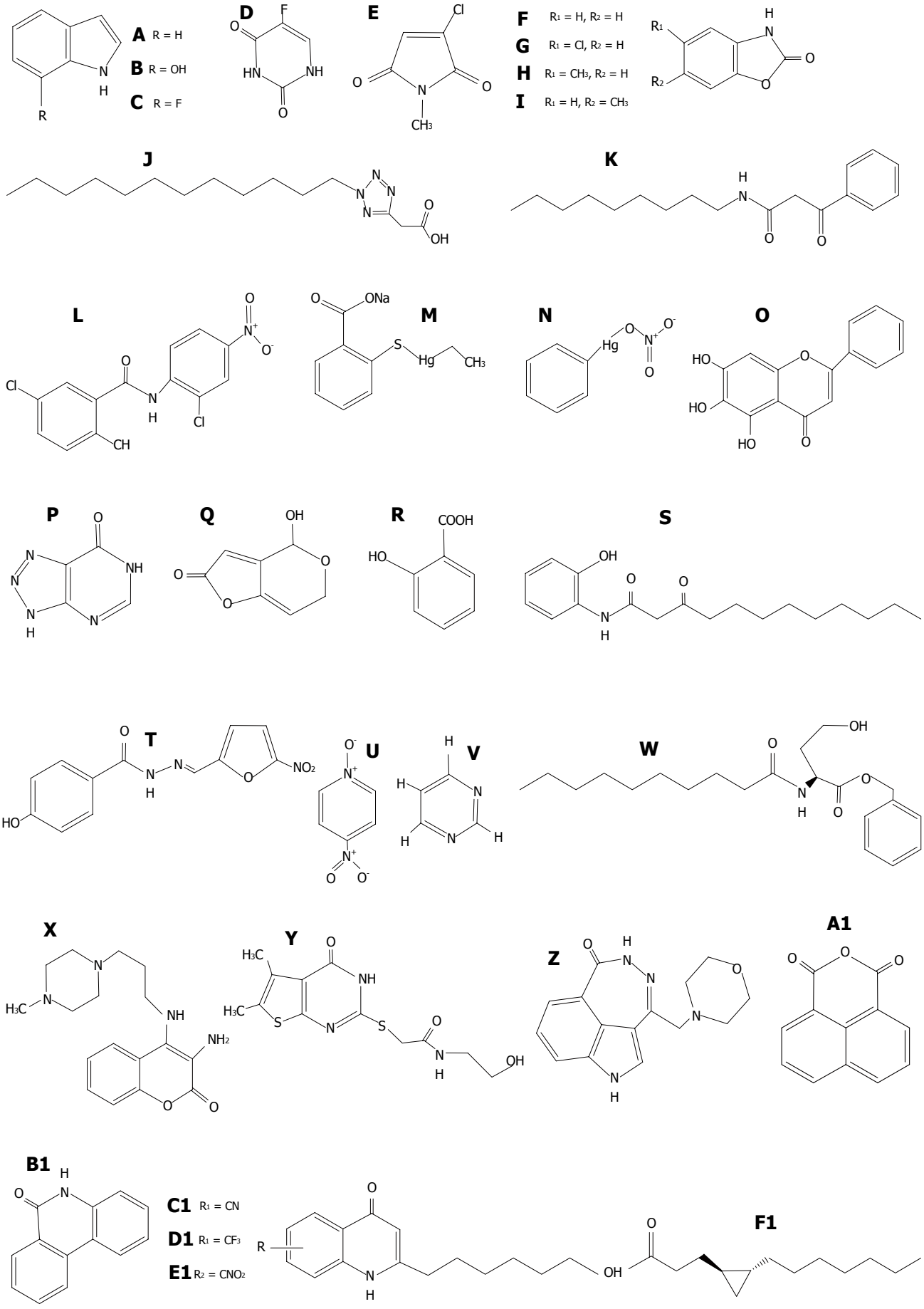
Halogenated compounds

One of the best characterized QQ compounds is the synthetic brominated furanone 4-bromo-5-(bromomethylene)-2(5*H*)-furanone known as C-30 (Figure 2E)[104]. This compound was synthetically modified from the natural brominated furanone (5*Z*)-4-bromo-5-(bromomethylene)-3-butyl-2(5*H*)-furanone of the algae *Delisea pulchra*. Another furanone compound is 5-(bromomethylene)-2(5*H*)-furanone, also called furanone C-56 (Figure 2F) which is a derivative of the secondary metabolites produced by the algae[105]. Interestingly, although C-30 is effective for the attenuation of several QS-dependent virulence factors *in vitro* and in animal models, resistance against this compound has been found both in laboratory PA14 derived mutants and in clinical isolates; to date, the only resistance described mechanism is the active efflux of this compound by the MexAB-OmpR pump but the existence of other mechanisms cannot be ruled out[72,106,107]. Furanone C-56 affects the processes of biofilm formation and dispersal although it does not influence initial attachment to abiotic substrata. In addition, indole (Figure 3A) produced from L-tryptophan by a variety of bacteria and 7-hydroxy indole (7HI) (Figure 3B), an oxidized compound of indole created by bacterial oxygenases, are extracellular signals that attenuate the production of biofilm and virulence factors in *P. aeruginosa*[108]. In addition, among 31 natural and synthetic indole analogs, 7-fluoroindole (7FI) (Figure 3C) was identified to be a QQ compound capable of reducing the production of virulence factors such as 2-heptyl-3-hydroxy-4(1*H*)-quinolone, pyocyanin, rhamnolipid, pyoverdin, and pyochelin[109]. 7FI shows higher inhibition toward biofilm formation than indole or 7HI.As another fluorine compound which shows the QQ ability, 5-fluorouracil (5-FU) (Figure 3D), an anticancer uracil analog, also is a potent inhibitor of *P. aeruginosa* virulence[110]. Since 5-FU is basically used for clinical purposes as a chemotherapeutic approach in patients with cancer, it holds promise as a QQ compound for clinical use. However, clinical strains resistant against this compound have been identified[72,107].

Based on the previous report that chlorolactone (CL) is an inhibitor to the QQ receptor[111], three other synthetic CL analogs were tested for QQ effects. As a result, meta-bromo-thiolactone (mBTL) (Figure 2A) was the most effective QQ compound since pyocyanin production and biofilm formation were inhibited in the presence of mBTL, in addition, mBTL moderately protected *C. elegans* and human lung epithelical cells from killing by *P. aeruginosa*[48]. Other halogenated QQ compounds include the derivatives of anthranilic acid which is the primary precursor of 4-hydroxy-2-alkylquinolines (Figure 2H-J)[61]. The halogenated anthranilic acid analogs inhibit quinoline biosynthesis and the expression of QS-related genes. Beyond that, halogenated maleimide analogs also are QQ compounds; in particular, bromo- and iodo-substituted maleimides decrease bacterial attachment and biofilm formation whereas chloro-N-methyl-maleimide has bacteriocidal action rather a QQ effect[112]. In addition to this, 5-chloro-1,3-benzoxazol-2(3H)-one[113] also called chlorzoxazone[114] are QQ compounds that contain a halogen group (Figure 3G).

Lactonases and acylases

Degrading enzymes such as lactonases and acylases are another class of QQ compounds. Their effect on QQ is due to the degradation of AHL-based autoinducers. To date, some unique lactonases have been characterized; for example, the halotolerant lactonases derived from *Bacillus* spp[115] and a thermally-stable lactonase from *Bacillus weihenstephanensis* P65[116], which may be useful for future applications. Lactonase itself or in combination with ciprofloxacin prevented systemic spread of the bacteria in murine burn wounds infected with *P. aeruginosa*, while for the combination mice mortality was completely abolished and skin regeneration was promoted[117]. In addition, immobilized esterases and acylases embeded on medical plastic materials inhibit biofilm formation[118]. Another unique approach using a lactonase is to utilize an engineered *Lactobacillus plantarum* strain expressing the lactonase AiiA from *Bacillus thuringiensis* 4A3[119]. Extracellular virulence factors such as pyocyanin, protease, elastase, and rhamnolipids of multi-drug resistant clinical isolates of *P. aeruginosa* were inhibited and the attachment to uroepithelial cells was reduced by co-culturing *P. aeruginosa* with the engineered strain. The original trial was performed using a *P. aeruginosa* strain capable of expressing a lactonase derived from *Microbacterium testaceum*, which led to reduced production of virulence factors and attenuated cytotoxicity against human lung epithelial cells[120]. A new trial using genetic engineering has been recently reported; this is by an engineered T7 bacteriophage expressing a lactonase with activity for a broad-range of bacterial hosts[121]. The engineered T7 bacteriophage was able to inhibit the biofilm formation of a consortium of *P. aeruginosa* and *Escherichia coli*.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B R = OH
C R = F
D
E
F R1 = H, R2 = H
G R1 = Cl, R2 = H
H R1 = CH3, R2 = H
I R1 = H, R2 = CH3
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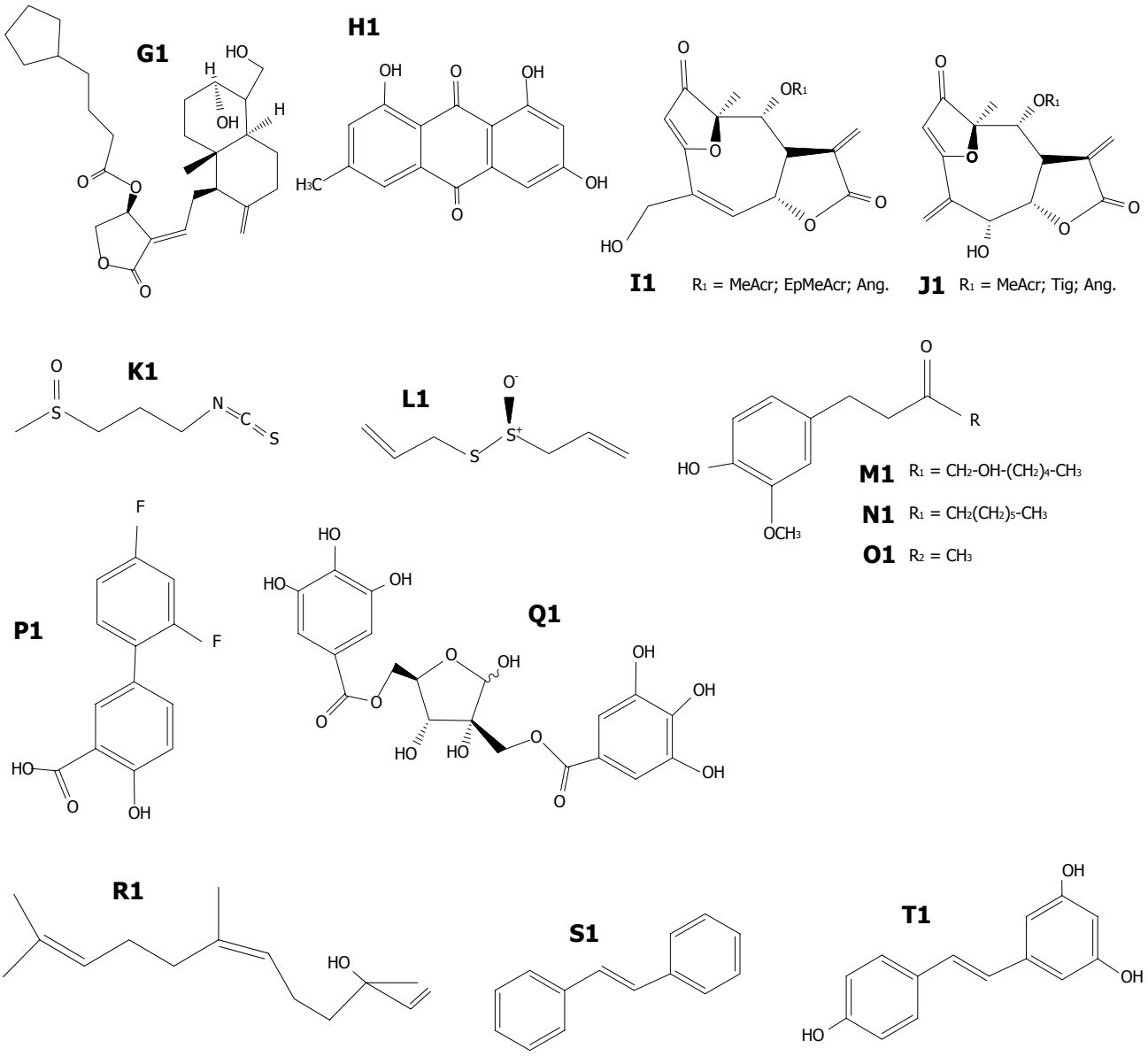

Figure 3 Structures of representative quorum quenching molecules of *Pseudomonas aeruginosa*. A: Indole; B: 7-hidroxy indole; C: 7-fluoroindole; D: 5-fluorouracil; E: 2-chloro-N-methyl-maleimide; F: 1,3-benzoxazol-2(3H)-one; G: 5-cloro-1,3-benzoxazol-2(3H)-one (clorzoxazone); H: 5-methyl-1,3-benzoxazol-2(3H)-one; I: 6-methyl-1,3-benzoxazol-2(3H)-one; J: PD12; K: V-06-018; L: Niclosamide; M: Thimerosal; N: Phenylmercuric nitrate; O: Baicalein; P: 5-imino-4,6-dihydro-3H-1,2,3-triazolo[5,4-*d*]pyrimidin-7-one; Q: Patulin; R: Salicylic acid; S: 3-oxo-C12-(2-aminophenol); T: Nifuroxazide; U: 4-nitropyridine-N-oxide; V: Pyrimidine; W: N-decanoyl-L-homoserine benzyl ester; X: V23; Y: V30; Z: P1; A1: NAP; B1: PJ97A; C1: 6-CN; D1: 6-CF3; E1: 6-NO2; F1: Lyngbyoic acid; G1: Andrographolide 14-(5-cyclopentylvaleryl); H1: Emodin; I1: Goyazensolide-type; J1: Isogoyazensolide-type; K1: Iberin; L1: Allicin; M1: [6]-gingerol; N1: [6]-shogaol; O1: Zingerone and *S. aureus*; P1: Diflunisal; Q1: Hamamelitannin; R1: Cis-nerolidol; S1: Trans-stilbene; T1: Resveratrol.

QQ compounds found by several screening approaches

For searching for novel QQ compounds, structure-based computational screens and high-throughput screens have been conducted. An ultra-high-throughput, cell-based assay to screen a library of approximately 200000 compounds was used to find an inhibitor which can decrease the gene expression regulated by the Las system[122]. As a result, PD12 (Figure 3J), a tetrazole with a 12-carbon alkyl tail and V-06-018, a phenyl ring with a 12-carbon alkyl tail (Figure 3K), which have both similarity with the structure of 3OC12-HSL, were identified as QQ compounds.In addition, a compound having QQ ability was also found among a series of 1,3-benzoxazol-2(3H)-one derivatives[113]; thereby, 1,3-benzoxazol-2(3H)-one (Figure 3F), 5-chloro-1,3-benzoxazol-2(3H)-one (Figure 3G), 6-methyl-1,3-benzoxazol-2(3H)-one (Figure 3I), and 5-methyl-1,3-benzoxazol-2(3H)-one (Figure 3H) have QQ ability. As another approach for clinical application of QQ compounds, the thousands of drugs clinically used in the treatment of different diseases were screened to find drugs with QQ properties which can be applicable to humans. By the screening, it was found that an anthelmintic drug, niclosamide (Figure 3L) strongly inhibits the QS response by *P. aeruginosa*[123], although the active compound was demonstrated to be 5-FU

which was already described as a QQ agent[107,110]. Moreover, since antibiotics are also robust compounds for clinical use, inhibition of QS by antibiotics was surveyed. As a result, it was found that low concentrations of azithromycin, ceftazidime, and ciprofloxacin inhibit QS in *P. aeruginosa*[124]. In addition, QS in a *P. aeruginosa* environmental isolate was inhibited at sub-inhibitory concentrations of tobramycin[125] although other studies demonstrated that a low concentration of tobramycin induces biofilm formation[126].

Screening using a computational approach and molecular docking analysis has also been useful for evaluating the binding capacity of QQ compounds to receptor proteins; thereby, new potential QQ compounds were identified. Pharmacophore modeling and *in silico* screening to find an antagonist for QS in *P. aeruginosa* indicated that a compound with tetravalent lead has QQ ability[127]. Another two compounds thimerosal (Figure 3M) and phenyl mercuric nitrate (Figure 3N) were selected as QQ compounds based on their similarity to the Pb-QQ compound. Also, the automated docking program by which the docking capability of a ligand to a receptor can be analyzed identified 5 potential new QQ compounds; among the candidates, baicalein (Figure 3O) has the strongest QQ ability as it inhibits biofilm formation of *P. aeruginosa* and the QQ effect by baicalein increases synergistically in the presence of ampicillin[128]. Also, another 5 compounds were identified to be QQ by using a structure-based virtual screening approach targeting the QS receptor LasR; of the 5 compounds, the most promising was 5-imino-4,6-dihydro-3H-1,2,3-triazolo[5,4-d]pyrimidin-7-one also called G1 (Figure 3P)[129].

Other AHL antagonists

Some of QQ compounds described above are antagonists of AHL molecules; hence their QS inhibition effect is triggered by interrupting the binding (interaction) between AHL molecules and receptors. To date, there are a large number of AHL antagonists; for example, patulin (Figure 3Q)[130], salicylic acid (Figure 3R)[114], 3-oxo-C12-(2-aminophenol) (Figure 3S)[131], and nifuroxazide (Figure 3T)[114] as well as C-30 (Figure 2E)[104]. In addition, 4-nitropyridine-N-oxide (Figure 3U) is a QQ compound[132], which also reduces bacterial adhesion to silica-coated surfaces[133]. Other QQ compounds are pyrimidine (Figure 3V)[134], N-decanoyl-L-homoserine benzyl ester (C2) (Figure 3W)[135], 2,5-piperazinedione (Figure 2C)[52], and phenylacetic acid (Figure 2B)[51]. The bacterial sensitivities to several antibiotics (tobramycin, gentamycin, cefepime, and meropenem) in the presence of C2 were higher than those without C2[135]. This may be due to the synergistic interactions between C2 and the antibiotics. In addition, QQ by the cyclic dipeptide 2,5-piperazinedione (Figure 2C) might be due to interference with the binding of the natural ligand 3-oxo-C12-HSL to its receptor protein based on the molecular docking analysis[52]. Phenylacetic acid (Figure 2B), which is similar to salicylic acid, has been reported to be a QQ compound[51].

Inhibitors with different QS targets

There are some reports on inhibitors with QS targets different than AHLs and receptors.A new class of antivirulence compounds was reported by Shouldice *et al*[136]; the QQ compounds interact with the bacterial periplasmic protein DsbA, which is essential for the folding and function of exported virulence factors. Another target of QQ compounds is mono-ADP-ribosyltransferase which functions as a bacterial toxin[137]. Some newly-identified QQ compounds were found by using a virtual screen of commercially available compounds combined with a directed poly(ADP-ribose) polymerase; thereby, V23 (Figure 3X), V30 (Figure 3Y), and P1 (Figure 3Z) compounds as well as NAP (Figure 3A1)[137] and PJ97A (Figure 3B1)[138] were identified as inhibitors of toxin production[137]. Other antagonists are a series of compounds targeting PqsR, the receptor of the *pqs* system[139]. Among the analogs of 2-heptyl-4-hydroxyquinoline (HHQ) synthesized, three HHQ analog with 6-CN (Figure 3C1), 6-CF_3 (Figure 3D1), or 6-NO_2 (Figure 3E1) along with n-C_7H_{15} are the best competitors[139], which are promising starting compounds for further drug design.

Cell extracts and secretion products from isolated microorganisms

Based on the concept that microbial interaction (inhibition, repression, acceleration, and dependence) is a complex phenomenon due the large numbers of microbes, a new approach to isolate unique microorganisms with QQ ability and to utilize cell extracts and secretion products has been recently reported. Among the 46 marine bacterial isolates, 11 extracts from *Bacillus*, *Marinobacter*, *Halobacillus*, *Staphylococcus*, or *Ferrimonas* species showed antibiofilm activity against *P. aeruginosa*[140]. The partially-purified antibiofilm compound from S6-15 (similarity with *Bacillus pumilus*) is stable up to 60 ℃ and under neutral and alkaline conditions. In addition, its QQ ability was inactivated by the treatment by enzymes such as proteinase K, trypsin and lysozyme[140]. Also, bacteria able to utilize AHL molecules as a sole source of carbon and nitrogen have been isolated and characterized as AHL-degrading bacteria[141]. Among 41 isolates which retained QQ activity after heat treatment, some of the isolates showed impaired QS inhibition after the treatment by proteinase K whereas the other isolates remained active. In addition, actinomycetes with QQ activity were also isolated from marine sponge. In this study, methanol extracts of 12 actinomycetes had an inhibitory effect on the production of QS-mediated virulence factors[142]; in particular, of the three strains which showed very good anti-QS activity, the most promising strain is NIO 10068 (*Streptomyces* sp.) that secretes cinnamic acid and/or linear Pro-Gly dipeptide which may be QQ compounds. Further bacteria capable of having QQ ability were also isolated from healthy coral species[143]; of 120 bacterial

isolates, up to 24% of the isolates showed anti-QS activity. In particular, a *Favia* sp. coral isolate inhibits the biofilm formation of *P. aeruginosa* by secreting a low-molecular mass compound which is not inactivated by heat and proteinase K[143]. Also, a cell-free lysate of endophytic bacteria isolated from *Pterocarpus santalinus* Linn. also showed QQ activity[144]. *Bacillus firmus* PT18 and *Enterobacter asburiae* PT39 isolated as the endophytic bacteria exhibit potent AHL degrading ability by inhibiting about 80% violacein production in a biosensor strain. QQ activity by the cell lysate was effective against biofilm formation rather than to planktonic cells, and the QQ activity was due to the presence of AHL lactonase in cell-free lysate[144].

Moreover, a small cyclopropane-containing fatty acid, lyngbyoic acid (Figure 3F1), a major metabolite produced by the marine cyanobacterium, *Lyngbya* cf. *majuscule* has been identified to be a QQ compound capable of strongly inhibiting Las-QS system[145]. In addition, the biosurfactant, lunasan produced by *Candida sphaerica* UCP 0995 is also a QQ compound[146]. Recently, it was discovered that a conditioned high density lipoprotein is also a QQ compound capable of reducing the virulence of *P. aeruginosa* by influencing *las*-and *rhl*-QS systems as well as biofilm formation[147]. Furthermore, ultra-small solid lipid nanoparticles for the pulmonary delivery, which are prepared by using various pharmaceutical lipids, are fabricated to deliver QQ compounds to a target site without any penetrable cellular barrier[148]. In this study, plain small solid lipid nanoparticles exhibited anti-virulence properties themselves.

QS inhibitors from food and plant sources

Since biocompatibility of QQ compounds to higher organisms is one of the important requirements for clinical use, there are a lot of trials to find QS inhibitors from food and plants. The anti-QS activity of aqueous extracts from edible plants and fruits, like pineapple, plantain, and sapodilla, was evaluated; most of these extracts showed QQ activity without inhibiting bacterial growth in *P. aeruginosa*[149]. Also, analogs from a natural bicyclic diterpeniod lactone, andrographolide which is the main phytoconstituent from *Andrographis paniculata* Nees (herb), were screened to evaluate QQ activity[150]. An andrographolide-based compound, 14-(5-cyclopentylvaleryl) andrographolide (compound 11b) (Figure 3G1) had the best QQ activity among all the new compounds. In addition, some QQ compounds were found from traditional Chinese medicine by using a molecular docking analysis and QS assays[151]. As a result, emodin (Figure 3H1) had a certain antibiofilm activity as well as the ability to increase the activity of ampicillin against *P. aeruginosa*.

Furthermore, five sesquiterpene lactones of the goyazensolide (Figure 3I1)and isogoyazensolide-type (Figure 3J1)isolated from the Argentine herb *Centratherum punctatum*[152], iberin (Figure 3K1)from horseradish[153], allicin (Figure 3L1)from garlic[154], and phenolic components {([6]-gingerol (Figure 3M1),[6]-shogaol (Figure 3N1), zingerone (Figure 3O1)} from ginger[155] and anacardic acids[156] are QQ compounds, which might be suitable for further development of antivirulence and antibacterial agents.

S. aureus

Since QS regulates the expression of multiple *S. aureus* virulence determinants, and since the frequency of drug resistant clinical strains causing infections is rising (like methicillin-resistant *S.aureus* "MRSA"), several compounds aiming to disrupt these regulatory interactions have been identified; among them, perhaps the best characterized is the QS inhibitor RIP (Figure 2L), an endogenous *S. aureus* peptide that is able to decrease the damage of *S. aureus* in several animal models as discussed before[94,157]. At the molecular level, the production of several toxins is activated in a cell density manner by the RNAIII-activating protein (RAP) and by the autoinducing peptide (AIP), and is inhibited by RIP and by inhibitory AIPs; RAP participation in the pathogenesis consists in inducing the phosphorylation of a 21-kDa protein (known as target of RAP or TRAP). While RIP inhibits its phosphorylation, the phosphorylation of TRAP is essential to create the autoinducing loop since it leads to the activation of RNAIII synthesis[94]. In addition to decreasing the damage of *S. aureus* during infection, RIP treatment also is able to prevent its adhesion to human kidney cells and its biofilm formation on dialysis catheters[158]. Other effective peptides analogues to RIP are FS3 and FS8 (Figure 2M) discussed previously[98,99]. Moreover, recently four AIP non-functional peptide analogues were identified; these peptides have an ample spectrum since they can repress many AgrC receptors (type I-IV) and have a very high affinity. For example, treatment with the peptides block hemolysis (at picomolar concentrations)and attenuate the production of toxic shock syndrome toxin-1 by 80% at nanomolar concentrations; hence, these compounds are the most potent synthetic inhibitors of QS in *S. aureus* to date[159].

Beyond the QS inhibitory peptides, several other interesting molecules able to block the expression of *S. aureus* virulence factors have been discovered, among them, the small molecule biaryl compounds in which the aromatic rings either are either fused or separated by a short linker. This result is particularly interesting, since they are able to inhibit the production of the alpha-hemolysin and the modulin-α toxin in a dose-dependent manner without inhibiting bacterial growth, since they are effective against methicillin-resistant *S. aureus*, and since one of the effective compounds is diflunisal (Figure 3P1), an Federal Drug Administration-approved nonsteroidal anti-inflammatory drug[160]. Diflunisal has the clear advantage that it could be easily repurposed for treating *S. aureus* infections or could be used to coat catheters and other medical devices just as was recently done for 5-FU (Figure 3D)[161], which has QS inhibitory activity against *P. aeruginosa*[110], *Escherichia coli* (*E. coli*)[162] and perhaps several other

pathogens. In addition, some natural products with QS inhibition activity against *S. aureus* like 2,5-di-*O*-galloyl-dhamamelose (hamamelitannin) (Figure 3Q1), a non-peptide analog of RIP found in the bark of the plant *Hamamelis virginiana* had been identified. This compound is effective *in vitro* to inhibit virulence without affecting growth, and *in vivo* in a rat graft model, preventing device-associated infections[163]. Moreover, recently the screening of 83 essential oils led to the identification that black pepper, cananga, and myrrh oils and their common constituent cis-nerolidol (Figure 3R1) strongly attenuate *S. aureus* biofilm formation, its hemolytic activity, and protect *C. elegans* against its infection. Although their mechanism is not fully understood yet, transcriptional analyses showed that at least black pepper oil treatment inhibited the expression of the α-toxin gene (*hla*), nuclease genes, and QS regulatory genes[164]; similar effects can be observed with treatments with trans-stilbene (Figure 3S1)and resveratrol (Figure 3T1)[165].

QS SYSTEMS ARE PRESENT IN SEVERAL OTHER IMPORTANT BACTERIAL PATHOGENS

In addition to *P. aeruginosa* and *S. aureus,* several other bacterial pathogens utilize QS systems to control the expression of multiple virulence factors during infection. Among the more relevant for human health are *Vibrio* spp, *Acinetobacter* spp**,** *Burkholderia cepacea,* and enteric bacteria like *Escherichia* spp. and *Salmonella typhimurium*. The following section is an overview of their known QS systems and their relationship with virulence.

Vibrio spp.

Vibrio is a genus of facultative anaerobic Gram-negative bacteria possessing a curved rod shape (comma shape) typically found in saltwater. Several species are pathogenic to animals including humans and are responsible for food borne infections that are usually associated with eating contaminated food or water. In addition, they also cause wound infections and septicemia. The first QS system was described in the bioluminescent marine bacterium *Vibrio fischeri*, considered the paradigm for QS found in most Gram-negative bacteria. *Vibrio fischeri* colonizes the light-emitting organs of the squid *Euprymna scalopes*, in which it multiplies and reaches a high population density and induces the expression of luminescence genes. This gene expression occurs in a coordinated fashion[166]. The squid uses the light conferred by the bacteria to hide its own shadow in shallow waters and thus avoid predators[167]. To date several QS systems have been described in *Vibrio* spp.

In Vibrio harveyi, the following three QS systems are known: (1) LuxM (synthase), LuxN (receptor) and 3OHC4HSL (signal); (2) LuxS (synthase), LuxP (receptor) and AI-2 (signal); and (3) CqsA (synthase), CqsS (receptor) and CAI-1 (signal).

These systems are associated with bioluminescence, siderophores, protease and extracellular polysaccharide (EPS) production, and other virulence factors[168-170].

In Vibrio cholerae, two QS systems have been described: (1) LuxS (synthase), LuxP (receptor) and AI-2 (signal); (2) CqsA (synthase), CqsS (receptor) and CAI-1 (signal).

These systems have been associated with biofilm formation, EPS production, and other virulence factors[169].

Finally, in *Vibrio fischeri* three QS systems are known: (1) LuxI (synthase), LuxR (receptor) and 3OC6HSL (signal); (2) AinS (synthase), AinR (receptor) and C8HSL (signal); and (3) LuxS (synthase), LuxP (receptor) and AI-2 (signal).

These systems are associated with bioluminescence, host colonization, and motility[168,170]. Other QS systems found in various *Vibrio* spp. and in *Legionella pneumophila* utilize hydroxyketones (AHKs) as signalling molecules[170].

Acinetobacter spp.

Acinetobacter is a genus of aerobic, non-motile Gram-negative bacteria that are widely distributed in nature, commonly occurring in soil. Among them, some species like *Acinetobacter baumannii* (*A. baumannii*) are frequently isolated in nosocomial infections, especially in intensive care units, since they attack debilitated and immunocompromised patients; in addition they have a high tolerance against antibiotics and an inherent ability to acquire antibiotic resistance genes, being therefore a serious emergent health problem. Their QS systems consist of homologues of the LuxR and LuxI proteins of *Vibrio fischeri* known as AbaR (receptor) and AbaI (synthase) and play a role in biofilm formation and motility in *Acinetobacter* spp[171] and in *Acinetobacter baumannii*[172]. This QS system is an important virulence factor responsible for the outstanding antibiotic resistance and survival properties in the latter species[173]. However, the role of QS systems in the regulation of other virulence factors implicated in the development of infection has not yet been established[174].

Synthesis of N-(3-hydroxydodecanoyl)-L-HSL (3-hydroxy-C12-HSL) is catalyzed by AbaI from *Acinetobacter* strain M2 (initially characterized as *Acinetobacter baumannii*, although genomic sequencing studies have distinguished this strain as *Acinetobacter nosocomiales*[174,175]). The completed genome sequence of *A. baumannii* strain ATCC 17978 indicates that autoinducer synthase AbaI (gene *A1S_110*) and acyltransferases may be the sole participants in the synthesis of AHL signals of variable chain length by the organism[176]. Many strains of *Acinetobacter* (63%) produce more than one AHL. However, none of the AHL signals can be specifically assigned to a particular species of the genus[177]. *Acinetobacter* quorum signals are not homogenously distributed, and therefore

distinction between virulent and non-virulent strains on the basis of QS signals is difficult. Communication between bacteria with respect to cell density is integral to the maturation of *Acinetobacter* spp. Biofilm[176,178]. Mutation of *abaI*, which produces the acyl-homoserine lactone molecule, resulted in a 30%-40% reduction in biofilm production relative to that of the isogenic parental strain[173]. Exogenous addition of purified *Acinetobacter* acyl homoserine lactone restored biofilm maturation in the *abaI* mutant[176].

Burkhordelia cepacea

The *Burkholderia cepacia* complex is a group of Gram-negative bacteria composed of at least 18 different species; they are important human pathogens which produce pneumonia in immunocompromised individuals that are affected by lung diseases such as cystic fibrosis. All *Burkhordelia cepacea* complex members encode at least one QS system that consists of homologues of the LuxR and LuxI proteins of *Vibrio fischeri* [CepI(synthase), CepR (receptor), and AHLs C8-HSL and C6-HSL (signal)]. AHL production in the *Burkhordelia cepacea* complex is strain-dependent with respect to both the quantity and type of AHL molecules[179,180]. Another QS system in the *Burkhordelia cepacea* complex is the CciIR system [CciI (synthase), CciR (receptor), and/AHLs C8-HSL and C6-HSL (signal)][181]. Phenotypic assays and global transcript and protein analysis with *cepIR* and *cciIR* mutant strains have shown that AHL-mediated QS controls various functions, including swarming motility, biofilm formation and the production of virulence factors, such as proteases (*e.g.,* the metal proteases ZmpA and ZmpB), siderophores, toxins and antifungal agents[179].

In 2008, Boon *et al*[182] reported the identification of a novel fatty acid signal molecule that is produced by several *B. cenocepacia* strains. The structure of the molecule synthesized by *B. cenocepacia* J2315 was identified as *cis*-2-dodecenoic acid, referred to as BDSF (*Burkholderia* diffusible signal factor). BDSF is structurally related to DSF (diffusible signal factor, *cis*-11-methyl-2-dodecenoic acid), which was first isolated from supernatants of *Xanthomonas campestris* pv *campestris.* The BDSF-regulated QS system is involved in the control of several functions. Mutation of *rpfFBc* resulted in decreased motility, reduced adherence to porcine mucin, diminished exopolysaccharide (EPS) production and lowered protease activity. In addition, the BDSF mutant strains were found to be more susceptible to antimicrobial agents, and their ability to form biofilms was shown to be strongly reduced[179].

Escherichia spp./Salmonella typhimurium

E. coli and *S. typhimurium* are related enteric Gram-negative, facultative anaerobic bacteria. Although most *E. coli* strains are commensal for warm-blooded organisms, such as mammals, some serotypes cause serious food poisoning and other kinds of infections like urinary tract infections and neonatal meningitis, while *S. typhimurium* and other *Salmonella* pathogenic serovars are responsible for Salmonellosis, an infection that causes diarrhea, fever, vomiting, and abdominal cramps. Although usually the illness resolves after four to seven days without medical treatment, several million people are infected by this bacterium each year. In *E. coli* and *S. typhimurium*, three QS systems have been described: (1) Unknown (synthase), SdiA (receptor) and 3OC8HSL (signal). This system has been associated with motility and acid resistance[183]; (2) LuxS (synthase), LsrB (receptor) and AI-2 (signal). Lsr operon expression (AI-2 uptake)[184]; and (3) Unknown (synthase), QseC (receptor) and AI-3 (signal). This system has been implicated in virulence, motility and biofilm formation[185].

QS SYSTEMS BEYOND BACTERIA

QS systems have been extensively studied in bacteria and are of great interest for understanding the development of clinically-significant infections, but whether eukaryotes have cell signaling systems similar to bacterial QS mechanisms is a question that has recently drawn the attention of research worldwide. In this section, we will discuss some examples of eukaryotic microorganisms and human cells that use QS for the development of certain biological functions. The first report of a QS system in eukaryotes was carried out more than 40 years ago, when it was observed that dense cultures of the fungi *Candida albicans* show a reduced tendency towards the morphological transition from yeast to hypha, which is considered a key virulence factor for this opportunistic fungal pathogen[186]. To date, several compounds have been identified as responsible for this phenomenon, such as 2-phenylethanol, tryptophol, farnesol, farnesoic acid, and tyrosol[187]; these QS molecules are secreted by *C. albicans* and when they accumulate over a threshold level, they trigger changes in: (1) fungal dimorphisms[186]; (2) biofilm formation[188]; and (3) expression of virulence genes[189]. In addition, in other dimorphic fungi (*Mucor rouxii, Histoplasma capsulatum, Ceratocystis ulmi*), the "inoculum size effect" is usually observed; however, QS autoinducers in these organisms have not been identified[187]. In 1997, it was discovered that in the protozoan parasite of humans *Trypanosoma cruzi,* the differentiation of replicating and slender forms to non-dividing and stumpy ones is also a density-dependent (quorum) response that limits the population size[190]. This phenomenon is mediated by the soluble factor SIF (stumpy induction factor) that is released by trypanosomes, and a recent study revealed that the QS signaling in *T. cruzi* shares components with the quiescence pathways of mammalian stem cells, providing novel therapeutic targets *via* QS interference[191]. Like those parasitic species described thus far, in 2006 it was demonstrated that the budding yeast *Saccharomyces cerevisiae* endure morphological transition from the yeast form to

Table 1 Quorum sensing systems and quorum sensing-virulence associated phenotypes in the reviewed organisms

Organism	QS systems	Regulated phenotypes
Gram (-) bacteria		
Pseudomonas aeruginosa	(1) LasI (S)-LasR (R)-HSL (s) (2) HSL RhlI-RhlR (3) Alkyl quinolones (PQS)	Expression of several virulence factors including: Pyocyanin, pyoverdine, elastase, alkaline protease, HCN, rhamnolipids and biofilm formation
Vibrio harveyi	(1) LuxM (S)-LuxN (R)- 3OHC4HSL (s) (2) LuxS (S)-LuxP (R)-AI-2 (s) (3) CqsA (S)-CqsS (R)-CAI-1 (s)	Expression of bioluminescence genes and several virulence factors including: Siderophores, protease, EPS production
Vibrio cholerae	(1) LuxS (S)-LuxP (R)-AI-2 (s) (2) CqsA (S)-CqsS (R)-CAI-1 (s)	Expression of several virulence factors including: Biofilm formation and EPS production.
Vibrio fischeri	(1) LuxI (S)-LuxR (R)-3OC6HSL (s) (2) AinS (S)-AinR (R)-C8HSL (s) (3) LuxS (S)-LuxP (R)-AI-2 (s)	Expression of bioluminescence, host colonization and motility genes
Acinetobacter spp	(1) Abal (S)-AbaR (R)-3OHC12HSL (s)	Expression of virulence factors including biofilm formation.
Burkhordelia cepacea	(1) Cep1 (S)-CepR (R)-C8HSL,C6HSL (s) (2) Ccil (S)-CciR (R)-C8HSL,C6HSL (s)	Expression of swarm motility genes and several virulence factors including: proteases, siderophores, toxins, antifungal agents and biofilm formation
Escherichia coli *Salmonella typhimurium*	(1) Unknown (S)-SdiA (R)-3OC8HSL (s) (2) LuxS (S)-LsrB (R)-AI-2 (s) (3) Unknown (S)-QseC (R)-AI-3 (s)	Expression of motility genes, acid resistance and virulence factors including biofilm formation
Gram (+) bacteria		
Staphylococcus aureus	(1)AgrB (S)-AgrC (R)-AIP(s)	Expression of several virulence factors including: hemolysins, leukocidins, cell surface adhesins, exoenzymes, and biofilm formation
Fungi		
Candida albicans	2-phenylethanol, tryptophol, farnesol, farnesoic acid, and tyrosol as (s)	Fungal dimorphism, biofilm formation and expression of virulence genes
Mucor rouxii, Histoplasma capsulatum, Ceratocystis ulmi	QS (s) unknown	Fungal dimorphism
Saccharomyces cerevisiae	Phenylethanol and tryptophol as (s)	Transition from yeast to filamentous form
Protozoa		
Trypanosoma cruzi	SIF soluble factor as (s)	Differentiation of replicating to non-dividing forms
Mammal cells		
$CD4^+$T cells	IL-2 (s) and IL-2Rα (R)	Regulation of the $CD4^+$T cells population
Cancer cells	Multiple (S) and (R) and paracrine factors as (s)	Regulation of the metastatic process

IL-2: Interleukin 2; SIF: Soluble inhibitory factor; QS: Quorum sensing; PQS: Pseudomonas quinolone signal; EPS: Exopolysaccharide.

a filamentous form in response to both cell density and the nutritional state of the environment. This induction is mediated by the phenylethanol and tryptophol auto signaling molecules, that regulate the transcription of a set of approximately 150 genes and which include *FLO11*, an essential gene for filamentous growth as well as several others genes that may play a role in the transition from the exponential to the stationary growth phase[192,193]. However, not only parasitic infections or traits present in unicellular eukaryotes are controlled by QS, since surprisingly in our body the number of cells of the immune system is maintained throughout a similar mechanism, where IL-2 is produced and secreted by activated $CD4^+$ T cells and sensed with high affinity (IL-2Rα) by a population of $CD4^+$ Treg, which in turn can regulate the number of total $CD4^+$ T population[194] by competition for the IL-2 factor[195]. Failure of QS due to the absence of IL-2 or by defects on the sensor IL-2Rα leads to lymphoid hyperplasia and autoimmune diseases[196]. Furthermore, in 2009 Hickson *et al*[197] proposed that cancer cells may use QS mechanisms to operate as communities and regulate different multicellular functions as the metastatic process resembles bacterial biofilm formation and dispersion. This idea emerged based on several lines of evidence that suggested a close relationship between high cancer cell densities and high metastatic ability[197]. This relationship can be possibly explained by the fact that the cells secrete paracrine factors or autoinducers that increase their metastatic efficiency; these observations have been made since the 90's[198] and recently by combining mathematical modeling with experimental evidence, the presence of QS systems in cancer was confirmed[199]. Such interesting findings are now opening new areas of study, including the development of future clinical applications (a summary of all the QS reviewed is provided in Table 1).

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REVIEW

Epidemiology, management, and economic evaluation of screening of gallstone disease among type 2 diabetics: A systematic review

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Abstract

The knowledge of gallstone disease (GSD) is crucial to manage this condition when organizing screening and preventive strategies and identifying the appropriated clinical therapies. Although cholecystectomy still be the gold standard treatment for patients with symptomatic GSD, expectant management could be viewed as a valid therapeutic method for this disorder. If early treatment of GSD decreases the morbidity or avoids further cholecystectomy, it may save clinical care costs in later disease periods sufficiently to offset the screening and early treatment costs. In addition, whether routine screening for GSD is worthwhile depends on whether patients are willing to pay the ultrasonography screening cost that would reduce the risk of cholecystectomy. In this review we discuss the epidemiology, management, and economic evaluation of screening of GSD among type 2 diabetics.

Key words: Gallstone disease; Epidemiology; Management; Economic evaluation; Type 2 diabetes

Core tip: According to the willingness-to-pay viewpoint, this review indicated that from the societal perspective but not from consumer viewpoint, it is worthwhile to organize a routine ultrasonography screening for gallstone disease in diabetic population for further cholecystectomy prevention.

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INTRODUCTION

Gallstone disease (GSD) is a common gastrointestinal condition with crystalline deposits in the gallbladder and impaired excretion of bile into the intestine throughout the world[1]. GSD yields a relative lower mortality rate, however, a relative higher risk of mortality in GSD patients is not totally explained by the high mortality rate of related cancer. This high morbidity of GSD substantially affects the economy and public health[2]. The increasing incidence of GSD over the past several decades is due to parallel modifications in personal dietary habits and physical activity associated with the Western lifestyle[3]. In the absence of an organized screening program for symptomatic GSD, treating GSD and related complications yields substantial medical burden[1].

Based on the Wilson criteria, GSD is needed to screen due to it is one of essential health issues; the disease natural course should be known; there should be a recognizable latent or early symptomatic state; a screening process is easy to do and interpret, accurate, acceptable, reliable, and has good sensitivity and specificity; there should be an acceptable treatment recognized for this disorder; treatment is much better if began early; there should be a clinical policy on who should be treated; both diagnosis and treatment have good efficacy; and this condition should be a continuous disease process[4]. Both obesity and the metabolic syndrome have been viewed as risk factors related to GSD formation[3,5,6]. Epidemiologic evidence suggested that people with diabetes mellitus were at higher risk of stone formation[3,7]. Academic studies indicated an increased morbidity of GSD in diabetic patients[7-9]. In addition, hypertriglyceridemia, hyperinsulinemia, and autonomic neuropathy (leading to gallbladder hypomotility and biliary stasis) were also revealed as associated factors to the incident GSD diabetic population[7,8]. This implies that GSD formation and diabetes development may share pathophysiologic pathways[3]. However, how diabetes predisposes to GSD is still not well known[8].

The choice of ultrasound scanning in GSD evaluation is ideal as it is cheap, non-invasive, safe, and repeatable without known adverse effect on the patients in clinical scenarios[10]. For symptomatic GSD subjects, expectant management may also indicate a valid clinical therapy although cholecystectomy still represents the gold standard[11]. From the viewpoint of preventive medicine, early detection of GSD by routine ultrasonography screening followed by appropriate therapy could avoid the further cholecystectomy. This review aims to explore the epidemiology, management, and economic evaluation of screening of GSD among type 2 diabetics.

THE CLINICAL DIAGNOSIS OF GSD

It is often a diagnostic challenge to determine which abdominal symptoms are related to GSD. Typically, GSD pain occurs in the right upper quadrant of the abdomen, but pain is not specific in this area[12]. Fewer than 50% of those with GSD actually have clinical symptoms, and fewer than 10% further develop potentially life-threatening complications[13]. The physicians must depend on the patient's description of the pain and results of laboratory examinations and diagnostic imaging to decide a appropriate diagnosis[12]. The physical examination also may show mild epigastric or right upper quadrant tenderness, but most patients do not have significant physical characteristics[13]. The majority of asymptomatic GSD will remain asymptomatic for a long time period.

Mechanisms underlying GSD formation include supersaturation of bile with cholesterol, consequent sedimentation, crystallization, and stone formation and abnormal gallbladder motor function with resultant delayed emptying and stasis of bile[10]. The availability of ultrasonography as viewed a valid tool for GSD diagnosis has allowed the evaluation of GSD morbidity[2]. It is safe, fast, and not expensive and involves no radiation exposure[13]. Positive findings include single or multiple stones, a positive Murphy sign on contact with the ultrasonographic probe, thickening of the gallbladder wall, and pericholecystic fluid[14]. Patients are usually left feeling unwell for as much as one or two days. If obstruction persists, it worsens movement and palpation, is associated with fever, and is localized to the right upper guardant part of abdomen, with the pain becoming sharp, which will result in acute cholecystitis[12,15]. Clinical studies showed higher positive (0.99-1.00) and negative (0.90-0.96) predictive values regarding the diagnostic efficacy, indicating that ultrasonography is a reliable technology for GSD screening[1,16]. However, a drawback is that its accuracy is dependent on the people who perform and interpret it[13].

Biliary pain occurs when the neck of the gallbladder is hindered by a gallbladder or stone pressure rises, producing a visceral foregut pain[15]. Factors that relate to choledocholithiasis include tests of abnormal liver function, common bile duct dilation of eight mm or more, and common bile duct stones identified by ultrasonography[17]. In addition, the abdominal plain radiography or computer tomography (CT) scan should also exclude the presence of calcified stones[18-20].

THE MORBIDITY OF GSD

Epidemiological studies in both Eastern and Western countries showed that ultrasonography is an reliable diagnostic tool for GSD morbidity[2,21-23]. The mechanisms of GSD have been implicated in type 2 diabetes. Some controversy exists regarding the association between diabetes and GSD, although population-based epidemiologic studies have demonstrated a positive relationship between type 2 diabetes and increased morbidity of GSD[1,24-26]. The possible pathogenic mechanism for this is that type 2 diabetic population

Table 1 Prevalence of gallstone disease in various populations

Ref.	Study year	Screened number	Setting	Prevalence of gallstone disease	Associated factors
Elmehdaw *et al*[8]	2009	327	Benghazi, Libya	DM: 9.75% Non-DM: 17.5%	Age, obesity
Pradhan *et al*[28]	2009	80	Nepal		Non-vegetarian
Acalovschi *et al*[29]	2009	1332	Romania	19% with chronic hepatitis C; 17% controls	Abdominal obesity, steatosis
Khan *et al*[30]	2009	9175 (5050 males, 4125 females)	England	Male fell from 20.2% to 19.1%, females fell from 30.4% to 29.0%	Diabetes, not for CHD, BMI to females, elderly
Friedrich *et al*[31]	2009	9206 (5559 from Danish, 3647 of German)	Denmark, Northeast Germany		Higher BMI, unfavorable lipid levels, higher prevalence of diabetes
Walcher *et al*[32]	2010	2147	Germany	8%	Protective effect: alcohol consumption
Ruhl *et al*[33]	2011	14228	United States	7.10%	Cardiovascular disease, cancer
Al-Bayati *et al*[34]	2012	200	Iraqi	33% of diabetics, 17% of non-diabetics	BMI > 25 kg/m^2, increased duration of DM, increased HbA1C, multiparous females
Jiang *et al*[35]	2013	1270	Shanghai, China	CAD (+): 19.5% CAD (-): 11.3%	CAD
Agunloye *et al*[10]	2013	400	Ibadan, Nigeria	17.5%	Age, BMI, DM, duration of the disease
Yilmaz *et al*[36]	2014	441	Turkey	12.2%	Age, BMI, Gender, metabolic syndrome
Shen *et al*[37]	2014	6511	Taiwan	13.2%	Age, Gender, metabolic syndrome
Ibitoye *et al*[38]	2014	1283	Nigerian	2.9%	

BMI: Body mass index; CHD: Coronary heart disease.

with GSD may cause acute cholecystitis more obvious and make higher likelihood of progression to septicemia compared with non-diabetic subjects, who exhibit functioning gallbladders normally. Type 2 diabetic patients may show a higher lithogenic bile index compared with non-diabetics after adjustment for sex and age[9]. The association between type 2 diabetes and GSD is stronger among patients who have a history of treated diabetes mellitus than it is among those with a single disease history of diabetes, that is, hyperglycemia may affect gallbladder motility[21]. The linkage between obesity, diabetes, and GSD most likely originate from metabolic syndrome[16,27]. In addition, diabetic patients represent cases of hyperglycemia that reflect relevant effects on gallbladder motility[9].

Tables 1 and 2 indicate that many evidence-based studies of the prevalence, incidence, and risk factors for GSD have been conducted. However, it is difficult to appropriately compare the results of some studies because the heterogeneous nature of these studies (for example, patient selection), which varied significantly. The prevalence of overall GSD was higher than the general Chinese population in Taiwan when using the same methodology of GSD assessment[8,42]. Previous population based studies had resulted in disparate findings on diabetes mellitus and GSD[24,25]. In Italy, the estimated prevalence of GSD is significantly higher in diabetic patients than in the general population (24.8% *vs* 13.8%)[43]. In New Zealand, the prevalence of GSD in diabetics was estimated to be 32.7% as compared to 20.8% in the control group[44]. An epidemiological study in Nigeria concluded that 17.5% of the diabetic patients had GSD on ultrasound[10]. The study about the prevalence of GSD in Chinese type 2 diabetics is rare or lack of appropriate statistical methods. The overall prevalence of GSD among type 2 diabetics in Kinmen was 14.4%, including single stone 8.0%, multiple stones 3.2%, and cholecystectomy 3.2%[42]. Further, the overall prevalence among elderly type 2 diabetics was 17.1% (men: 14.5%, women: 19.0%), which included the presence of single stone, 9.1%; multiple stones, 4.4%; and cholecystectomy 3.7%[45]. Upon international comparison, the prevalence of any type of GSD falls within the range of 10%-32% in type 2 diabetics and is higher than that in non-diabetic patients[44,46-49].

Cross-sectional study designs only reveal useful information of disease prevalence, but reveal nothing about the incidence or temporality in the study population. To explore the incidence and causal relationships between predictive factors and disease, the population needs to be re-examined regarding follow-up time. The morbidity of GSD increases as age increases, noticeably elevating in people aged 40 years and older and becoming from 4- to 10-fold more likely[4,16]. The incidence of GSD appears to vary among test diabetic populations and differs among studies conducted in disparate countries[1]. In the general and elderly Chinese type 2 diabetes population, the incidence of GSD was 3.56% per year (95%CI: 1.78%-6.24% per year) and 4.17% per year (95%CI: 2.22%-7.05% per year), respectively[9,26]. Previous epidemiologic studies showed that the annual incidence of overall GSD in type 2 diabetics was higher than that in other general population-based studies[9,22]. In addition, evidence-based studies exploring GSD in the elderly sub-population have focused almost entirely on the consequences of

Table 2 Incidence of gallstone disease in various populations

Ref.	Study year	Screened number	Setting	Incidence of gallstone	Associated factors
Festi *et al*[2]	2008	9611	Italy	0.67% (0.66% in males, 0.81% in females)	Risk factors: In men: increasing age, high BMI, history of diabetes, peptic ulcer and angina, and low cholesterol and high triglyceride levels; In females: increasing age and high BMI Predictors: In men: increasing age and pain in the right hypochondrium In females: increasing age
Halldestam *et al*[39]	2009	621	Sweden	1.39 per 100 person-years	Length of follow-up and LDL-cholesterol levels Inversely: alcohol consumption
Jonas *et al*[40]	2010	8901	Sweden	Surgical group: 122.2/10000 person-years controls: 22.2/10000 person-years	After antiobesity surgery (A fivefold increased risk)
Liu *et al*[25]	2012	108850 (60734 diabetic patients and 48116 control patients)	Taiwan	0.632% per year	Risk factor: increased age Associated: high body mass index, elevated fasting plasma glucose levels, and nonalcoholic fatty liver disease
Chen *et al*[1]	2014	1296	Taiwan	0.632%	High body mass index, elevated fasting plasma glucose levels, nonalcoholic fatty liver disease
Heida *et al*[41]	2014	288	Dutch	5.9%	BMI

BMI: Body mass index; LDL: Low density lipoprotein.

interventions such as percutaneous cholecystostomy and endoscopic retrograde cholangiopancreatography, or on the management of elderly patients with symptomatic biliary disease at hospitals[50,51]. The annual incidence of GSD in elderly type 2 diabetics was also higher than that in younger diabetic patients or the general population using the same methodology of GSD assessments[2,26]. To explore the incidence and risk factors for GSD is essential to prevent its development and avoid the further cholecystectomy caused by complications, which is often insidious in nature.

Gallstone formation is multifactorial, and involves constitutional and environmental factors. People with GSD have increased mortality, overall mortality, and mortality from cardiovascular disease and cancer. This relationship exists for ultrasound diagnosed GSD and cholecystectomy[33]. GSD with complications, especially cholecystitis and cholangitis, in the elderly is related to higher morbidity and mortality rates[52].

THE NATURAL COURSE OF GSD

The natural course of GSD is usually not malignant, but complications contribute substantially to medical care costs and may even be life threatening[40]. One of the essential advantages of early detection of GSD is that ultrasonography could diagnose asymptomatic stages, which incurs early treatment and the prevention of major complications such as acute pancreatitis or gallbladder cancer[53,54]. The increasing magnitude and epidemiologic shifts in the natural history of GSD worldwide qualify for the need of research in different geographical areas, and also to explore the predictive factors[55,56]. This is particularly because the majority of risk factors associated with GSD are potentially modifiable[41]. In addition, cholecystectomy could be used to treat GSD, that is, the estimated utility value in subjects with GSD will be a 0.09 increase from this therapy. Thus, the number of quality-adjusted life years obtained from cholecystectomy would be 1.8 (0.09 × 20) if subjects had a life expectancy of 20 years[57]. Screening regimes for GSD depend on the incidence and progression rates as well as the risk factors that change these rates. An understanding of the disease progression of GSD would appropriately determine the benefits of prevention strategies.

A chronic disease model according to the epidemiologic information of GSD is necessary to allow the benefits of intervention to be modeled. Since GSD may only persist a short duration before cholecystectomy, a shorter desirable interscreening interval may be warranted. The disease progression of GSD affects the decision of a screening interval for the surveillance of this patient population. In addition, the effectiveness of screening strategy for GSD is determined by the progression of GSD[58]. Since the natural history of GSD may not be homogeneous across study countries, assumptions of disease progression parameters could not be directly compared from previous results[11,59]. Several evidence-based studies on the natural history of GSD also have been conducted[11,58]. A clearer understanding of the risk factors associated with GSD may help us to identify cases and to reduce the risk in some patients[60].

For the disease natural course of GSD, the four-state Markov chains model following the pathway of

proliferative phase is showed as follows:

$$\underset{\text{(State 1)}}{\text{No GSD}} \xrightarrow{\lambda_{12}} \underset{\text{(State 2)}}{\text{single stone}} \xrightarrow{\lambda_{23}} \underset{\text{(State 3)}}{\text{multiple stones}} \xrightarrow{\lambda_{34}} \underset{\text{(State 4)}}{\text{cholecystectomy}}$$

To estimate the progression rates, let λ_{12}, λ_{23}, and λ_{34} indicate the annual progression rate from state 1 to state 2, from state 2 to state 3, and from state 3 to state 4, respectively. The annual progression rates from single stone to multiple stones and from multiple stones to cholecystectomy are estimated as 0.114 (95%CI: 0.015-0.173) and 0.148 (95%CI: 0.101-0.242), respectively. Corresponding average durations in single stone state and multiple stones stage are 8.77 (95%CI: 5.78-66.67) years and 6.76 (95%CI: 4.13-9.90) years, respectively. The application of parameters to the annual transition probabilities from single stone state to multiple stones state and from multiple stones state to cholecystectomy state are 10.00% and 13.76%, respectively. An annual screening program could reduce cholecystectomy by 82.9% (95%CI: 75.7%-90.4%) compared with the non-screening group. Comparatively, biennial screening, 3-year screening, 4-year screening, and 5-year screening could reduce cholecystectomy by 71.6% (95%CI: 57.0%-88.8%), 64.8% (95%CI: 46.1%-81.5%), 49.6% (95%CI: 23.9%-75.3%), and 32.1% (95%CI: -2.8%-66.7%), respectively[58]. However, one problem is in four-state Markov chains model, we should be aware that single stone might not always consequentially develop into multiple stone.

Many factors such as obesity and type 2 diabetes have been indicated to be significant risk factors related to GSD[1,9,11], and the transition state is probably unstable over time. The screening efficacy of preventing cholecystectomy associated with GSD depends on early diagnosis. To choose the frequency of sonographic check-ups as well as sensitivity and specificity, it is helpful to know the disease scenario and progression from the asymptomatic state to the symptomatic state. This characteristic will provide early diagnosis and therapeutic strategies for GSD[57].

THE MANAGEMENT OF GSD

The treatment options for GSD are according to few crucial steps such as typical symptoms, further complications, and gallbladder function, as well as size and composition of GSD[18]. Cholecystectomy remains the reliable operation for patients with symptomatic GSD. It is safe because the lowest risk of recurrence and more than 90% of patients with complete biliary pain relief[12]. Currently, it is also under argumentation if cholecystectomy may be also used for pre-symptomatic GSD. It is generally presumed that surgical procedures are not suggested routinely in symptom-free subjects due to the low rate of complications[18].

Statins used could relieve hepatic cholesterol biosynthesis and may reduce biliary cholesterol secretion, consequently causing decreased cholesterol concentration in bile[61]. A larger observational study showed academic evidence that long-term use of statins is related to a decreased rate of diagnosed GSD requiring advanced cholecystectomy[62]. Another population-based case-control study also indicated that long-term sustained statin use decreases incident GSD in both men and women[63]. The results may be one of clinical relevancies given that GSD represents a major burden for medical care systems[62]. In addition, a previous study showed that ezetimibe could not only prevent cholesterol GSD through obstructing intestinal cholesterol absorption so that biliary cholesterol secretion is decreased, and gallbladder motility function is reserved by desaturating bile in gallstone-susceptible C57L mice disputed to the lithogenic diet, but also promote the dissolution of cholesterol GSD through a greater capacity to develop an abundance of unsaturated micelles[64]. For both prevention and treatment of cholesterol GSD, ezetimibe is viewed as a novel and potential cholelitholytic agent[65].

Oral bile acids have successfully dissolved GSD in an extremely limited patient population for the nonsurgical treatment of GSD[12]. The clinical effectiveness of bile acid therapy was determined in symptomatic GSD smaller than 15 mm within a functioning gallbladder[12]. Oral bile acids could be only selected in symptomatic GSD patients who are unsuitable for cholecystectomy and have small, uncalcified, and cholesterol-enriched stones with a patent cystic duct in a functioning gallbladder[18,66]. Currently, bile acid therapy is revealed only for patients unsuitable or unwilling to receive cholecystectomy[12,67].

A previous study showed that acute cholecystitis grows in up to 10% of symptomatic GSD patients and leads to the entire obstruction of the cystic duct[68]. Once acute cholecystitis is found, patients should be revived with intravenous fluids, accompanying medical conditions should be stabilized, and surgery should be performed at the earliest time[12]. In addition, GSD could migrate from their primary site in the gallbladder through the cystic duct and into the common bile duct[12]. An essential treatment for choledocholithiasis includes gallbladder removal and clearance of retained common bile duct stones. The findings of a prospective, multi-center, randomized controlled trial comparing single-stage laparoscopic cholecystectomy (LC) and laparoscopic stone extraction with preoperative endoscopic retrograde cholangiopancreatography followed by LC indicated that the procedures were equally effective in the clearance of common bile duct stones[69].

THE ECONOMIC EVALUATION OF SCREENING OF GSD

Based on the welfare economic theory, the maximum value of individual's willingness-to-pay (WTP) is defined as the benefit to an individual receiving medical service or intervention[70]. For the WTP perspective, the payment vehicle not only refers to the means of payment by

a patient, but also is assumed as total cost of both copayment for health insurance scheme and out-of-pocket money which is not covered by health insurance benefit[71]. An evidenced-based study indicated that 24.4% of subjects would not like to pay a screening cost for GSD detection, implying that they did not think GSD status would influence their daily quality of life. The WTP values were significantly higher in individuals with more advanced GSD than in those with mild GSD. This also suggests that GSD associates with impaired quality of life and thus such patients would pay more to reduce the sequelae of further cholecystectomy[70].

Economic evaluations are criticized commonly by decision makers for ignoring budget impacts, about which decision makers are desperately concerned. Payers could get into financial difficulty if they adopt too many cost-effectiveness interventions and affordability, which depends on the overall volume of patients, is therefore a prime concern[11,72]. Few well-organized population-based studies have been conducted to explore the cost and effectiveness of GSD screening regimes. The cost-benefit analysis was used in one study to discuss whether a GSD screening regime compared with non-screening group is worthwhile in Taiwan from different viewpoint. The findings revealed that indirect costs play a main role in the routine GSD screening regime. Annual screening program could save the most discounted indirect costs per case (NTD220345) (US$6995.1; 31.0NTD = 1USD) compared with non-screening group. Based on the health care payer's viewpoint, annual screening discounted net cost was NTD24893 (US$790.3) per case. This implied that from health care payer's viewpoint, the clinical efficacy from the routine annual screening regime could not exceed the cost incurred in the GSD screening strategy. To consider the indirect cost, the NTD245238 (US$7785.3) net saving per case indicates that from the societal perspective, the annual screening program is rather constructive ($P < 0.0001$)[72]. In Chile, a screening program for GSD in a high risk sub-population reveals significant cost-effectiveness. The incremental cost-effectiveness ratio of universal screening compared with elective intervention, high risk intervention, and selective screening programs were estimated US$180, US$147, and US$481, respectively[73]. Preventive strategies aimed at GSD screening incur both substantial medical budgetary savings and highly cost-effective clinical care.

METHODOLOGICAL CONSIDERATION

Since GSD has a more complicated clinical aspect, it is not the rule for people with GSD definitely to progress towards more serious complications. In clinic, 60% of the patients with GSD are asymptomatic throughout their life. In these people, early detection may not help them to avoid possible healthy problem. In addition, up to now, there has been no effective early treatment for GSD that could prevent the resulting cholecystectomy. What we could and we should do now for GSD patients is to find the patients more likely to have a serious outcome in the future and perform an early cholecystectomy to avoid the secondary common bile duct stone, gallstone pancreatitis and possible cancerization. That is why some asymptomatic patients are indicated for cholecystectomy. Also, some asymptomatic patients with diabetes and cardio-cerebrovascular complications should be treated by cholecystectomy in a stable condition to avoid unpredictable attack of GSD. That is currently the aim for GSD screening. Furthermore, since the natural history of GSD may be heterogeneous and more complicated, according to current knowledge, there is no relationship between the number of gallstones and cholecystectomy. We still have no clear idea about how the gallstone produces and what is the progression factors for GSD.

CONCLUSION

In conclusion, GSD is an escalating major health problem and involves constitutional and environmental factors. Considering the fact that oral bile acids could be only selected in an extremely limited patient population (symptomatic gallbladder disease patients who are unsuitable for surgery and have small, uncalcified, and cholesterol-enriched stones), the majority of patients with or without complications will need surgery. Without suitable screening programs for symptomatic GSD, treating GSD and related complications yields substantial medical care costs. Whether routine screening for GSD is worthwhile depends on whether subjects are willing to pay the ultrasonography screening costs that would reduce the risk of further cholecystectomy.

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MINIREVIEWS

How to use magnetic resonance imaging following neoadjuvant chemotherapy in locally advanced breast cancer

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Abstract

Magnetic resonance imaging (MRI) is highly sensitive in identifying residual breast cancer following neoadjuvant chemotherapy (NAC), and consequently is a commonly used imaging modality in locally advanced breast cancer patients. In these patients, tumor response is an important prognostic indicator. However, discrepancies between MRI findings and surgical pathology are well documented. Overestimation of residual disease by MRI may result in greater surgery than is actually required while underestimation may result in insufficient surgery. Thus, it is important to understand when MRI findings are reliable and when they are less accurate. MRI most accurately predicts pathology in triple negative, Her2 positive and hormone receptor negative tumors, especially if they are of a solid imaging phenotype. In these cases, post-NAC MRI is highly reliable for surgical planning. Hormone receptor positive cancers and those demonstrating non mass enhancement show lower concordance with surgical pathology, making surgical guidance more nebulous in these cases. Radiologists and surgeons must assess MRI response to NAC in the context of tumor subtype. Indiscriminate interpretations will prevent MRI from achieving its maximum potential in the pre-operative setting.

Key words: Breast; Magnetic resonance imaging; Neoadjuvant chemotherapy; Biomarkers; Phenotypes

Core tip: Following neoadjuvant chemotherapy, breast magnetic resonance imaging (MRI) most accurately predicts surgical pathology in triple negative, Her2 positive and hormone receptor negative tumors, especially if they are of a solid imaging phenotype. In these cases, post-neoadjuvant chemotherapy (NAC) MRI is highly reliable for surgical planning. Hormone receptor positive cancers and those demonstrating non mass enhancement show lower concordance with surgical pathology, making surgical guidance more nebulous in

these cases. Radiologists and surgeons must assess MRI response to NAC in the context of tumor subtype.

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INTRODUCTION

Breast cancer is a heterogeneous disease consisting of many different tumor subtypes, each with its own biology, prognosis, and treatment options. These subtypes are characterized by distinct molecular profiles, proliferation rates, and tumor receptors, including estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2). In today's paradigm of personalized medicine, biomarker profiles allow tailoring treatment strategies to the individual tumor. Current treatment of locally advanced breast cancers includes chemotherapy, hormone therapy [if hormone receptor (HR) positive] and surgical resection. Increasingly, chemotherapy is given prior to surgery. Neoadjuvant chemotherapy (NAC) offers advantages in terms of adding prognostic information and improving surgical options. Tumor response to NAC is an important prognostic indicator. Patients who have a pathologic complete response (pCR) following NAC have improved overall survival, disease-free survival and recurrence-free survival[1-6]. NAC can also facilitate breast-conserving surgery in patients whose initial presentation may have warranted mastectomy[7-9]. Even if patients still have residual disease, especially if they need radiation, breast conservation will have fewer complications than mastectomy and radiation. As treatments improve and responses to NAC become more common, a new challenge arises - accurately determining the extent of surgical resection needed to excise residual tumor. Magnetic resonance imaging (MRI) is highly sensitive in identifying residual disease following NAC, with multiple studies demonstrating it to be more accurate than mammography, ultrasound or physical examination[10-16]. Consequently, MRI is a commonly used imaging modality in locally advanced breast cancer patients.

In these patients, pre-operative MRI is an important addition to the decision-making armamentarium. The appearance of breast cancer on MRI can be classified by its morphology into phenotypic categories[17], which are associated with response to NAC and ability to offer breast-conserving surgery[17,18]. Overall, MRI has been shown to be the most sensitive imaging modality by which to follow a patient's response to NAC and to be more sensitive than clinical examination[11-16,19-23]. While an excellent test, MRI is far from perfect. Discrepancies between MRI findings and surgical pathology are well documented. Overestimation of residual disease by MRI may result in greater surgery than is actually required (larger lumpectomies, wider margins, mastectomy)[1,24]. Underestimation may result in insufficient surgery, resulting in positive margins and re-excisions[1]. Thus, it is important to understand when MRI findings [particularly radiologic complete responses (rCR)] are reliable and when they are less accurate.

The general question of the accuracy of an rCR to predict a pCR may be overly broad - accuracy needs to be considered in the context of tumor subtype. Literature has shown that the accuracy of post-NAC MRI is related to tumor subtype, with the strongest evidence arising from multi-institutional trials like I-SPY[18] and Translational Breast Cancer Research Consortium Trial 017[25], as well as additional support from multiple single-institution studies[26-29]. A smaller literature base suggests that MRI phenotype is also related to the accuracy of MRI in the post-NAC, pre-operative setting.

In this manuscript, we review the evidence for accuracy of post-NAC MRI findings and focus on how best to use MRI in this setting, specifically for the evaluation of extent of disease and pCR. In particular, this review will evaluate the association between the diagnostic performance of MRI in the post-NAC setting and the biomarker profile of the tumor, as well as the association between pre-NAC phenotypic tumor appearance on MRI and diagnostic accuracy. A clear understanding of these relationships can be valuable in setting appropriate treatment goals and expectations[18]. In much the same way each breast cancer requires a tailored treatment strategy, a strategy for tailored imaging interpretation should also be employed and would enable more accurate recommendations to be made for individual patients.

ASSOCIATIONS WITH MRI PHENOTYPE

The relationship between phenotypic MRI appearance of breast cancers and response to NAC has been studied[17,29]. Although phenotypic categorizations vary slightly, in general, phenotypes tend to focus on the separation of solid and well-contained unifocal (Figure 1A) and multifocal masses from more diffuse and infiltrative non-mass enhancement (NME) (Figure 1B)[17,18,29]. These phenotypes impact NAC response, with well-defined mass phenotypes more likely to have a response sufficient to allow for breast conserving surgery[17,18]. Well-defined masses also show higher concordance between MRI and surgical pathology, with an rCR in the setting of solid phenotypes (particularly hormone-negative tumors) predictive of a corresponding pCR at surgery[18]. On the other hand, MRI is less accurate in predicting pCR in tumors presenting as non-mass/diffuse enhancement, with larger discrepancies between post-NAC MRI and surgical pathology[18].

Studies have also suggested that these differing phenotypic appearances have particular patterns of

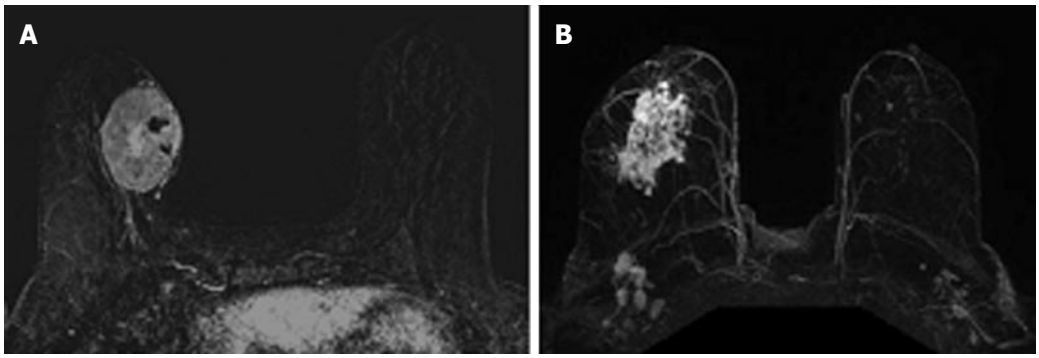

Figure 1 Magnetic resonance imaging phenotypes solid unifocal mass (A) and more diffuse non-mass enhancement (B).

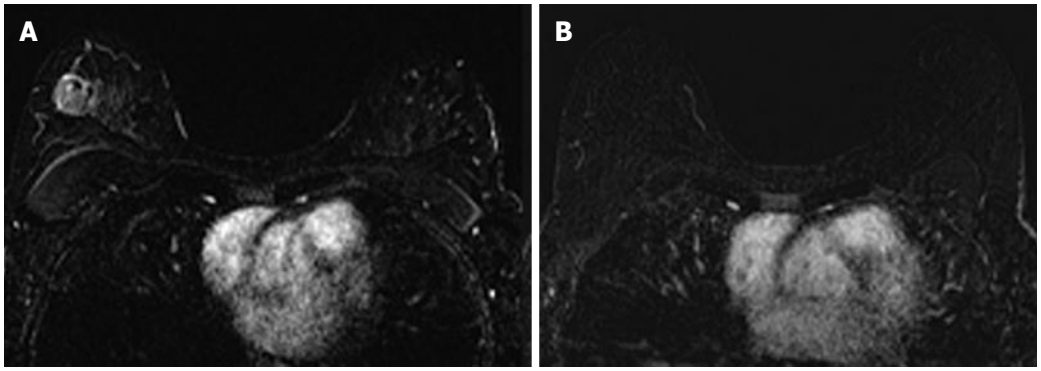

Figure 2 Forty-two years old woman with triple negative right breast cancer. A: Baseline axial T1-weighted post-gadolinium fat-saturated magnetic resonance image demonstrates a 3.6 cm unifocal mass in the upper outer quadrant; B: Post-neoadjuvant chemotherapy magnetic resonance imaging demonstrates complete resolution of the mass seen previously. Surgical pathology demonstrates biopsy site changes and expected changes related to chemotherapy with no evidence of residual cancer.

response to NAC[17,24,29-31]. Locally advanced malignancies presenting as a mass lesion often shrink in a concentric pattern to a smaller mass. Following NAC, NME often diminishes to a scattered pattern of residual disease that can extend throughout the original area of involvement, though as small foci that are difficult to detect on MRI. Residual infiltrating single cells will likely not be visible on MRI.

The associations between MRI accuracy and phenotype are likely confounded by tumor biomarker status. Comparisons of MRI phenotypes relative to tumor biomarker profiles[18,24,28,29,32,33] have shown a number of trends, with an association between unifocal mass presentation and triple negative tumors (TN: ER negative, PR negative, Her2 negative) (Figure 2). Multifocal mass presentation is more common in HER2+ (and questionably in HR positive) tumors. Although they do not have a characteristic phenotypic presentation, HR positive cases, especially ER positive tumors, are more likely to present as non mass/diffuse enhancement compared to other subtypes (Figures 3 and 4). Although these relationships have been demonstrated, all phenotypes are seen in all biomarker profiles[18].

ASSOCIATIONS WITH TUMOR BIOMARKERS

Extent of disease evaluation

The impact of tumor biomarkers on the accuracy of MRI for detecting the extent of disease must be considered when interpreting post-NAC MRI in anticipation of surgical resection. In addition to the individual status of receptors, biomarkers can categorize tumors into different subtypes. Tumor subtypes include luminal (ER/PR positive, Her2 negative), Her2 positive, and basal (ER/PR/Her2 negative; analogous to TN.) Multiple studies have demonstrated that in the post-NAC setting, the MRI assessment of extent of residual disease is most accurate in tumors that are either TN (Figure 2) or Her2 positive.

McGuire *et al*[26] retrospectively reported their institutional experience and found that MRI was most accurate in estimating pathologic size of residual disease in the Her2 positive and TN subtypes. Additionally, they found that MRI was more likely to underestimate the amount of residual disease in the luminal subtype (ER/PR positive, Her2 negative) when compared with TN or Her2 positive tumors. In a study done by Loo *et al*[29], MRI findings correlated well with the pathologic findings in the TN and Her2 positive breast cancers, but not with ER positive or Her2 negative breast cancers. Kuzucan *et al*[24] evaluated only Her2 negative cancers, and report similar findings-higher concordance between post-NAC tumor size on MRI and pathologic size in HR negative tumors compared to HR positive tumors. In Kuzucan's study, MRI accuracy was also increased in tumors expressing high levels of the proliferation marker Ki-67 (defined as > 40% positive). A study by

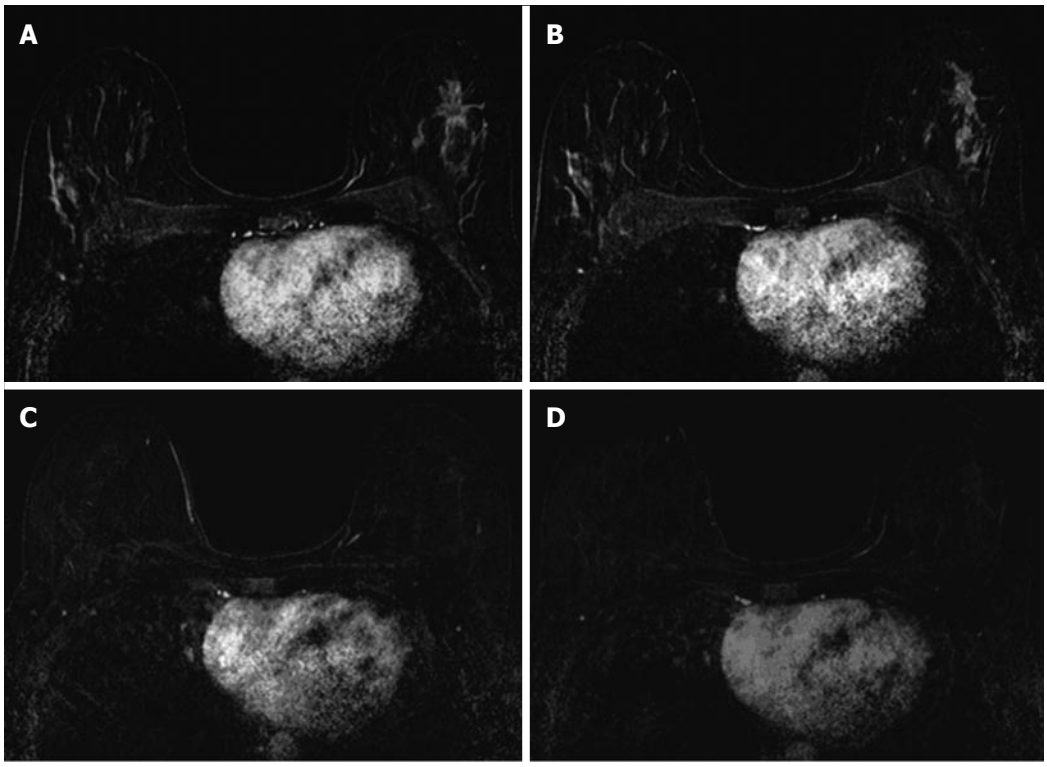

Figure 3 Thirty-seven years old woman with HR+ left breast cancer. A and B: Baseline axial T1-weighted post-gadolinium fat-saturated magnetic resonance image demonstrates a speculated mass and contiguous non-mass enhancement extending posteriorly for a total of 7 cm of disease in the upper outer breast; C and D: Post-neoadjuvant chemotherapy magnetic resonance imaging demonstrates decrease in size and degree of enhancement of prior findings. Surgical pathology demonstrates 6.9 cm of invasive ductal carcinoma.

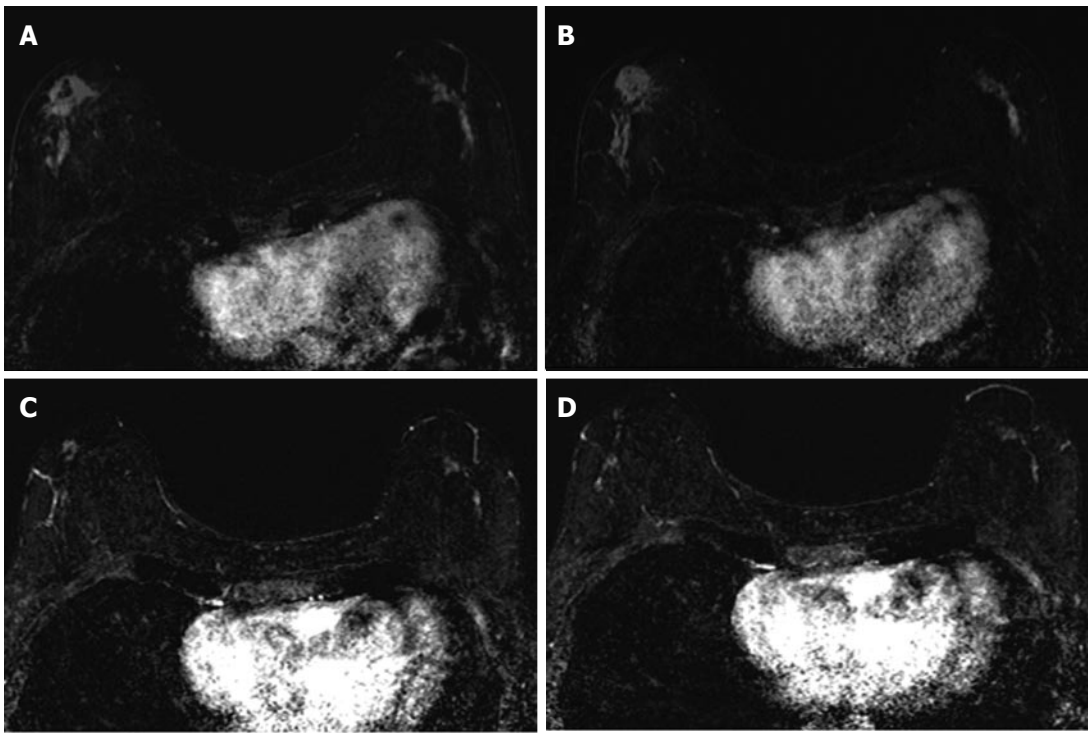

Figure 4 Sixty-four years old woman with bilateral HR+ breast cancer. A and B: Baseline axial T1-weighted post-gadolinium fat-saturated magnetic resonance image demonstrates 3.2 cm irregular mass and contiguous non-mass enhancement (NME), spanning up to 7.2 cm, in the right central outer breast and 3.5 cm of clumped linear NME in the central outer left breast; C and D: Post-neoadjuvant chemotherapy magnetic resonance imaging demonstrates decrease in size of the right breast mass and NME. NME in the left breast demonstrates only mild improvement. Surgical pathology demonstrates 4.3 cm of residual disease on the right and 3.7 cm of disease on the left.

Kim *et al*[34], which investigated TN cancer, also found that Ki-67 affects the diagnostic accuracy of MRI, with higher correlation between MRI and residual tumor size at surgery in Ki-67 positive patients.

In I-SPY, a multicenter neoadjuvant trial with serial MRIs over the course of therapy, there were the fewest discrepancies between the post-NAC MRI tumor size and pathologic size in Her2 positive, HR negative, and TN tumors[18]. Overall, 38% of patients analyzed had a size discrepancy of at least 2 cm between MRI and surgical pathology, with two thirds of these discrepancies being an overestimation of disease on MRI. These size discrepancies were significantly more common in HR+/Her2- tumor subtypes. Additionally, size discrepancies differed by MRI phenotype; among the solid phenotypes, underestimation of disease by 1.5 cm or more was rare. These Her2+, HR-, and TN tumors were also the tumor subtypes most likely to have a substantial response to NAC. The experience at our institution is in accordance with other published reports. In cases of Her2 positive, HR negative, and TN tumors, if there is residual disease on MRI, it is highly likely that there will be residual disease in the surgical specimen. Underestimation of disease in these subtypes is rare, particularly in the triple negative group where no false negative MRI's were seen.

pCR evaluation

Apart from measuring residual disease for the purposes of surgical planning, the ability of MRI to predict a pathologic complete response (pCR), a surrogate for improved outcome, is of particular importance in breast cancer management. Attaining pCR gives prognostic information that can be used for decision making, including the type of surgical procedure and/or reconstruction to recommend, and is also used as an immediate endpoint in evaluating the efficacy of NAC. Data show that pCR is associated with improved outcomes, and is more predictive when assessed by individual tumor subtype than for all subtypes combined[1,35]. A non-invasive method to accurately determine whether or not a pCR had been achieved would potentially change how trials are designed, and could eventually change surgical management of breast cancer.

While MRI accuracy depends on both its positive predictive value (PPV), and its negative predictive value (NPV), the NPV becomes the most important variable if the goal is to spare a patient invasive treatment in the setting of an rCR. That is, one must be able to trust that a negative MRI is a true negative in order to safely omit surgical resection or other treatment. In the reported papers looking at the accuracy of MRI for predicting pCR in the post-NAC setting, one must note that relatively high accuracy is possible with a low NPV. This can occur when MRI has a very high PPV, ultimately leading to high accuracy despite low NPV. In tumor subtypes that are less likely to respond to NAC, such as luminal tumors, the likelihood of residual disease is high, resulting in high PPV. However, the chance of a false negative is also highest in this group, so despite high apparent accuracy (driven by PPV), an rCR should be interpreted with caution (Figures 3 and 4).

Just as the accuracy of MRI in predicting extent of disease differs by tumor subtype, it appears that the ability of MRI to accurately predict pCR also differs by tumor subtype[24]. The NPV of MRI for predicting pCR differs by tumor subtype, highest in HR negative/Her2 positive tumors and triple negative (Figure 2) tumors[18,24-26,36]. In our report of I-SPY patients, when the post-NAC MRI underestimated residual disease (which occurred 4.3% of the time), all the discordant cases were either HR positive (Figures 3 and 4) or had diffuse phenotypes (Figure 4)[18].

Recently, several groups have reported on the accuracy of post-NAC MRI for correctly identifying pCR. Of note, some groups define pCR as the absence of any invasive tumor cells (the preferred definition of the FDA)[37], while others require the absence of both invasive and *in situ* disease - this definition must be noted when interpreting study findings, as residual *in situ* disease may lead to higher local recurrence rates[38].

One retrospective multicenter study of 746 women undergoing NAC found overall NPV for MRI of 47% and accuracy of 74% for predicting pCR[25]. The NPV for MRI varied by tumor subtype, and was highest amongst HR-/Her2+ tumors (62%) and TN tumors (60%). The overall accuracy was highest for HR+/Her2 negative tumors, likely because the PPV in this group was 91%. This likely reflects the fact that because this subtype is the least likely to respond to NAC, the pretest probability for having residual disease is higher.

Single institution studies have shown similar results. Chen *et al*[27] demonstrated the vast difference in MRI accuracy by tumor subtype, with accurate prediction of pCR in 95% of Her2 positive tumors, but only in 50% of Her2 negative tumors. Kim *et al*[34] found MRI accurately predicted pCR in 91% of TN cases. Kuzucan *et al*[24] focused on Her2 negative patients, and also found higher accuracy in HR negative tumors, with a PPV of 88% and NPV of 88%. In HR positive tumors, MRI had a PPV of 100% but an NPV of only 56%. The authors noted that the higher NPV in the HR negative tumors may have been related to a higher prevalence of solid tumor phenotypes, acknowledging that tumor phenotype impacts MRI accuracy and response to NAC[24]. In Ko *et al*[28]'s 2013 report, overall PPV was 89.6% and NPV was 83.8%. Of the five false negative MRI's in their study, 3 were ER positive, 2 were Her2 positive, and 3 initially appeared as non mass enhancement. The most recent report, by Bufi *et al*[36] in 2014, shows the highest NPV rates to date. In the TN tumor subtypes, they report NPV of 100%. In the Her2 positive subtype, they report NPV of 100% using diffusion weighted imaging, suggesting that newer advanced MRI techniques may improve accuracy of MRI in different subtypes[36].

CONCLUSION

MRI most accurately predicts pathology in TN, Her2 positive and HR negative tumors, especially if they are of a solid imaging phenotype. In these cases, post-NAC MRI is highly reliable for surgical planning. Hormone receptor positive cancers and those demonstrating NME demonstrate lower concordance with surgical pathology, making surgical guidance more nebulous in these cases.

While MRI may not yet meet the necessary NPV threshold to safely allow for omission of surgical treatment, this may be feasible for specific tumor subtypes in the future. It is unclear whether or not the differential accuracy of MRI by tumor subtype is mediated by tumor phenotype, tumor response to NAC, or biological differences that affect imaging, or possibly by all of these factors. Regardless, it is clear at this point that radiologists and surgeons must assess MRI response to NAC in the context of tumor subtype. If imaging interpretations are not made in this context, pre-operative MRI will continue to be limited by both overestimation and underestimation of residual disease. The same way each breast cancer requires a tailored treatment strategy, tailored interpretation strategies should also be employed. Future work on redefining thresholds for enhancement interpretation based on tumor biology and on the development of receptor subtype-based imaging protocols may improve accuracy in the future.

With the understanding that pCR predicts recurrence free survival, if an rCR can confidently predict pCR (as in TN and Her2 positive tumors), then an rCR can predict recurrence free survival. As an imaging predictor for such important outcomes, MRI interpreted in the context of tumor subtype would be a tremendous asset in decision-making and patient counseling.

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MINIREVIEWS

Cervical cancer screening: A never-ending developing program

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Abstract

With the term "oncological screening", we define the overall performances made to detect early onset of tumors. These tests are conducted on a population that does not have any signs or symptoms related to a neoplasm. The whole population above a certain age, only one sex, only subjects with a high risk of developing cancer due to genetic, professional, discretionary reasons may be involved. Screening campaigns should be associated, when risk factors that can be avoided are known, with campaigns for the prevention of cancer by means of suitable behavior. The goal of cancer screening cannot however be limited to the diagnosis of a greater number of neoplasms. Screening will be useful only if it leads to a reduction in overall mortality or at least in mortality related to the tumor. Screening should then allow the diagnosis of the disease at a stage when there is a possibility of healing, possibility that is instead difficult when the disease is diagnosed at the appearance of signs or symptoms. This is the reason why not all campaigns of cancer screening have the same effectiveness. In Italy, every year there are about 150000 deaths due to cancer. Some of these tumors can be cured with a very high percentage of success if diagnosed in time. Cervical cancer can be diagnosed with non-invasive tests. The screening test used all over the world is Papanicolaou (Pap) test. This test may be carried out over the entire healthy population potentially exposed to the risk of contracting cancer. Public health has begun the screening campaigns in the hope of saving many of the approximately 270000 new cases of cancer reported each year. Screening is done following protocols that guarantee quality at the national level: these protocols are subject to change over time to reflect new realities or to correct any errors in the system. A simplified sketch of a possible route of cancer screening is as follows: (1) after selecting the target population, for example all women between 25 and 64 years (in the case of monitoring of cervical cancer), an invitation letter with the date and time of the appointment, planned according to the acceptance capacity of the hospital, is sent to all individuals; (2) an examination, which depending on the individual and the type of cancer to be monitored, for example, can

be a Pap smear, is performed and the patient can go home; (3) once available the results of examinations, if negative, they shall be communicated to the person concerned that will be notified by mail and will be recalled for a second test at a few years of distance, in the case of non-negativity, instead, the patient is contacted by telephone and informed of the need to carry out further examinations: it is said that the patient is in the "phase two" of the screening pathway; (4) in phase two, reached by only a small portion of the interested parties (usually less than 3%-5%), more in-depth tests are carried out, which, depending on the individual and the type of cancer, can be: cytological and colposcopic examinations, the removal of a fragment of tissue (biopsy) and subsequent histological examination, additional tests such as ultrasound, radiography, or others such as computerized tomography, magnetic resonance imaging, positron emission tomography, *etc.*, in case of negativity, the concerned person will be called for new control tests at a a few years of distance, in case of non-negativity, it will be proposed instead an oncologic therapeutic plan and/or surgery to treat the diagnosed tumor; and (5) once the treatment plan is completed, the individual enters the follow-up protocol, which is monitored over time to see if the tumor has been completely removed or if instead it is still developing. Cervical cancer is undoubtedly the most successful example of a cancer screening campaign. Paradoxically, its effectiveness is one of the strongest reasons to criticize the usefulness of vaccination against human papillomavirus (HPV) in countries where the screening service with Pap test is organized in an efficient manner. Cervical cancer screening protocols are directed to sexually active women aged 25-64 years: they provide the Pap test performed by examining under a microscope or by staining with a specific "thin prep" the material taken from the cervix with a small spatula and a brush. It is recommended to repeat the test every two or three years. It is important to emphasize that women vaccinated against HPV must continue the screening with Pap test. Although some screening programs (*e.g.*, Pap smears) have had remarkable success in reducing mortality from a specific cancer, any kind of screening is free from inherent limitations. The screening methods are in fact applied to large parts of the apparently healthy population. In particular, the limits for certain cancers may be as obvious as to prohibit the introduction of an organized screening program. Potential limitations of organized screenings are basically of two types: organizational and medical. The limits of organizational type relate to the ability of a program to recruit the whole target population. Although well organized, a screening program will hardly be able to exceed a coverage of 70%-80% of the target population, and in fact the results of the current programs are often much smaller. The limits of medical type are represented by the possibility of reducing the overall mortality, or specific mortality, using a specific screening campaign.

Key words: Cervical cancer; Screening; Papanicolaou test; Human papillomavirus test; Vaccination

Core tip: Most cases of cervical cancer are preventable and, if caught early, highly curable. Despite this, cervical cancer is the second most common cause of cancer death and a leading cause of morbidity in women worldwide. Unfortunately, cure is less likely when the disease is diagnosed at an advanced stage. Although the human papillomavirus is considered the major causative agent of cervical cancer, yet the viral infection alone is not sufficient for cancer progression.

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INTRODUCTION

Human papillomavirus (HPV) belongs to the diverse group of sexually transmitted viruses that manifest affinity to the squamous epithelia of the skin and mucous membranes. It has been proved that types 16 and 18 in particular could lead to cervical cancer. High-risk strains of HPV (HR-HPV) types have been found in cervical cancer worldwide[1]. The Papanicolaou (Pap) smear was the mainstay of screening in women for over 60 years[2]. All current guidelines recommend colposcopy for women with high-grade squamous intraepithelial lesions (H-SIL), with a view to performing a biopsy or conization. Randomized controlled trials and retrospective comparisons much more strongly suggest that regular well-organized smear testing prevents a number of deaths due to cervical cancer. It should be remembered that many cellular atypia found on cervical smears never progress to cancer. The frequency of overdiagnosis has not been studied. Smear-based screening appears to have very few serious adverse effects. In practice, despite the lack of solid evidence, it seems unreasonable not to recommend screening for cervical cancer. Organized screening is preferable to opportunistic screening performed without quality controls and without research to optimize screening strategy[3,4]. The available technology for prevention and its developments allows real opportunities for cervical cancer elimination in defined populations to be foreseen[5-8].

PHASE ONE: CYTOLOGY AND HPV DNA TESTING

Two quality metrics for gynecologic cytology are

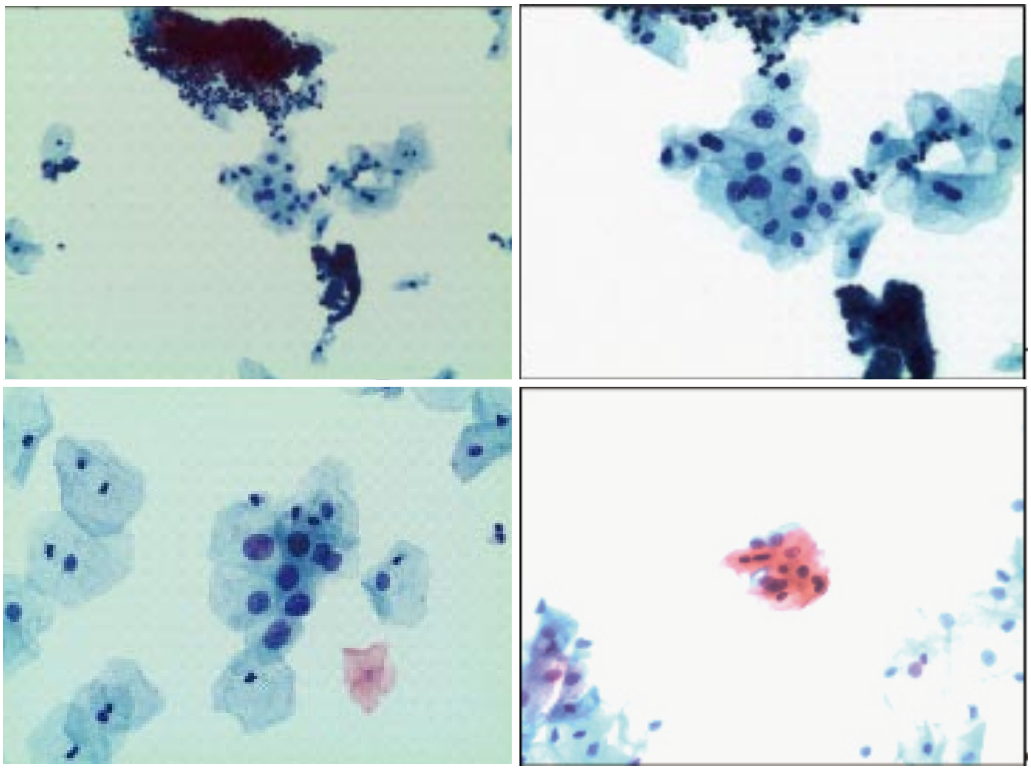

Figure 1 Atypical squamous cells of undetermined significance.

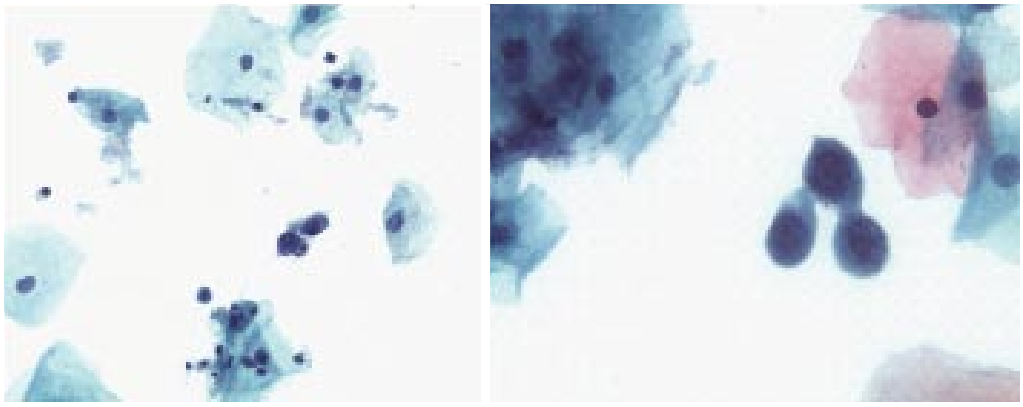

Figure 2 Atypical squamous cells, cannot exclude high-grade squamous intraepithelial lesion.

available: "prospective rescreening" and "retrospective rescreening". Most laboratories (> 85%) prospectively rescreen more than 10% of Pap tests interpreted as negative for intraepithelial lesion or malignancy. Most (72%) report inclusion of less than 20% high-risk cases. Most laboratories use multiple measures to define "high risk". Most laboratories (96.2%) retrospectively rescreen Pap tests from the preceding 5 years only. In most laboratories (71.4%), only Pap test results with H-SIL or worse prompt retrospective review. Upgraded diagnoses from negative for intraepithelial lesion or malignancy to atypical squamous cells (ASC), cannot exclude H-SIL (ASC-H), should be monitored (Figures 1-5)[9-18].

PHASE TWO: COLPOSCOPY AND HISTOLOGY

Though in the 1980s colposcopically-directed biopsy excluded over 90% of cervical intraepithelial neoplasia (CIN) 3 or worse (CIN3+), recent reviews found sensitivity of colposcopically-directed biopsy for CIN3+ of 50%-65%. Studies from China showed that the sensitivity of colposcopically-directed biopsy for CIN3+ is higher for large CIN3+ than for small CIN3+ and higher for associated high-grade cervical cytology than for low-grade cervical cytology. Colposcopically-directed biopsy excluded over 90% of CIN3+ in the 1980s because colposcopy clinics in the 1980s evaluated women with

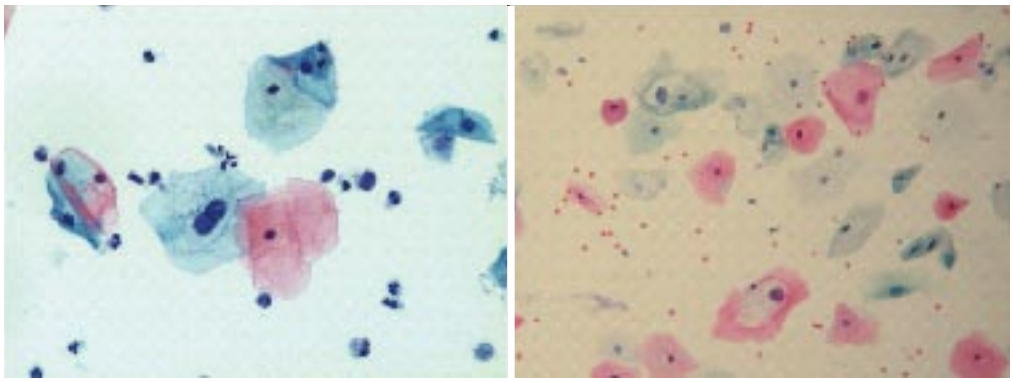

Figure 3 Low-grade squamous intraepithelial lesion.

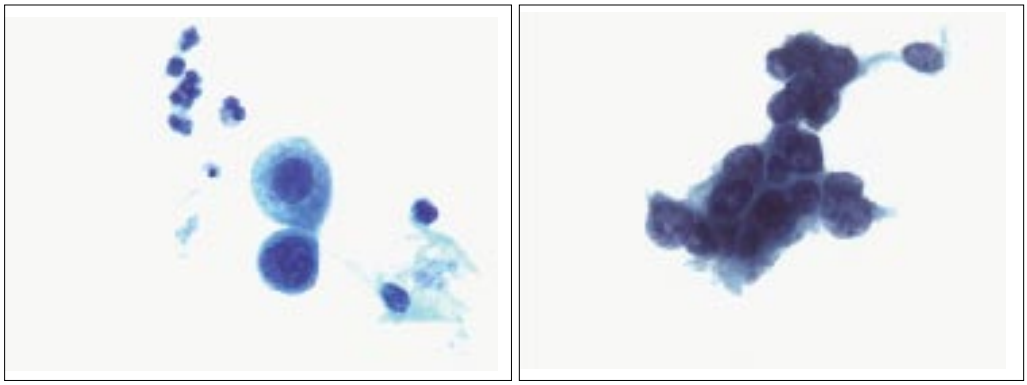

Figure 4 High-grade squamous intraepithelial lesion.

Figure 5 Atypical glandular cells.

high-grade cytology that had large CIN3+. It no longer excludes CIN3+ well because current colposcopy clinics evaluate women with low-grade cytology that have small CIN3+. When colposcopically-directed biopsy is used to exclude CIN3+, our understanding of the natural history of CIN is skewed, errors occur in defining appropriate screening practice, and inaccurate diagnosis results in incorrect treatment. The impression that CIN is more common on the anterior lip of the cervix is an artifact introduced by the inaccuracy of colposcopy. An unjustified enthusiasm for screening with visual inspection with acetic acid (VIA) occurred when the sensitivity of VIA for CIN3+ was inflated by screening studies using colposcopically-directed biopsy as the gold-standard for CIN3+. As the diagnosis of CIN3+ solely by endocervical curettage (ECC) is uncommon in women under age 25, the ECC may be omitted in women under age 25 years. If multiple cervical biopsies are performed, to limit discomfort, a bronchoscopy biopsy instrument which obtains 2-mm biopsies should be used (Figures 6-17)[19,20]. A novel technique uses a high-resolution microendoscope (HRME) to diagnose cervical dysplasia. HRME imaging reduces the limitations of existing cervical cancer screening methods currently in use in low-resource settings and so has the potential to contribute to cervical cancer prevention in the developing world[21,22].

PHASE THREE: TREATMENT AND FOLLOW-UP

Pre-cancerous lesions of cervix (CIN) are usually treated with excisional or ablative procedures. In the United Kingdom, the National Health Service cervical screening guidelines suggest that over 80% of treatments should be performed in an outpatient setting (colposcopy clinics). Treatment methods commonly used for precancerous lesions are conization,

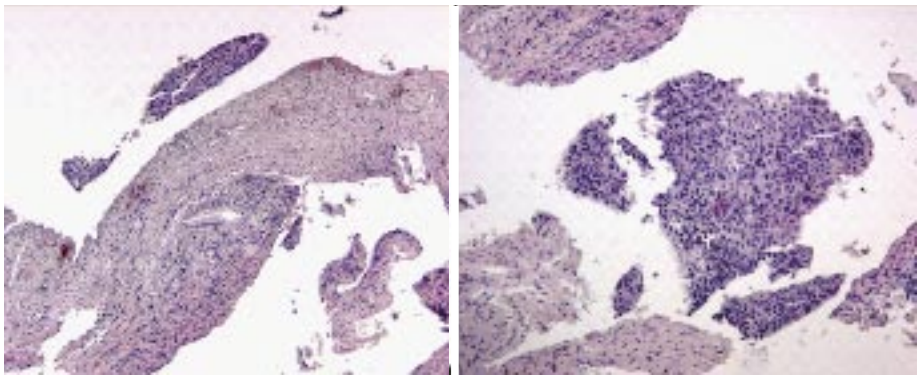

Figure 6 Dysplasia histologic samples.

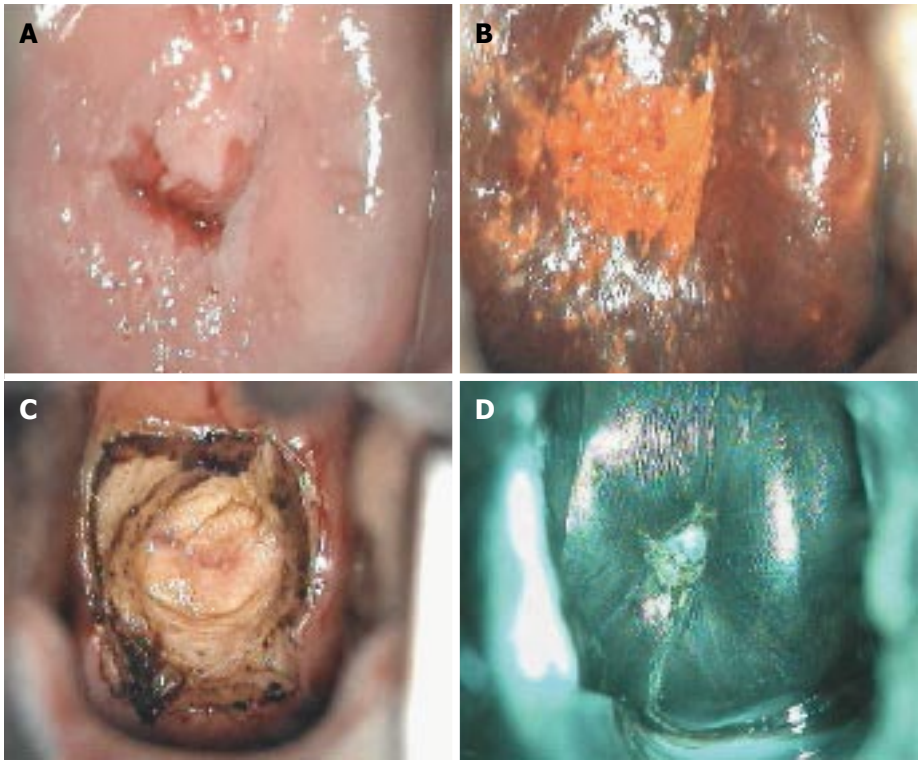

Figure 7 Cervical intraepithelial neoplasia grade 1 colposcopic appearance: (A) after acetic acid application; (B) after Lugol's iodine solution application; (C) after loop electrosurgical excision procedure; and (D) at 6-mo follow-up.

loop electrosurgical excision procedure (LEEP), laser ablation, and cryotherapy. Recently, outpatient LEEP has replaced cryotherapy in many countries. However, a greater awareness of the importance of cervical cancer in the developing world and a greater awareness of the long-term consequences of LEEP like cervical insufficiency, have renewed interest in cryotherapy. Among the trials, cure rates ranged from 56.8% to 96.6% in prospective controlled studies and from 70% to 95.5% in observational trials. Cryotherapy has very low rates of complication and serious complications that require medical therapy or affect the reproductive future results are extremely rare. Side effects include vaginal discharge and cramping which are temporary, usually self-limited, and well tolerated after preventive patient counseling. When surveyed, women highly accept cryotherapy. Compared to other methods of treatment, cryotherapy is very affordable and feasible to integrate screening programs and treatment for cervical cancer[23]. Often, due to improper judgment of interventional indications for cervical lesions, overtreatment to various degrees takes place, influencing patients' health and lives[24,25].

GUIDELINES

Cytopathology experts, interested stakeholders, and representatives from the College of American Pathologists (CAP), the Centers for Disease Control and Prevention, the American Society of Cytopathology

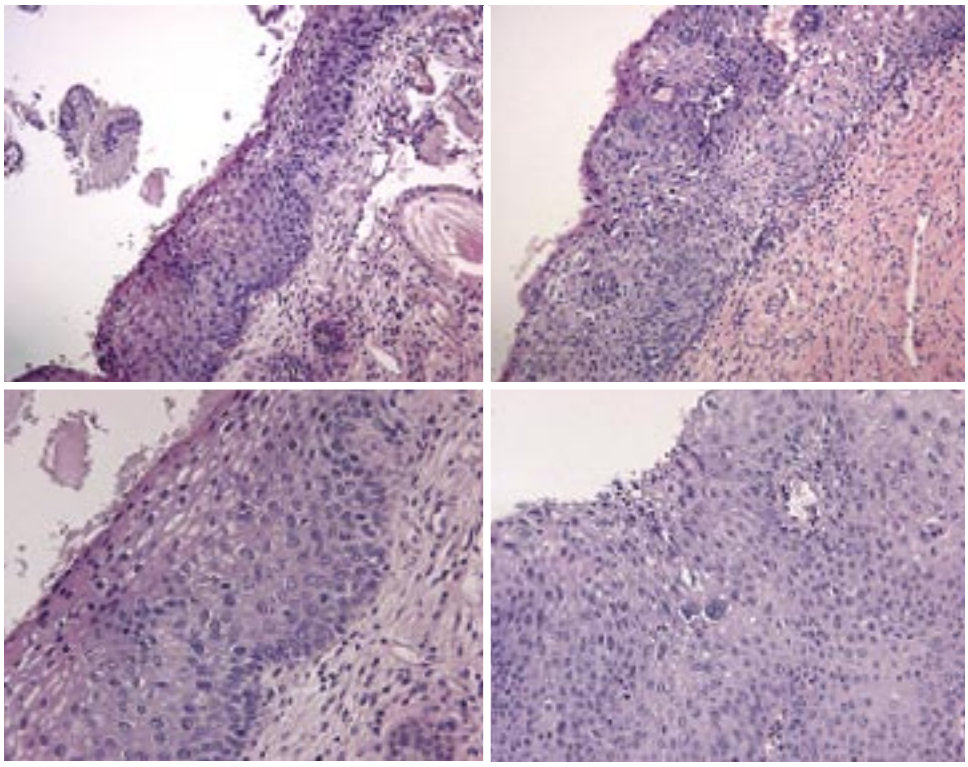

Figure 8 Cervical intraepithelial neoplasia grade 1 histologic samples.

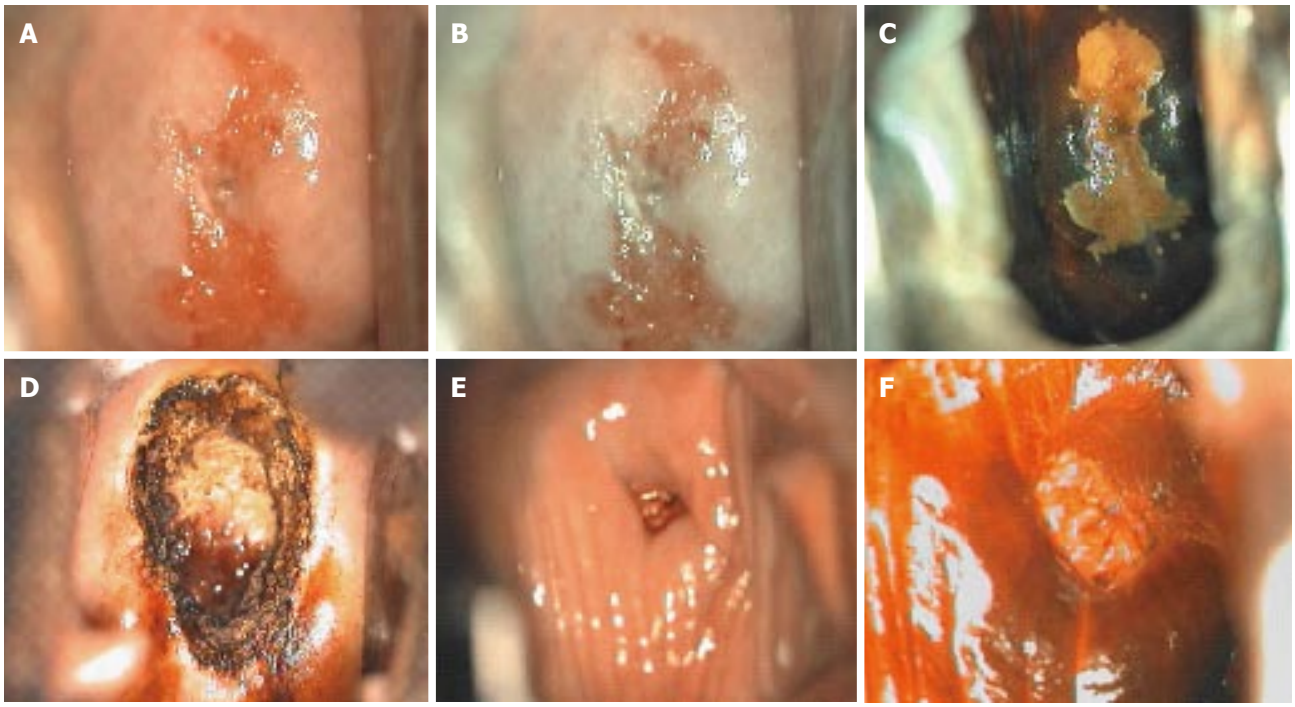

Figure 9 Cervical intraepithelial neoplasia grade 2 colposcopic appearance: (A) before application of any solution; (B) after acetic acid application; (C) after Lugol's iodine solution application; (D) after loop electrosurgical excision procedure; (E) at 6-mo follow-up before application of any solution; and (F) at 6-mo follow-up after acetic acid application.

(ASC), the Papanicolaou Society of Cytopathology, the American Society for Clinical Pathology, and the American Society of Cytotechnology convened the Gynecologic Cytopathology Quality Consensus Conference to present preliminary consensus statements developed by working groups, including the Cytologic-Histologic Correlations Working Group 4, using results from surveys and literature review. Conference

Table 1 The Bethesda system

Specimen type:
Conventional smear (Pap smear)
Liquid-based preparation
Other
Specimen adequacy:
Satisfactory for evaluation (describe presence or absence of endocervical/transformation zone component and any other quality indicators, e.g. partially obscuring blood, inflammation, *etc.*)
Unsatisfactory for evaluation… (specify reason)
Specimen rejected/not processed (specify reason)
Specimen processed and examined, but unsatisfactory for evaluation of epithelial abnormality because of (specify reason)
General categorization (optional):
Negative for intraepithelial lesion or malignancy conventional smear (Pap smear)
Other: see interpretation/result (*e.g.*, endometrial cells in a woman ⩾ 40 yr of age)
Epithelial cell abnormality: see interpretation/result (specify "squamous" or "glandular" as appropriate)
Interpretation/result:
Negative for intraepithelial lesion or malignancy: when there is no cellular evidence of neoplasia, state this in the general categorization above and/or in the interpretation/result section of the report, whether or not there are organisms or other non-neoplastic findings
Organisms:
Trichomonas vaginalis
Fungal organisms morphologically consistent with Candida spp.
Shift in flora suggestive of bacterial vaginosis
Bacteria morphologically consistent with Actinomyces spp.
Cellular changes consistent with HSV
Other non neoplastic findings (optional to report; list not inclusive):
Reactive cellular changes associated with:
Inflammation (includes typical repair)
Radiation
IUD
Glandular cells status post hysterectomy
Atrophy
Other:
Endometrial cells (in a woman ⩾ 40 yr of age): specify if "negative for SIL"
Epithelial cell abnormalities:
Squamous cell:
ASC:
Of undetermined significance (ASC-US)
Cannot exclude H-SIL (ASC-H)
Low-grade SIL (L-SIL) (encompassing: HPV/mild dysplasia/CIN1)
High-grade SIL (H-SIL) (encompassing: moderate and severe dysplasia, CIS/CIN2 and CIN3):
With features suspicious for invasion (if invasion is suspected)
SCC
Glandular cell:
Atypical:
Endocervical cells (NOS or specify in comments)
Endometrial cells (NOS or specify in comments)
Glandular cells (NOS or specify in comments)
Atypical
Endocervical cells, favor neoplastic
Glandular cells, favor neoplastic
Endocervical adenocarcinoma in situ
Adenocarcinoma:
Endocervical
Endometrial
Extrauterine
NOS
Other malignant neoplasms (specify)
Ancillary testing: provide a brief description of the test methods and report the result so that it is easily understood by the clinician
Automated review: if case examined by automated device, specify device and result
Educational notes and suggestions (optional): suggestions should be concise and consistent with clinical follow-up guidelines published by professional organizations (references to relevant publications may be included)

Adapted from: International Agency for Research on Cancer (IARC), 2013. AIS: Adenocarcinoma *in situ*; NOS: Not otherwise specified; SCC: Squamous cell carcinoma; CIN: Cervical intraepithelial neoplasia; CIS: Carcinoma *in situ*; IUD: Intrauterine contraceptive device; SIL: Squamous intraepithelial lesion; ASC: Atypical squamous cells.

participants voted on statements, suggested changes where consensus was not achieved, and voted on proposed changes. To document existing practices in gynecologic cytologic-histologic correlation (see

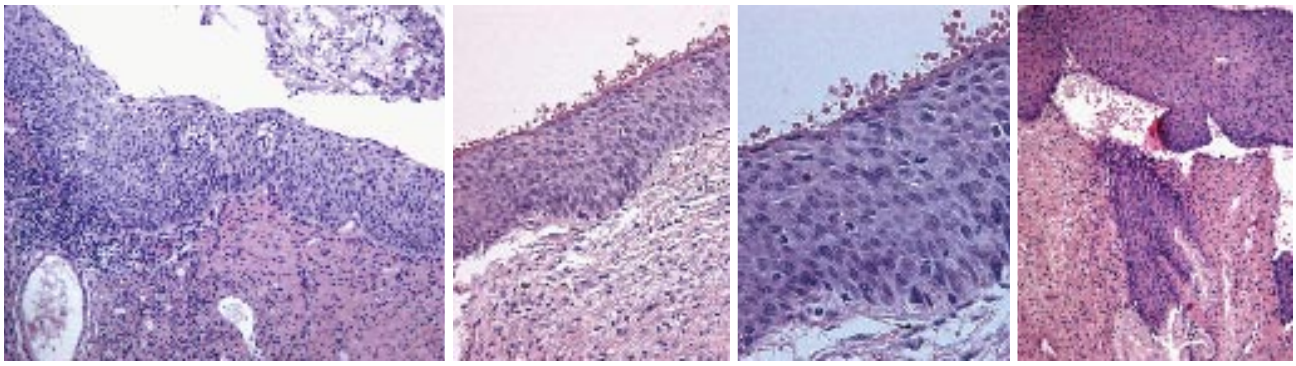

Figure 10 Cervical intraepithelial neoplasia grade 2 histologic samples.

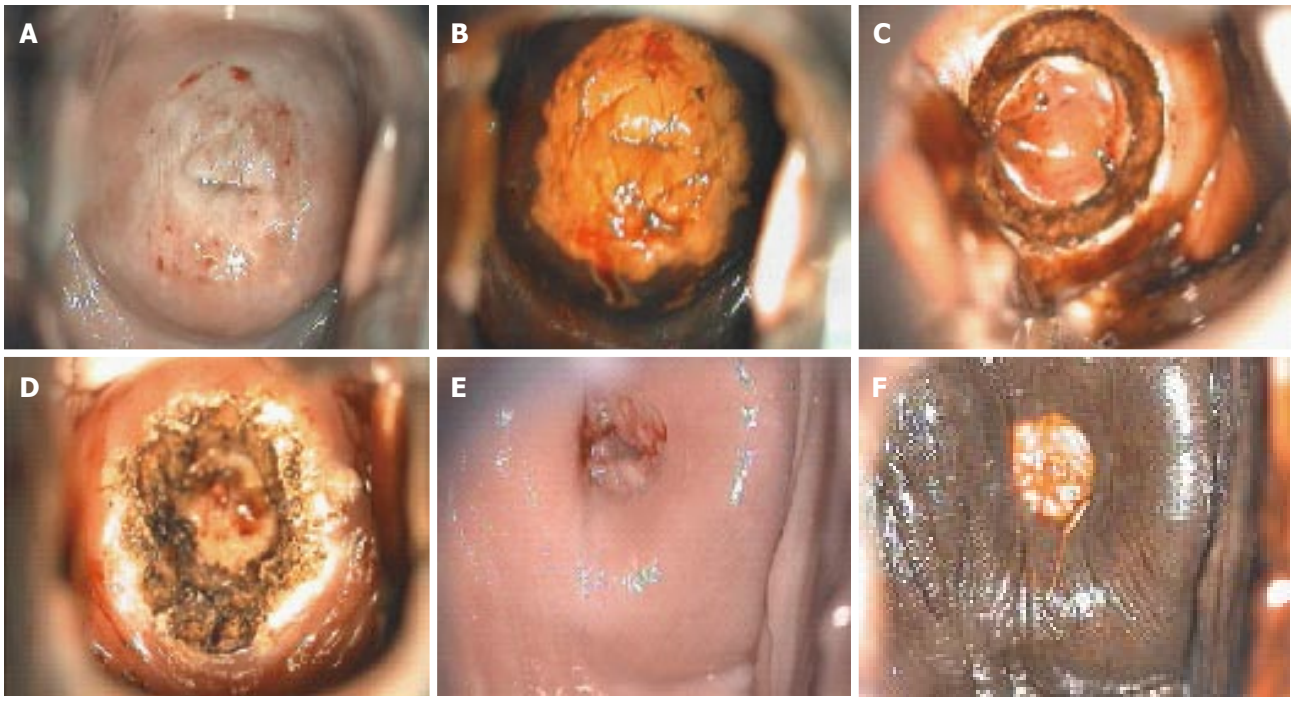

Figure 11 Cervical intraepithelial neoplasia grade 3 colposcopic appearance: (A) after acetic acid application; (B) after Lugol's iodine solution application; (C) during loop electrosurgical excision procedure; (D) after loop electrosurgical excision procedure; (E) at 6-mo follow-up after acetic acid application; and (F) at 6-mo follow-up after Lugol's iodine solution application.

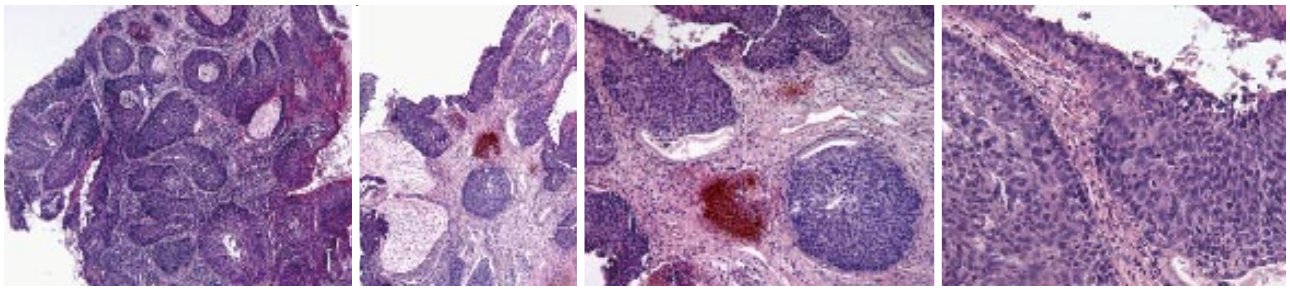

Figure 12 Cervical intraepithelial neoplasia grade 3 histologic samples.

the Bethesda System cytologic reports in Table 1), to develop consensus statements on appropriate practices, to explore standardization, and to suggest improvement in these practices, the material is based on survey results from US laboratories, review of the literature, and the CAP Web site for consensus comments and

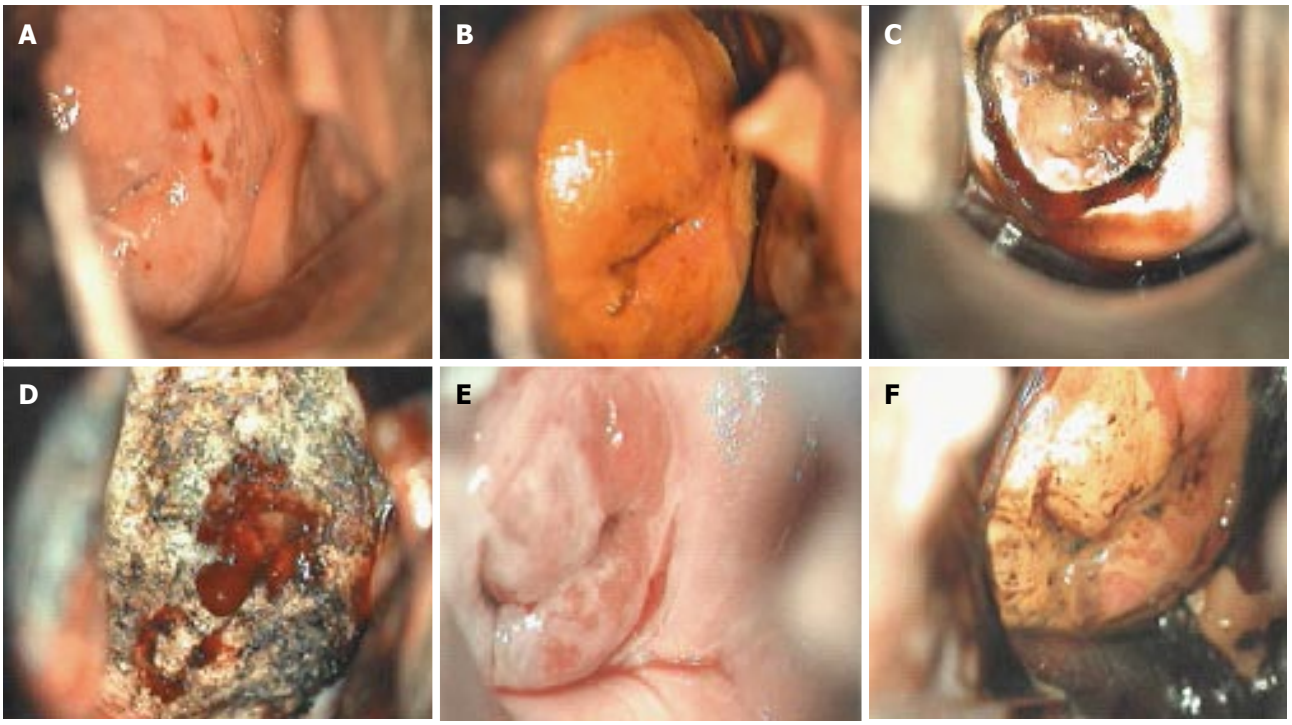

Figure 13 Carcinoma *in situ* colposcopic appearance: (A) after acetic acid application; (B) after Lugol's iodine solution application; (C) during loop electrosurgical excision procedure; (D) after loop electrosurgical excision procedure; (E) at 6-mo follow-up after acetic acid application; and (F) at 6-mo follow-up after Lugol's iodine solution application.

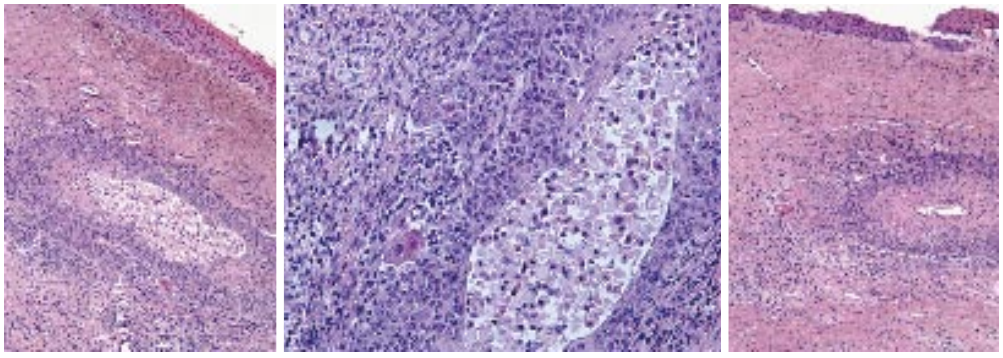

Figure 14 Microinvasive carcinoma histologic samples.

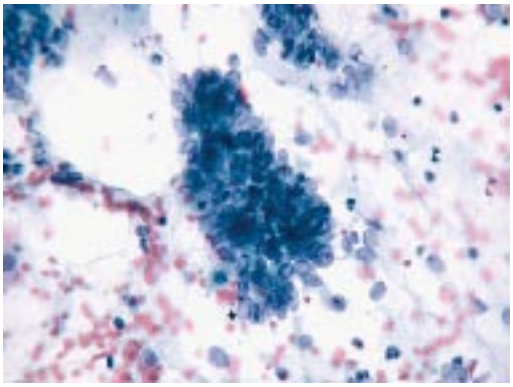

Figure 15 Adenocarcinoma.

additional survey questions[26-30].

CONCLUSION

Vaccination against HPV is expected to decrease the incidence of cervical cancer in most countries. However, it is also expected to influence the effectiveness of screening. In the future, maintaining Pap test as the primary test for cervical screening may become too expensive. As the prevalence of cervical dysplasia decreases, the positive predictive value of citology also decrease, and consequently, more women will undergo unnecessary diagnostic procedures and follow-up. The HPV deoxy ribonucleic acid (DNA) test has recently emerged as the best tool to replace cytology as primary screening. It is less subjected to human errors and much more sensitive than the Pap test in detecting high-grade cervical lesions. By incorporating

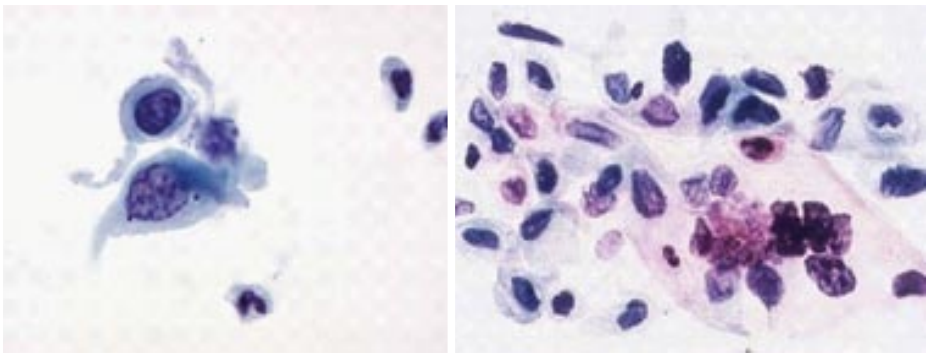

Figure 16 Squamous cells carcinoma.

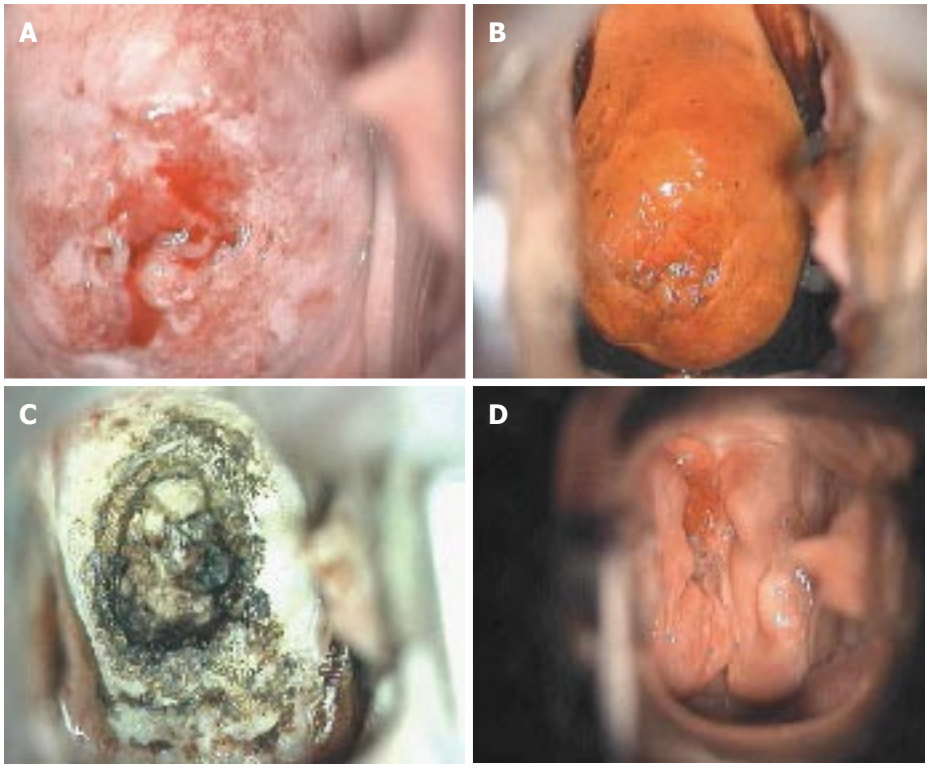

Figure 17 Adenocarcinoma *in situ* colposcopic appearance: (A) after acetic acid application; (B) after Lugol's iodine solution application; (C) after loop electrosurgical excision procedure; and (D) at 6-mo follow-up.

this test the overall quality of screening programs will improve and will allow spacing out the screening tests, while maintaining safety and reducing costs. Although HPV testing is less specific than Pap test, this problem could be solved by reserving the latter for triaging cases of HPV positivity. Since most HPV-positive smears contain significant anomalies, Pap cytology is expected to perform with sufficient accuracy in these cases. Pap triage of HPV-positive patients would also provide a low-cost strategy to monitor the effectiveness of the vaccine in the long term. Although HPV typing could be implemented as a screening tool for the population, further research is needed to determine the optimal age to begin screening, the role of the HPV test and other markers of disease progression, and adequate follow-up procedures for the HPV-positive and smear-negative women[31-33].

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MINIREVIEWS

Bayesian methods in reporting and managing Australian clinical indicators

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Abstract

Sustained clinical improvement is unlikely without appropriate measuring and reporting techniques. Clinical indicators are tools to help assess whether a standard of care is being met. They are used to evaluate the potential to improve the care provided by healthcare organisations (HCOs). The analysis and reporting of these indicators for the Australian Council on Healthcare Standards have used a methodology which estimates, for each of the 338 clinical indicators, the gains in the system that would result from shifting the mean proportion to the 20th centile. The results are used to provide a relative measure to help prioritise quality improvement activity within clinical areas, rather than simply focus on "poorer performing" HCOs. The method draws attention to clinical areas exhibiting larger between-HCO variation and affecting larger numbers of patients. HCOs report data in six-month periods, resulting in estimated clinical indicator proportions which may be affected by small samples and sampling variation. Failing to address such issues would result in HCOs exhibiting extremely small and large estimated proportions and inflated estimates of the potential gains in the system. This paper describes the 20th centile method of calculating potential gains for the healthcare system by using Bayesian hierarchical models and shrinkage estimators to correct for the effects of sampling variation, and provides an example case in Emergency Medicine as well as example expert commentary from colleges based upon the reports. The application of these Bayesian methods enables

all collated data to be used, irrespective of an HCO's size, and facilitates more realistic estimates of potential system gains.

Key words: Clinical indicators; Improvement; System gains; Bayesian; Statistical models

Core tip: The article's purpose is to bring attention to the increasing use of Bayesian methods in the clinical field to overcome shortcomings of previous analyses, and provide an application of how such methods are used in clinical management in Australia; in particular, on how to best report and use clinical indicator data for system improvement. The paper identifies flaws associated with traditional clinical indicator reporting techniques which are still often-used; describes part of current Australian clinical indicator reporting methods; and demonstrates how and why Bayesian methods are fundamental to the improved methods overcoming issues that would otherwise arise with such data.

Howley PP, Hancock SJ, Gibberd RW, Chuang S, Tuyl FA. Bayesian methods in reporting and managing Australian clinical indicators. *World J Clin Cases* 2015; 3(7): 625-634 Available from: URL: http://www.wjgnet.com/2307-8960/full/v3/i7/625.htm DOI: http://dx.doi.org/10.12998/wjcc.v3.i7.625

INTRODUCTION

Healthcare accreditation systems and quality measurement systems are internationally used for the purposes of improving clinical care and organisational outcomes. Accreditation in healthcare reflects the systematic assessment of hospitals against explicit predetermined standards[1,2] and consists of multiple means of assessment such as self-appraisal, peer-reviewed interviews, scrutiny of documentation, checking of equipment and investigation of key clinical and organisational data[3]. These systems involve considerable levels of resources from the participating agencies and healthcare organizations (HCOs) and are believed to facilitate improved levels of quality in healthcare[4-7].

The Australian Council on Healthcare Standards (ACHS) has a well-established national healthcare accreditation program. It provides robust support for Australian healthcare and is one of the four most commonly cited national healthcare accreditation programs in the world[1,8,9]. In addition to providing a national accreditation scheme, the ACHS supports HCOs by providing sets of clinical indicators (CIs) which HCOs may opt to utilize. HCOs may simply collect their own data, or they may additionally submit their data *via* the ACHS's online performance indicator reporting tool for analysis and reporting[9-13]. CIs measure performance in a clinical setting; the reporting of CIs in HCOs aims to detect suboptimal care either in structure, process or outcome, and can be treated as a tool to assess whether a standard in patient care is being met. They may provide evidence for accreditation purposes and guide the process of quality improvement in healthcare[14].

There is world-wide interest in how to integrate clinical indicators within the accreditation process and mechanisms for their collection differ across countries[9]. A comparison of the four most often referenced national accreditation programs internationally[1,8,9] identified the following key points: (1) the Joint Commission (JC) in the United States and Accreditation Canada are examples of accreditation bodies that have integrated the mandatory requirement that hospitals provide core indicators as part of the accreditation process in order to help focus on-site survey evaluation activities in accreditation[9,15-22]. The JC has done so through its ORYX® program and through its integration of measurement data into its Priority Focus Process for the on-site survey[16,17]. Accreditation Canada has done so through its Qmentum program and combining indicator data with their "instrument" data obtained through questionnaires completed by representative samples of clients, staff, leadership and/or other key stakeholders[9,22]; (2) Haute Autorité de Santé, France, has mandatory accreditation for all its hospitals and has connected many of its accreditation standards to indicators. There are 13 criteria that must be satisfied to achieve certification, of which four are linked to indicators. In total, there are 14 indicators connected with accreditation criteria[9,23,24]; and (3) the ACHS, Australia, provides 6-monthly and trend reports to hospitals which have elected to submit their CI data. The contribution of these reports to a hospital's self-evaluation and quality improvement efforts are relied upon for instigating the CI data collection within hospitals and thus their inclusion in the accreditation process[9].

Taiwan was the fourth country, following the United States, Canada, and Australia, to implement a healthcare accreditation project and the first country in Asia to do so. The reporting of CIs is now required by law for hospitals in Taiwan, and many internal and several nationwide clinical indicator systems have been launched, including three nationwide quality measurement systems: Taiwan Healthcare Indicator Series, Taiwan Clinical Performance Indicators, and Taiwan Community Hospital Association indicators[25]. These three clinical indicator systems are optional for hospitals to utilize. Their target participants are varied and with the diversity of indicators collected there has been difficulty integrating CIs into Taiwan's accreditation process[26], which is governed by Taiwan's Joint Commission on Hospital Accreditation. Taiwan's Ministry of Health and Welfare is currently assessing how best to integrate the CIs from the varied agencies and government departments for enhancing the efficiency and effectiveness of CIs on quality improvement in healthcare.

Since 1993, Australian HCOs preparing for accreditation have submitted data on sets of CIs. The ACHS routinely collates the data in six-month periods and generates reports which are provided to HCOs, along with de-identified reports which are provided to accreditation surveyors, national medical colleges and government bodies. In 2012 the ACHS received data from 670 Australian and New Zealand HCOs on 338 CIs across 22 specialties, or clinical indicator sets[13]. This is the largest source of CI data in the world. The ACHS clinical indicators are not mandatory for any organisation to submit. HCOs select CIs that are relevant to them at that time and where there is a need within that clinical area; for example, high cost procedures, high patient thoroughfare, or a new clinical area to that HCO.

For a given CI, the i^{th} HCO provides the observed number of patients who incur the "event of interest" (O_i) and the number of patients at risk of the event (D_i). Traditional methods of analysis and reporting of such data have been flawed, failing to account for sampling variation and focusing on comparing individual HCO proportions with the mean proportion across all HCOs or with an externally set benchmark value determined by experts, with the primary intention of identifying "outliers". The approach employed in the reporting of the ACHS CIs, as part of the ACHS's Clinical Indicator Program, shifts the focus towards the potential benefits from system-wide improvements of clinical areas rather than simply comparing individual HCO performances within a clinical area which occurs with other traditional approaches. Further, the new approach has required the application of Bayesian hierarchical models to address issues of small samples, which arise in six-monthly data collection, and to preclude overestimation of the potential system improvements. Accounting for sampling variation through Bayesian hierarchical models additionally reduces HCOs' concerns of being misrepresented as extreme as a consequence of a small sample size in a given period.

This paper outlines flaws associated with traditional reporting techniques which are still often used elsewhere; describes part of the current ACHS CI reporting methods; provides examples of annual clinical comments and perspectives based on the reports; and demonstrates how and why Bayesian methods have been fundamental to the improved reporting methods overcoming issues that would otherwise arise with such data.

PROBLEMS WITH TRADITIONAL METHODS OF REPORTING CIS

The implementation of league tables which rank CIs within and across HCOs is a common practice which aims to establish an increased level of accountability and competition, and thus provoke individual strategies towards improved performance[27-29]. Deming's philosophy and systems theory identifies, however, how co-operation rather than competition is required to foster genuine quality improvement and how the system's components and the interdependencies of these components must be foremost in one's mind during the improvement cycle[29,30]. The increased focus upon "competition" between HCOs that occurs as a result of publishing league tables can lead to perverse incentives being created[31]. HCOs may, for example, be motivated towards manipulating their data or taking patients that are considered a "low risk" in order to improve their perceived performance, even if this is at the expense of other HCOs in the system[27,31-33].

There is limited, if any, value reporting league tables of HCO performances. Such presentations are likely to mislead[31,32,34,35] even when statistical techniques have been utilised that adjust for differences that arise due to varying sample sizes, as there will inevitably be a top-ranked HCO and bottom-ranked HCO even if all HCOs were providing outstanding service. Whilst confidence intervals are often introduced to determine where statistically significant differences in the ranks exist, the calculation of multiple intervals will increase the risk of identifying differences due to chance. Employing a conservative significance level to compensate will increase the confidence intervals' widths. In some cases the intervals for HCOs ranked first and last overlap rendering the publication of such tables meaningless[10,32,36,37].

Further, any variations in rank that may be observed with time may be a result of the "regression to the mean" phenomenon[38] rather than reflecting fundamental change in quality. Andersson *et al*[34] produced a measure of the "…expected change in the rank order if one were to repeat the study" to assess the validity of ranking and demonstrated the "…tremendous uncertainty in the ordering…"[34].

Fundamentally, the league table approach is flawed as it focusses attention on individual HCOs and, in particular, those deemed to be poorer performers requiring improvement rather than addressing issues that may help bring systemic advances to the system of HCOs[27,39]. Thus the analysis of clinical indicators must report more than a simple proportion and rank.

Setting thresholds and performing significance tests using p-values is also commonly practiced, and was previously employed by the ACHS. Comparing individual HCO proportions with a nominal threshold value provides minimal assistance to the HCO system as a whole since most HCOs will be within the tolerance level, potentially reducing motivation to undertake improvement, and HCOs with larger volumes of patients (larger sample sizes) are more likely to yield proportions, or rates, which are statistically significant. Consequently, the principal result of such analyses is the classification of individual HCOs as either satisfactory or not[10,27], rather than highlighting system variation or focusing attention on necessary system-wide improvements.

AN IMPROVED APPROACH FOR ANALYSING AND REPORTING CLINICAL INDICATORS

Focusing upon the system of HCOs helps with identifying clinical areas where investigation and improvement activity may produce the most benefit. An approach was applied that uses the data arising from the system of HCOs to identify a potentially achievable mean proportion and identifies clinical areas with large "potential gains" resulting from achieving such a mean proportion. The ACHS reports achieved this by introducing, for each CI, a measure of the gains (or reduction in the number of undesirable events) that could be achieved if the mean proportion was shifted to the 20th centile. The calculation of these potential gains is based on the amount of variation in the system (represented by the difference in the mean proportion, π, and 20th centile proportion, p_{20}, across all HCOs) and the impact upon the system, or volume effect, (represented by the summed D_i, where D_i represents the number of patients at risk of the event at the i^{th} HCO, across all n HCOs providing data for the CI) as shown in equation (1).

$$\text{Potential Gains} = (\pi - p_{20}) \sum_{i=1}^{n} D_i \quad (1)$$

The estimated potential gains facilitates and motivates scientific investigation within clinical areas by providing a relative measure between CIs of the potential improvement. Smaller variation and smaller potential for system impact [in terms of potential for events occurring, represented by ΣD_i in equation (1)] are reflected in a smaller value for the potential gains. Reported as part of the annual Australasian Clinical Indicator reports, this measure enables comparisons of clinical areas for improvement activity rather than allocating responsibility solely to individual HCOs[11].

The use of the 20th centile to calculate potential gains in the system has great appeal as the estimated gains don't rely upon a subjective target but instead is influenced by the system and the data it has produced; the estimated gains are being guided by the existing between-HCO level of variation in proportions. The 20th centile is approximately one standard deviation from the overall mean proportion and may be considered a practicable goal[10]. Since the distribution of proportions is often not symmetric, using standard errors is less useful.

The potential gains as a measure considers the HCOs as part of an holistic system that may have potential for improvement rather than focusing on individual HCOs' performances. Such an approach enables healthcare professionals and governing bodies "…to determine those clinical areas where there are potentially greater gains and hence funding for quality improvement activity would be of a higher priority"[10].

In the case of CIs where higher proportions are desired, potential gains are calculated using the 80th centile, p_{80}, as $(p_{80} - \pi) \Sigma D_i$. This represents the number of additional events that would occur if the mean proportion were equal to p_{80}.

The 20th (or 80th) centile and ensuing calculation of the potential gains, however, should not be obtained simply by using the observed proportions (O_i/D_i) since they are affected by sampling variation. Further, since the HCOs report their data across six-month periods the observed proportions will be based on large and small sample sizes, affecting the precision and reliability of the estimated proportions[10]. Consider for example an HCO that reports only three individuals at risk of a particular event in a given period. A difference of one in the number incurring the event of interest would correspond in a change in the estimated proportion of 33%. Additionally, it would be more likely that HCOs with such small D_i will report the extreme proportions (0% and 100%) despite this most likely not reflecting the true underlying proportion at the HCO.

Rather than exclude HCOs with small data, and thus lose data, a statistical technique known as Bayesian hierarchical modelling and the associated empirical Bayesian shrinkage estimator have been used to better estimate the proportions for the HCOs in a given six-month period. The approach essentially utilizes and combines an individual HCO's proportion and the summary results from the system of HCOs to produce a better estimate of the individual HCO's true underlying proportion.

Empirical Bayes models

"Medical research applications often involve hierarchical data structures as data are collected on random samples of patients nested within each hospital"[10]. When data are collected from many HCOs there is usually substantial variability among the HCOs in addition to variability within the HCOs due to sampling.

Many readers would be more familiar with the non-Bayesian approach to analyses, known as the frequentist or classical approach, which uses only the information provided by the sample data to make wider inference. In contrast, the Bayesian methodology uses additional prior knowledge or belief, presented in the form of probability distributions, in making wider inference. Bayesian methods essentially assess the appropriateness of the prior knowledge given the new data and quantifies this in the form of probability statements or density functions for the values we are wishing to estimate[40,41]. The "Bayesian methodology has been shown to be particularly useful in both the clinical setting and the area of public health policy when the results of a study must subsequently be used to facilitate a decision"[10]. In the Bayesian paradigm, a two-stage hierarchical model representing the nesting of the patients within HCOs may be used to make inferences[42-48], with the first stage representing the distribution of the HCO-specific proportions (CI proportions among HCOs) and the

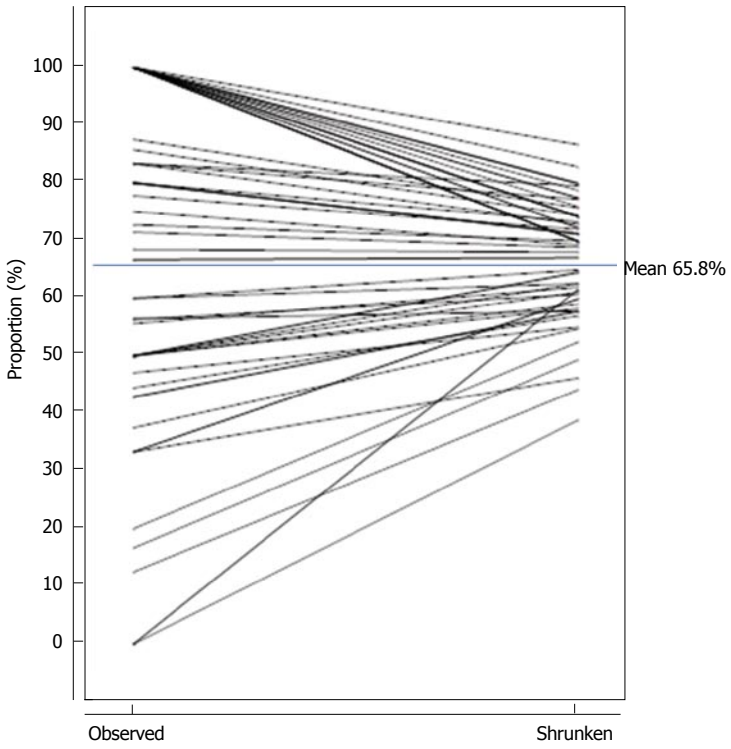

Figure 1 Shrinkage Plot showing Observed and Shrunken Proportions for 62 Healthcare Organisations.

second stage modelling the additional sampling variation associated with what we observe for the patients within HCOs.

The approach borrows strength from the ensemble[49] of HCOs to estimate any individual HCO's true proportion better than an individual HCO's data alone. Essentially the approach uses statistical models for: (1) the conditional probability of the observed counts of events given the unknown true CI proportions (we are attempting to estimate the latter); and (2) a *prior* distribution for the true CI proportions given their overall mean and variance (the latter may be estimated from the data, in which case the approach is referred to as an *empirical* Bayes approach). The two statistical models combine *via* Bayes' rule[45,50] to produce the probability distribution for the unknown true CI proportions given the observed counts and overall mean and variance; this is known as the posterior distribution.

The expectation of this posterior distribution is the empirical Bayes shrinkage estimator for an HCO's CI proportion. The estimated proportion for a given HCO is effectively a weighted average of the individual HCO's observed proportion and the overall mean proportion across all HCOs. The weighting depends upon the systematic variation between HCOs and sample size of the individual HCO, as well as the particular two-stage model's family of distributions.

To reflect the between-HCO and within-HCO sources of variation in the CI proportions, the ACHS reports use the gamma-Poisson hierarchical model. This model was applied since the gamma distribution could represent the distribution of the ratios of observed to expected numbers of events[10]. Further, using the gamma-Poisson model instead of a beta-binomial model for proportions was shown to result in more conservative estimates of the 20th centile-based potential gains due to a relatively greater shrinkage using the former rather than the latter model[10].

The gamma-Poisson model assumes that the O_i follow a Poisson distribution with mean $\lambda_i E_i$, where E_i is the expected number of events at the i^{th} HCO obtained by multiplying D_i by the mean proportion, π, and the true ratios of observed and expected numbers of events, λ_i, are obtained from a gamma distribution with mean, μ, and variance, S_r^2. That is, we have $O_i \sim Poisson\ (\lambda_i E_i)$ and $\lambda_i \sim Gamma\ (\mu, S_r^2)$ which combine using Bayes' rule[45,50] to provide the estimated ratios of an HCO, which are interpretable as proportions by multiplying a ratio by the mean proportion.

The effects of empirical Bayesian shrinkage estimators

Figure 1 visually demonstrates the changes to the individual HCOs' estimated proportions and the distribution of proportions following "shrinkage" for a particular clinical indicator having 62 HCO submissions of data; for this indicator a high proportion was desirable. Each HCO returned data enabling the observed proportion (O_i/D_i) to be obtained and a shrunken proportion was calculated by the afore-mentioned two-stage models and Bayesian methods and multiplying by π (the overall mean proportion). Figure 1 joins each HCO's corresponding observed and shrunken proportions. Several HCOs had common observed and shrunken proportions, hence Figure 1 does not show 62 distinct lines.

The D_i for the CI ranged from 1 to 78. The observed proportions ranged from 0% to 100%. There were 20

Table 1 Report showing Statistics and Estimated Gains for clinical indicators in Emergency Medicine[13]

CI	Desired level	Number HCOs	20th centile (%)	Mean proportion (%)	80th centile (%)	Numerator	Denominator	Potential Gains	Stratum Gains	Outlier Gains
1.1	High	309	99.2	99.1	99.9	26344	26577	219	-	177
1.2	High	323	75.6	79.8	93.7	342984	429896	59829	34972	15420
1.3	High	323	61.0	63.7	93.1	943806	1482555	436635	164974	96017
1.4	High	323	64.6	69.9	96.1	1308074	1870202	488979	77692	100353
1.5	High	317	85.1	87.9	98.6	355355	404382	43560	-	14029
2.1	High	62	58.8	65.8	74.4	338	514	44	29	-
3.1	Low	102	9.9	28.3	47.3	199881	706869	129557	94225	53779
3.2	Low	39	30.1	60.6	82.0	6451	10639	3246	861	1119
3.3	Low	40	19.2	48.5	70.7	7518	15502	4546	2826	2032
4.1	Low	7	0.0	0.1	0.2	938	6742	646	-	241
4.2	Low	6	0.0	0.1	0.1	261	5024	259	-	119
5.1	Low	2	23.9	23.9	23.9	358	15	-	-	-
5.2	Low	3	54.5	54.5	54.5	61	112	-	-	-
6.1	High	7	66.4	71.5	92.7	10276	14363	3033	-	1143
6.2	High	4	20.0	37.0	46.0	2783	7519	678	-	290
7.1	High	10	26.4	44.4	91.8	3395	7653	3630	-	1509
7.2	High	7	21.9	51.4	84.8	183	356	119	-	38
7.3	High	5	21.9	17.7	33.9	854	4818	778	-	-
7.4	High	4	40.8	83.6	99.8	244	292	48	-	33
8.1	Low	28	0.9	4.1	8.8	1547	37888	1203	600	606
8.2	Low	53	1.7	4.9	6.9	49389	1008385	31885	-	10066

CI: Clinical indicator; HCO: Healthcare Organisation.

HCO submissions with observed proportions equaling 100%; the D_i for these HCOs ranged from 1 to 7. There were 3 HCO submissions with observed proportions equaling 0%; the D_i for these HCOs ranged from 1 to 8. The 20th and 80th centiles of the observed proportions were 43.8% and 100% respectively. The 20th and 80th centiles of the shrunken proportions were 58.6% and 74.9% respectively. The mean proportion was 65.8%.

The key points to observe for this example are: (1) HCOs with extreme observed proportions (0% and 100%) are shrunken more greatly towards the mean; a consequence of having smaller D_i; (2) not all HCOs having the same observed proportion will have the same shrunken proportion; a consequence of having differing D_i; (3) the spread of the distribution of shrunken proportions is far less than the spread of the distribution of observed proportions, reflecting the spread of the true underlying proportions; and (4) the 20th and 80th centiles of the shrunken proportions are closer to the mean proportion than the respective centiles of the observed proportions and hence facilitate better estimates of the potential gains.

The potential gains for a CI are then calculable using the shrunken proportions and 20th or 80th centiles using equation (1), or its equivalent for the 80th centile, and are presented along with other summary information for CIs. Table 1 presents an example of such a report for the clinical area Emergency Medicine.

The potential gains provided in Table 1 provide a measure to help prioritise investigation and improvement activity. Consider CIs 1.2 and 2.1; each are desired to have a high proportion. CI 1.2 has a mean proportion of 79.8% whilst 2.1 has a mean proportion of 65.8%. On this alone, it may seem 2.1 should be the priority for improvement; however, the potential gains incorporates the variation between HCOs as well as the size of the potential impact upon the system represented by "Denominator" which is the total number at risk of an event across all HCOs. When this information is considered we observe potential gains for 1.2 that is some 1360 times the potential gains for 2.1, reprioritising where investigation and improvement activity may be best undertaken.

Other estimated gains and funnel plots

In addition to the 20th centile-based potential gains, the ACHS reports present estimates of stratum gains and outlier gains, as shown in Table 1. Stratum gains represent the gains that would be achieved from moving the mean proportions of the poorer performing strata to the mean proportion of the best performing stratum. The strata for the HCOs are: public or private; metropolitan or non-metropolitan; and State (region of Australia).

Outlier gains present the gains that may be achieved from improving the outlier HCO proportions to equal the overall mean proportion. Outlier HCOs have differences in observed and expected numbers of events exceeding three times the standard error of the difference, after shrinkage. A funnel plot, as shown in Figure 2, plots for a given CI the HCOs' differences in observed and expected counts, or *Excess* count, ordered by D_i. Doing so allows a visual check for any patterns that would suggest a volume effect (a pattern due to D_i). In this particular case there were no outliers or an effect due to sample size. Whilst traditional methods may have resulted in little more than the reporting of no outliers,

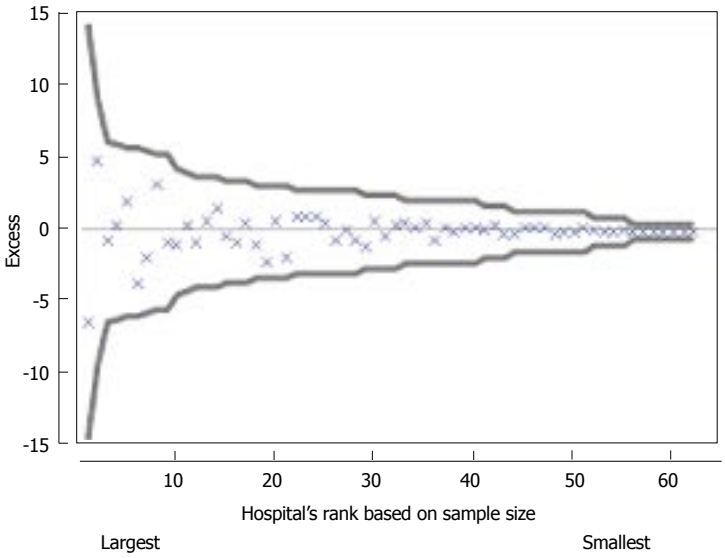

Figure 2 Funnel Plot of Shrunken Estimates of Excess (difference in Observed and Expected) counts for 62 healthcare organisation submissions for clinical indicator 2.1.

the 20th centile gains and stratum gains presented in the ACHS reports focus attention on potential for improvement in the system.

How the reporting system is used

The annual reports described in this paper are part of a two-tiered reporting system. In addition to these annual reports, HCOs receive individual six-monthly reports which identify the individual HCO's performance (rate) compared with both the entire system's rate and themselves based on trend analysis of their six-monthly rates. The latter reports are used by individual HCOs to self-assess whilst the annual report is provided to the relevant Colleges before being published and the Colleges are invited to comment on their set of CIs. In earlier editions of this report the response was less than hoped for, but the annual edition, currently in its 15th year, has more recently received and incorporated clinical comments and perspectives on all results.

Vignettes of the types of comments are provided below[12]. Importantly, Colleges and State Governments are reading reports and thus engaging more with the data.

Example 1: Response from the College of Nursing to the CI representing falls for those aged 65 years or more: "There is no significant change to the data reported in 2013 compared to 2012, despite the fact that the HCO population is aging, with higher numbers of complex and higher acuity patients–particularly within the public HCOs….HCOs with outlier rates within this CI need to review their falls management protocols and falls prevention education, to reduce the current rate of falls–which is nearly double the fitted rate for all reporting HCOs"[12].

Example 2: "The Australian Faculty of Rehabilitation Medicine (AFRM) and the Australasian Rehabilitation Outcomes Centre (AROC) are proud of the continued high standard of compliance with the ACHS CIs by all participating HCOs. The AFRM has included the ACHS CIs in the AROC dataset to encourage HCOs to participate in this important collection and thereby promote continued improvement in these processes and outcomes. The quality of the data collected is of a high standard, with well-established, nationally consistent education programs *in situ*. On that basis, the AFRM and the AROC are confident about the results reported here. HCOs are encouraged to continue reviewing their CI collections to help inform processes and practices in order to maintain the high rates achieved in previous years"[12].

Example 3: Expert commentary from Royal Australian and New Zealand College of Obstetricians and Gynaecologists (RANZCOG) on outcome of selected primipara: "There has been a small increase in the number of spontaneous vaginal births in the selected primipara (CI 1.1), but it remains at around 45.0%. There are several reasons why the number of spontaneous vaginal births will be expected to continue to lessen: (1) women becoming more risk averse and therefore more often requesting obstetric procedures in order to minimize risk. This applies to all women, but particularly in relation to common issues such as how long to tolerate pregnancy progressing beyond the due date; (2) increasing maternal age; and (3) Reducing maternal parity with the consequential reduced morbidity from caesarean section in subsequent pregnancies.

Stratum differences were demonstrated in relation to private and public HCOs (36.4% *vs* 50.8% respectively). This is expected as the above factors are more prevalent in the private sector than public sector"[12].

Example 4: Expert commentary from RANZCOG on Intrauterine growth restriction: "The rate of CI 8.1 has been steadily improving, but this appears to have plateaued at around 1.64%. Failure to diagnose intrauterine growth restriction remains the most obvious

preventable factor in perinatal mortality at term. It has been rewarding to see this statistic fall over the 5 year period and it could be suggested that introducing this CI is partly responsible for this highly desirable improvement"[12].

Example 5: Expert commentary from the Australian and New Zealand College of Ophthalmologists on Cataract Surgery: "Unplanned overnight admission rates following cataract surgery were lower in 2013 with a reduction in the annual rate (CI 1.3). There are known factors such as older age groups, medical co-morbidities and surgery in the latter part of the day that may be contributing. In 2013, there were eleven outlier submissions from eight different HCOs. The outlier organisations can consider a grading/points system based on case complexity to identify cases that should be done in the morning or by an experienced surgeon"[12].

Example 6: Expert commentary from the Australian College of Midwives: General Comment, "The number of services reporting for each CI indicates the ease or difficulty of recording and reporting data defined and possibly the usefulness of the measure to local monitoring. Comparison of local results with the mean and stratified groups would enable health services and professionals to determine areas for policy, practice and research attention"[12].

DISCUSSION

The ACHS CIs aim to get refined data, controlling for casemix, *e.g.*, infection control for each of coronary-artery bypass, hip replacement, knee surgery, *etc.* Since there are 22 specialties (sets of indicators) representing the main Colleges in Australia, and hence some 338 CIs, an individual HCO has to select those sets, or subsets of CIs, that are appropriate to their needs. For example, sets based upon paediatrics, obstetrics, oncology, gynaecology or day only procedures or patients will not be relevant to many HCOs. Even for sets that are relevant some of the CIs within the set may not be collected due to costs of obtaining the data from the medical records, or the CI may not be seen as important to the individual HCO. The non-mandatory nature of the CIs is consistent with the non-punitive and non-invasive nature of the reporting methods described. The data collection and reporting method is designed to assist the healthcare system, not place greater burdens upon the system or HCOs unable to sustain the costs of widespread data collection. Whilst the CIs provide a means for HCOs to more easily provide necessary evidence to warrant accreditation, the aforementioned reasons along with nuances that exist within any HCO warrants the optional nature of their use. This further attracts some HCOs towards the ACHS accreditation process and to provide data honestly rather than be forced to provide data and be motivated towards manipulating their data or taking patients that are considered a "low risk" in order to improve their perceived performance, even if this is at the expense of other HCOs in the system[27,31-33].

The simplistic ranking of HCOs does not quantify the gains that could be achieved and has many disadvantages. The reporting of indicators which measure clinical and healthcare processes should quantify the potential gains to encourage action. Estimating the gains across many indicators enables the comparison and identification of areas with greater potential improvement and thus prioritisation of resources for investigation and improvement efforts. The required tools and resources to investigate and address those areas with the greatest gains must then be provided.

Bayesian hierarchical models and empirical Bayes shrinkage estimators borrow strength from the collection of HCOs to estimate any individual HCO's true proportion better than an individual HCO's data alone, accounting for sampling variation and addressing issues surrounding small sample sizes[49]. Shrinkage estimators are more beneficial in situations where denominators vary in size and some are small[10,51], as is the case for the CI data.

The shrinkage estimator effectively modifies an individual HCO's observed proportion by drawing it closer to the prior mean. The amount of modification (shrinkage) is less for those HCOs having larger sample sizes (reflecting the increased information being reported by those individual HCOs reducing the effects of sampling variation) and for systems exhibiting large systematic, or between-HCO, variation (reflecting lower strength of knowledge about the prior mean since the prior variance is large in such cases). Thus HCOs having smaller denominators will have their observed proportions shifted more closely towards the overall mean than HCOs having larger denominators. The implementation of the shrinkage estimators not only provides better estimates for each HCO, in particular those that would otherwise be identified as extreme due to small sample sizes, but additionally enables all HCOs to be included in the reports irrespective of size. Further, the approach is reflecting the reality that there is a level of dependence between the HCOs as they are all part of the one system.

The use of CIs as flags for required investigation towards system improvement is a valuable area of research. The development and application of appropriate statistical methods for analysing and reporting CIs is important and should focus on improving the healthcare system. Estimating the potential gains achievable through investigation and quality improvement that reduces the mean proportion to the 20th centile focusses efforts on system-wide improvements rather than assigning blame and onus on individual HCOs. In combination with the empirical Bayesian shrinkage estimators, the estimated potential gains support practicable reports on CIs for healthcare providers.

CIs are screening tools, so just as positive blood tests or breast cancer screening result in review and

further investigation so too positive results from the CI analysis must result in further investigation. There are three types of positive results, namely, large variation between HCOs, large variation between strata and outlier HCOs (large variation from expected). This paper has described the use of the difference between the mean and 20th centile proportions to estimate the impact of between HCO variation and stratum and outlier gains which estimate the impact of between stratum variation and variation from expected for individual HCOs. These gains are reported by the ACHS for all 338 CIs[13]. Bayesian methods play a key role in ensuring such measures are not overestimated.

The presented potential gains quantify reductions, or increases for certain CIs, in the numbers of "events of interest". Whilst it is possible to attribute monetary costs to each event in order to estimate potential financial changes, the problem remains of comparing the vast range of outcomes such as delays due to intensive care unit (ICU) access block, readmissions and out of hours discharges from ICU, adverse events related to medication errors, wound infections and failure to administer venous thromboembolism prophylaxis. Whilst the measures may be converted to indicate potential costs to the healthcare system doing so would take a restricted view of these measures of quality. Further, there remains the issue of how one may compare losses of life with additional waiting times. The simplest approach involves identifying CIs where significant numbers of patients were not receiving the standard of care implied by the CI as being acceptable; however, this remains an area of ongoing research.

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MINIREVIEWS

Scourge of intra-partum foetal death in Sub-Saharan Africa

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Abstract

Intra-partum foetal death has been variously defined. However, a definition adopted at a technical consultation in 2006 is employed in this review. The quality of intra-partum care is a crucial factor for pregnancy outcome for both mothers and new-borns. Intra-partum stillbirth is defined as late foetal death during labour, which clinically presents as fresh stillbirth. The largest proportion of the world's stillbirths occurs in the late preterm, term and intra-partum periods. The Western Pacific region has the greatest reduction in stillbirth with a 3.8% annual decline between 1995 and 2009; however, the annual decline in the African region is less than 1%. Caesarean delivery is still uncommon, especially in rural areas: 1% of births in rural Sub-Saharan Africa and 5% in rural South Asia are by caesarean delivery; 62% of stillbirths occurred during the intra-partum period; 61.4% of stillbirths are attributable to obstetrical complications. Preventive measures aimed at reducing the incidence of intra-partum foetal death entail all measures aimed at improving quality antenatal care and preventing intra-partum asphyxia. This review discusses intra-partum foetal deaths from a Sub-Saharan African perspective. It explores the contribution of research within the region to identifying its impact on new-born health and potential cost-effective policy interventions.

Key words: Intra-partum; Foetal; Death; Sub-Saharan; Africa

Core tip: Intra-partum foetal death includes intra-uterine deaths that occur within 12 h of delivery of a new-born weighing more than 1000 g, or that had more than 28 wk of gestation, but could not be resuscitated. Sub-Saharan Africa has the lowest recorded decline of intra-partum foetal deaths; however, this region recorded a doubling of her annual rate of reduction to 3.1% during 2000-2011, from 1.5% during 1990-2000. Impacts of research within the region towards improved new-born health and cost-effective policy interventions are examined.

Adekanbi AOA, Olayemi OO, Fawole AO, Afolabi KA. Scourge of intra-partum foetal death in Sub-Saharan Africa. *World J Clin Cases* 2015; 3(7): 635-639 Available from: URL: http://www.wjgnet.com/2307-8960/full/v3/i7/635.htm DOI: http://dx.doi.org/10.12998/wjcc.v3.i7.635

INTRODUCTION

Intra-partum foetal death is a subset of perinatal mortality: macerated stillbirths; lethally and congenitally malformed neonates as well as new-borns that died after the first 24 h of life are excluded. It basically refers to intra-uterine foetal deaths that occurred within 12 h of delivery of a new-born whose weight is more than 1000 g or which has gone beyond 28 wk of gestation[1]. It is a health indicator which measures the quality of obstetric care on one hand, and the association between maternal and neonatal health on the other hand; it is a determinant of the quality of intra-partum care.

According to the International Classification of Diseases, Revision 10, a stillbirth or late foetal death is that which occurs after 22 wk of gestation, or when the crown-heel length is 25 cm or more, and the weight is at least 500 g[2]. For the purpose of international comparison, stillbirth is better described as foetal death at a gestational age of 28 completed weeks or a crown-heel length of 35 cm or more and birth weight is at least 1000 g[3]. Gestational age measures are not as reliably documented as birth weight, especially in the low resource countries.

The greatest risk to life for the mother and baby is noted during childbirth[4]. Intra-partum related death accounts for over 2/5 of the world's annual maternal deaths; the death of 1.02 million babies during labour, and 904000 neonatal deaths around delivery are interrelated[1,4,5].

The quality of intra-partum care is therefore a crucial factor for the pregnancy outcome for both mothers and new-borns. Timely and appropriate care rendered by skilled attendants in an atmosphere that is conducive will prevent or at least reduce morbidity for both mothers and newborns.

The foetal mortality rate for gestations of at least 20 wk (6.2 foetal deaths per 1000 live births)[6] and infant mortality rate (6.9 infant deaths per 1000 live births)[7] in the United States in the year 2005 were similar. Depending on the definition used, 40% to 60% of perinatal mortality is due to foetal mortality[8]. According to the World Health Organization (WHO)[9], 8 out of every 1000 babies die during labour worldwide. In 2000, intra-partum mortality rate was estimated at 15 per 1000 births in Middle and Western Africa, while it was only 0.6 per 1000 births in developed countries[9].

Annually, there are 287000 maternal deaths[10] and over 3 million stillbirths, of which about a million die in the course of labour and about four million neonatal deaths, half of which happen on the day of birth[11].

Although stillbirths are underreported in developing countries, 97% of the cases occur in the region, and it accounts for 50% of worldwide perinatal deaths. It is hoped that the problem of stillbirths will be given due attention in these regions with a view to reducing the effect on the society[9,12]. A Nigeria based study posited that of the recorded perinatal deaths, 51.2% were fresh stillborns, while 39.1% were macerated[13]. This finding conforms to the Wigglesworth classification of perinatal death (Table 1). The high proportion of intra-partum foetal death is a reflection of poor-quality intra-partum care in the study-country, however, another study conducted in Nigeria recorded 73% macerated and 27% fresh stillbirths[14], which reflects variations noted from one centre to the other, even within the same country.

The neonatal period is a crucial part of the infant's life, as up to 40% of deaths of children younger than 5 years occurred within the period; also, neonatal death was observed to increase in relation to a rapid fall in postnatal deaths[1,15]. The need for concerted efforts from health practitioners and policy makers can never be overemphasized.

Twenty-five percent of neonatal deaths in low income countries and 8% of all deaths among children younger than 5 years during the 4-year period of 2000 to 2003, were attributed to birth asphyxia by the WHO[16]. A major focus on birth asphyxia is important in reducing child mortality; this will immensely contribute to the attainment of the Millennium Development Goal[17]. Tracking of stillbirths, however, is often incomplete and variable.

The global burden of disease literature gives insight into the prevalence of death during the peri-partum period[17]. The relatively insufficient resource allocation to the health sector, especially in Africa, which is identified as a bane, was addressed by the World bank in 1993; the bank issued a guide on resource allocation in the health sector to assist the developing countries in that perspective.

Intra-partum related neonatal death deserves prominence in global health programming and policy because it has a significant contribution to the under-5 child mortality rate. Early neonatal deaths are intertwined with maternal health, therefore, effective maternal cum neonatal health services are key to reversing the poor outcomes. The reduction of intra-partum-related neonatal deaths is a daunting challenge and success will depend on provision of effective and efficient care delivery[3,11,16].

Vital registration systems are weak in developing countries where more than 97% of neonatal deaths occur[12], therefore little is known about the causes of most of these deaths. Furthermore, autopsies on the dead foetuses, as well as placenta histological studies are rarely carried out in the region. This review discusses intra-partum foetal deaths from a Sub-Saharan African perspective. It explores the contribution of research within the region to identifying its impact on new-born health and potential cost-effective policy interventions.

Table 1 Perinatal deaths by Wiggles worth classification[a]

Birth weight (g)	No. of perinatal deaths					Total (%)
	Macerated stillbirths	Congenital malformations	Immaturity	Asphyxia	Other	
1000 (n = 34)	18	2	14	0	0	34 (5.2)
1001-1500 (n = 42)	16	7	16	3	0	42 (6.4)
1501-2000 (n = 65)	35	4	18	8	0	65 (9.9)
2001-2500 (n = 55)	20	7	11	16	1	55 (8.3)
N2500 (n = 464)	151	5	49	250	9	464 (70.3)
Total (%)	240 (36.4)	25 (3.8)	108 (16.4)	277 (41.9)	10 (1.5)	660 (100.0)

[a]Birth weight data missing for 61 neonates. Adapted from Fawole *et al*[13], Determinant of Perinatal Mortality in Nigeria.

PATHOLOGIC CONSIDERATIONS AND TERMINOLOGIES

Most foetal deaths, intra-partum or immediately post-partum, are caused by birth asphyxia, which is mainly due to mismanaged obstetric conditions. The causes of foetal deaths are: obstructed labour, infections, asphyxia, maternal haemorrhage, severe pre-eclampsia and eclampsia, maternal/foetal malnutrition, congenital anomalies and umbilical cord complications[18,19].

Congenital anomalies, diabetes, and infections associated with preterm birth and post-term pregnancy, which are preventable causes of stillbirth, contribute immensely to a high foetal death rate, however, the causes have been virtually eliminated in high income countries (HIC)[20]; the contributions from these important causes in the Sub-Saharan Africa region are scarcely documented.

"Birth asphyxia" according to WHO in 1997, is a condition in new-borns who had breathing abnormality at birth[21]. Non-specific terminologies such as foetal distress, Apgar score and foetal acidosis are not favoured; these terminologies are not predictive of outcome on the long run[22].

Post–asphyxia encephalopathy, birth asphyxia, hypoxic-ischaemic encephalopathy, foetal distress or perinatal asphyxia should not be used loosely, except when there is evidence of acute intra-partum causation[22-24]. Intra-partum stillbirth presents as fresh stillbirth when it is due to intra-partum hypoxic injury[25].

Intra-partum neonatal death is the death of a baby born alive within 28 d of life from evidenced intra-partum injury, with or without neonatal encephalopathy[3,11,20,21]; acute hypoxia superimposed on chronic hypoxia in a growth restricted foetus could easily lead to foetal death[22]. There is a limit to the ability of the foetus to tolerate excessive reduction in oxygen partial pressure, though a healthy foetus is conditioned to physiological hypoxia that regulates foetal circulation through unhindered oxidative metabolism[26].

The immediate response to acute hypoxia by the foetus and the new-born is shallow breathing followed by cessation of respiration, called primary apnoea, which leads to deep gasping and irregular respirations. It progresses to terminal apnoea when all respiratory efforts cease[26].

In the course of apnoea, the heart rate progressively decreases to a halt few minutes after the onset of secondary apnoea. Primary and secondary apnoea presents with bradycardia and dyspnoea[26].

Prompt tactile stimulation and/or assisted ventilation always lead to a restoration of the heart rate to normalcy; any delay in resuscitation is accompanied by slow recovery. The importance of institutionalization of the concept of emergency obstetric care (EMOC), increased skilled birth attendance and neonatal intensive care in all facilities charged with pregnancy care cannot be over-emphasized.

DEMOGRAPHIC TRENDS

Over 1 million stillbirths occur during labour[23]; a number of studies from some low income countries showed that up to 70% of stillbirths occur in the intra-partum period and are due to obstetric emergencies[4,22], while in advanced countries, half of all stillbirths occur in non-anomalous babies at > 28 wk gestation[20]. A WHO review of vital registration showed that the estimated number of global stillbirths was 2.6 million in 2009 and 3.0 million in 1995[27]. The worldwide stillbirth rate has declined by 14% between 1995 and 2007[27].

The Western Pacific region recorded the greatest reduction in stillbirths, with a 3.8% annual decline between 1995 and 2009, while the African region posted only an annual decline of less than 1% (0.7%)[24]. There is a wide variation in stillbirth rates among countries; South-East Asia and Africa had two-thirds of all stillbirths.

In advanced countries, the third-trimester stillbirth rate is less than 4 per 1000 total births, and this amounts to 25% of the worldwide average and 11% of the average in South Asia and Sub-Saharan Africa[25]. Finland has the lowest reported rate of 2.0 per 1000 total births, while Nigeria reported 41.9 per 1000 total births and Pakistan at 46.1 per 1000 total births, had the highest rates[24].

Sub-Saharan Africa registered a 39% decline in the under-five mortality rate. However, the region had an annual rate of reduction to 3.1% during 2000-2011, from 1.5% during 1990-2000[26]. There was a dramatic acceleration in the rate of decline in Eastern and Southern Africa; this coincided with a recorded sub-

stantial improvement in effective interventions to combat major diseases, especially HIV, and also measles and malaria[27].

RISK FACTORS

Utilization of emergency obstetrics care where available, remained low and even worse in the remote areas; 5% of births in rural South Asia and 1% in rural Sub-Saharan Africa are by caesarean delivery[28]. Sixty-two percent of stillbirths occurred in the intra-partum period; obstetrical complications accounted for 61.4% of stillbirths. The following significant maternal risk factors were documented: un-booked status, illiteracy, age of 35 years and above in pregnancy, extremes of reproductive age groups, multiple pregnancies and prolonged labour; the foetal risk factors associated with stillbirth are mal-presentation, decreased foetal movement, foetal distress, prematurity, small for gestational age, and neonatal infection[28-30]. The following independent risk factors were identified: congenital malformations, true knot of cord, meconium stained amniotic fluid, oligo-hydramnios, poly-hydramnios, previous adverse perinatal outcome, placental abruption, advanced maternal age, and hypertensive disorders[30]. Jewish ethnicity, gestational diabetes, previous caesarean section, and recurrent abortions were negatively associated with intra-uterine foetal death[31].

An Africa based study identified the following risk factors for perinatal death: un-booked status, lack of prenatal care, duration of schooling, maternal age above 35 years, asphyxia, and prematurity[12]. However, the researchers found that when maternal and neonatal factors were considered together, the following are the determinants of perinatal mortality: un-booked status, free maternity service, mother's level of education, mother's age within the range of 26 and 30 years, and mothers older than 40 years, prematurity, asphyxia, lack of prenatal care, mode of delivery and so on[12]. Some of the risk factors peculiar to the Sub-Saharan African society are lack of prenatal care, un-booked status, and lack of quality care to mention a few.

The most common patient-related avoidable factors found in a South African study were un-booked patients, patients booked late in pregnancy, patients who delayed before seeking medical assistance, and patients with inappropriate responses to poor foetal movements[32]; whereas the most common avoidable factors related to medical care were underestimation of foetal size and lack of response by staff to patients with poor obstetric histories. However, the common administration-related avoidable factors were unavailable operating theatres and lack of transportation between institutions[32].

In a number of countries in the Sub-Saharan Africa, a considerable percentage of deliveries are not supervised by skilled workers, there is a dearth of nurses and midwives, the database in these places is weak for setting priorities, and political willingness to address the issue is suboptimal.

It is implied by these identified risk factors that developing an algorithm for the management of such conditions, especially for use in the Sub-Saharan African countries, will be a good step towards reducing the scourge.

PREVENTION

Preventive measures aimed at reducing the incidence of intra-partum foetal death entail all measures aimed at improving quality antenatal care and preventing intra-partum asphyxia.

Appropriate obstetric care in the prenatal and intra-partum periods is vital. Also, close monitoring with readily available appropriate care during labour to enable obstetrical providers recognize conditions such as prolonged labour, placental abruption, placental previa, foetal mal-position, and foetal distress, will allow for rapid intervention through caesarean section to further reduce the rate of intra-partum foetal deaths.

Every death counts; a version of mortality audit in South Africa focuses on saving mothers, babies and children[33]. Replication of the exercise in all African countries, with the intention of putting to use lessons learnt from the exercise, will help stem the tide of the repugnant scourge.

Intermittent auscultation for monitoring foetal heart rate in labour is preferred and should be promoted in the low and medium income countries, rather than continuous foetal heart rate monitoring devices which might appeal to policy makers in such climes. This recommendation is based on the outcome of a Dublin based study, which concluded that there is no difference in intra-partum stillbirth rates, as well as long term outcome between the intermittent auscultation group and the continuous foetal heart rate monitored group[33].

CONCLUSION

The high rates of intra-partum foetal death in Sub-Saharan Africa should be a cause for concern for all stakeholders.

Urgent and effective steps are needed to promote equitable distribution of health facilities, providing maternal and newborn health care with optimal capacity for EMOC.

Initiatives that seek to increase rates of facility births in Sub-Saharan Africa must address the issues of quality of maternity care and socio-cultural determinants of access to health care.

It is important that health systems identify the causes of intra-partum foetal deaths peculiar to their location and endeavour to audit all stillbirths with a view to improving pregnancy outcomes for both mothers and new-borns.

Increased research attention focusing on this subject in the region is also advocated.

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ORIGINAL ARTICLE

Observational Study

In vitro differentiation of human umbilical cord Wharton's jelly mesenchymal stromal cells to insulin producing clusters

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Data sharing statement: Technical appendix, statistical code, and dataset available from the corresponding author at Dryad repository, who will provide a permanent, citable and open-access home for the dataset.

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Abstract

AIM: To investigate the differentiation of human Wharton's jelly derived mesenchymal stromal cells (WJ-MSCs) to insulin producing clusters (IPC) this study was conducted.

METHODS: The umbilical cords samples were collected from full term caesarian section mothers and the WJ-MSCs were cultured from tissue explants in High glucose-Dulbecco's Modified Eagle Medium (H-DMEM); H-DMEM supplemented with 10% fetal bovine serum (FBS) and antibiotics. The expression of CD90, CD44, CD105, CD34 and CD133 as well as osteogenic and adipogenic differentiation of cells in appropriate medium were also evaluated. The cells were differentiated toward IPC with changing the culture medium and adding the small molecules such as nicotinic acid, epidermal growth factor, and exendin-4 during 3 wk period. The gene expression of *PDX1*, *NGN3*, *Glut2*, insulin was monitored by reveres transcription polymerase chain reaction method. The differentiated clusters were stained with Dithizone (DTZ) which confirms the presence of insulin granules. The insulin challenge test (low and high glucose concentration in Krebs-Ringer HEPES buffer) was also used to evaluate the functional properties of differentiated clusters.

RESULTS: WJ-MSCs were positive for mesenchymal surface markers (CD90, CD44, CD105), and negative for CD34 and CD133. The accumulation of lipid vacuoles and deposition of calcium mineral in cells were considered as adipogenic and osteogenic potential of WJ-MSCs. The cells also expressed the transcriptional factors such as Nanog and OCT4. During this three step differentiation, the WJ-MSCs morphology was gradually changed from spindle shaped cells in to epithelioid cells and eventually to three dimensional clusters. The clusters expressed PDX1, NGN3, Glut2, and insulin. The cells became bright red color when stained with DTZ and the insulin secretion was also confirmed. In glucose challenge test a significant increase in insulin secretion from 0.91 ± 0.04 μIu/mL (2.8 mmol/L glucose) to to 8.34 ± 0.45 μIu/mL (16.7 mmol/L glucose) was recorded ($P < 0.05$). The insulin secretion of undifferentiated *WJ-MSCs* was not changed in this challenge test.

CONCLUSION: WJ-MSCs have the ability to differentiate in to islet-like cells *in vitro*. However, this process needs further optimization in order to generate efficient and functional IPCs.

Key words: Mesenchymal stromal cells; Umbilical cord; Beta cell; Islet

Core tip: Diabetes is a major chronic metabolic disorder in the world. Mesenchymal stromal cells (MSCs) has the ability to differentiate in to functional insulin producing cells.In this study, human Wharton's jelly derived MSCs (The clusters expressed *PDX1*, *NGN3*, *Glut2*, and insulin. The cells became red color when stained with DTZ and the insulin secretion was confirmed Wharton's jelly derived MSCs were differentiated to insulin producing clusters (IPCs). More efficient differentiation protocoles for generation of functional IPCs will be a potential new source for cell transplantation in diabetic patients.

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INTRODUCTION

Diabetes mellitus is characterized by an absolute or relative lack of blood insulin and associated the impaired metabolism of carbohydrates, fats and proteins. Recent study reported that 382 million people had diabetes in the world; and by 2035, this number is expected to be increased to 592 million[1]. Pancreas or islet cell transplantation is considered as an effective therapy for diabetic patients[2]. However, the limited number of cadaveric donors and immunological rejection are two major obstacles to achieve effective long term results[3].

Human embryonic stem cell (ESC) is a good promising source for treating diabetes[4]. However, ethical concerns about the use of human embryos and the risk of tumourigenicity are problems regarding the use of these cells for clinical use[4]. Other studies have made efforts to differentiate pancreatic beta cells from other sources such as induced pleuripotent stem cells (IPSs)[5,6], bone marrow-derived mesenchymal stromal cells[7,8], or umbilical cord blood cells[9].

Mesenchymal stromal cells (MSCs) are isolated from the Wharton's jelly or umbilical cord, are easily obtained and processed compared to ESCs and bone marrow derived MSCs[10]. The cells have differentiated into adipogenic, chondrogenic, and osteogenic lineage and also have expressed the CD105, CD44 and CD90 and negative for hematopoietic and vascular markers such as: CD33, CD56, CD31, CD34, CD45[10]. Wharton's jelly derived MSCs (WJ-MSCs), have high proliferation capacity and do not induce teratomas after transplantation[10].

The potential of these postnatal stem cells to differentiate toward insulin-producing cells has been evaluated previously. However, the current protocols are not optimized for efficient transdifferentiation. Here, we describe the three step modified protocol for direct differentiation of WJ-MSCs into insulin producing clusters (IPC). These clusters produce insulin in response to different glucose concentration.

MATERIALS AND METHODS

Preparation of human umbilical cord

Human umbilical cord samples were aseptically collected from full-term delivery by cesarean section at the Hafez Hospital affiliated to Shiraz University of Medical Sciences. Informed consent was received from mothers and the study design was approved by ethical committee of our university.

Preparation of WJ-MSCs

Umbilical cord Wharton's jelly was processed within 2 h after aseptic collection and cut into pieces of 0.5-1 mm^2. These pieces were placed in 10 cm plates and cultured in High glucose-Dulbecco's Modified Eagle Medium; H-DMEM supplemented with 10% fetal bovine serum (FBS) and penicillin 100 U/mL, streptomycin 100 μg/mL. The plates were placed in an incubator with saturated humidity at 37 ℃ containing 5% CO_2. The medium was changed every three days; the cell migrated from the margins of explants. After reaching 70%-80% confluence, the adherent cells were harvested with 0.05% trypsin-Ethylene diamine tetra acetic acid (EDTA), (Gibco, Germany) and the cell suspension was used for subsequent experiments. The presence of transcription factors that regulate maintenance of pluripotent

state in ESC (OCT4, Nanog) were studied by reveres transcription polymerase chain reaction (RT-PCR).

Flow cytometry analysis

The WJ-MSCs was stained with monoclonal antibodies: CD90, CD44, CD105, CD34 and CD133 (BioLegend, San Diego, Calif., United States) and analyzed using the FACS Calibur flow cytometer (Becton Dickinson, NJ, United States).

In vitro osteogenic and adipogenic differentiation

To investigate their capacity for mesodermal differentiation adipogenic and osteogenic differentiation was carried out by culturing cells with appropriate differentiation medium. For adipocytes differentiation, the WJ-MSCs were cultured in L-DMEM supplemented with 10% FBS, 10 nmol/L dexamethasone, 0.5 μg/mL insulin, 2 mmol/L glutamine for 15 d. Then the cells were fixed with 0.4% paraformaldehyde (PFA) and stained with oil-red-O (Sigma). For osteoblastic differentiation, the WJ-MSCs were cultured L-DMEM supplemented with 10% FBS, 10 nmol/L dexamethasone (Sigma), 10 mmol/L b-glycerol phosphate (Sigma) and 50 mg/mL ascorbic acid-2 phosphate (Sigma). After fixation, the differentiated cells were stained with alizarin red stain and calcium deposition was confirmed.

In vitro differentiation of WJ-MSCs to insulin producing clusters

The cultured WJ-MSCs from 4th passage with 80% to 90% confluency were induced to differentiate into IPC in three separate stages.

In first sage, the MSC monolayer was treated for 24 h with high glucose Dulbecco modified Eagle medium-F12 (DMEM-F12) supplemented with10% FBS, 10^{-6} mol/L of retinoic acid (RA; Sigma-Aldrich) and 1% antibiotic; then the medium was switched to DMEM-F12 containing only 10% FBS for 2 d.

In second stage, the cells were detached with 0.5% trypsin-EDTA and seeded in 0.1% gelatin (Sigma-Aldrich) coated plates. The medium was switched to DMEM-F12, supplemented with 10% FBS, 10 mmol/L nicotinic acid (Sigma-Aldrich), and 20 ng/mL epidermal growth factor (EGF, Sigma-Aldrich) for one week.

In third stage, in order to mature the insulin-producing cells, the DMEM-F12 medium was supplemented with 10% FBS and exendin-4 (Sigma-Aldrich) for one week. Glucagon-Like Peptide-1 or Exendin-4 is an insulin secretagogue, with glucoregulatory effects.

The formation of islet like clusters was daily monitored and the DTZ staining confirmed the presence of insulin granules. The genes expression of pancreatic endocrine cell development was monitored in the end of each step. The cells which were cultured in L-DMEM containing only 10% FBS was considered as a control group.

RT-PCR

The total RNA was extracted from WJ-MSCs s before and after third stage of differentiation by using Mini-RNease RNA extraction kit (Cinnagen, Iran). The cDNA was synthesized using MMULV reverse transcriptase (Cinnagen, Iran). The reaction condition for cDNA synthesis was: pre-heating at 42 ℃ for 90 min; followed by annealing at 85 ℃ for 5 s. The synthesized cDNA was used to examine the expression of pancreatic specific transcription factors as listed in Table 1. Amplification used a fluorescence-quantitative PCR instrument (ABI Prism7500, step one plus) by using SYBER Green (TAKARA, Japan) following the manufacturer's instructions. Amplification was carried out as follows: denaturing at 95 ℃ for 5 min; followed by 40 cycle denaturation at 95 ℃ for 1 min; annealing for 1 min. The final stage was run to generate a melting curve for verification of amplification product specificity. Beta actin gene was used as a control. The products were also visualized by gel electrophoresis.

Dithizone staining

Diphenylthiocarbazone, Dithizone (DTZ), (Sigma, United States) is a zinc-chelating substance which is used for identification of insulin granules in pancreatic beta-cells as bright crimson red. The Acinar cells remain unstained.

In order to evaluation insulin production in IPC at the end of 3rd week, the differentiated clusters were examined.

Insulin secretion

The clusters, were rinsed twice in Krebs-Ringer HEPES (KRH) buffer (125 mmol/L NaCl, 4.74 mmol/L KCL, 1 mmol/L $CaCl_2$, 1.2 mmol/L KH_2PO_4, 1.2 mmol/L $MgSO_4$, 5 mmol/L $NaHCO_3$, 25 mmol/L HEPES, 0.1 mmol/L 0.1%BSA, pH = 7.4) containing 2.8 mmol/L glucose (Low glucose concentration) (Sigma-Aldrich, United States).

After 1 h incubation, the medium was collected for insulin assay and the cells were stimulated with KRH buffer containing 16.7 mmol/L glucose for 1 h (High glucose concentration). The insulin level was determined by Enzyme linked Immunoassay kit and the results were compared with undifferentiated MSC cells as control group.

Statistical analysis

The data were presented as means ± SD. Each experiment was repeated 3 times. Data was assessed by one-way ANOVA followed by Tukey's test for pair wise comparison. $P < 0.05$ was considered as statistically significant. The statistical analysis was performed using Graph Pad Prism 5 software.

RESULTS

Cell morphology and Immunophenotyping of WJ-MSCs

WJ-MSCs formed a homogenous monolayer of adherent spindle shaped cells (Figure 1). Flow cytometry ana-

Table 1 Primer pairs for amplification of transcription factors

Names	Forward (F) 5´-3´	Reverse(R) 5´-3´
Insulin	GCAGCCTTTGTGAACCAACA	TTCCCCGCACACTAGGTAGAGA
PDX1	GGATGAAGTCTACCAAAGCTCACGC	CCAGATCTTGATGTGTCTCTCGGTC
NGN3	CAATCGAATGCACAACCTCA	GGGAGACTGGGGAGTAGAGG
GLUT2	AGGACTTCTGTGGACCTTATGTG	GTTCATGTCAAAAAGCAGGG
NANOG	CAGAAGGCCTCAGCACCTAC	ATTGTTCCAGGTCTGGTTGC
Oct-4	CAGTGCCCGAAACCCACAC	GGAGACCCAGCAGCCTCAAA
β-actin	GATCGGCGGCTCCATCCTG	GACTCGTCATACTCCTGCTTGC

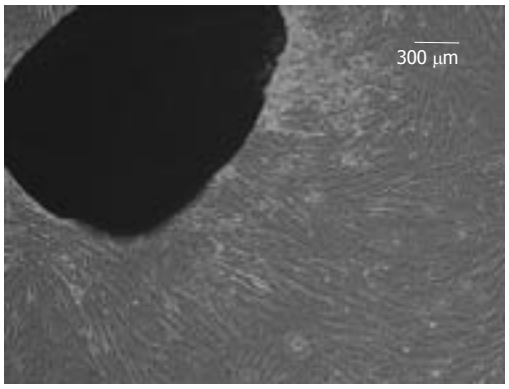

Figure 1 The Wharton's Jelly mesenchymal stromal cells were grown from the edge of tissue explants (× 100).

lyses showed that the WJ-MSCs were positive for mesenchymal markers (CD90, CD44, CD105), and negative for CD34 and CD133 (Figure 2). The ESCs transcriptional factors such as Nanog and OCT4, also expressed.

In vitro osteogenic and adipogenic differentiation of WJ-MSCs

The accumulation of lipid vacuoles (Stained red color in Oil Red O) in cells was considered as adipogenic differentiation. Deposition of calcium minerals (stained as orange red color with Alizarin Red) was also considered as osteogenic potential of these cells (Figures 3 and 4).

Morphological Changes before and after differentiation of WJ-MSCs

In passage 4 (P4), the WJ-MSCs were step wise differentiated toward insulin producing cells.

In stage 1, the cells have shown spindle-shaped morphology (Figure 5A). Cell morphology was gradually changed into round epithelioid cells and by addition of beta cell maturation factor such as nicotinamide in stage 3, three dimensional clusters were formed (Figure 5B-D). These clusters were stained red color with DTZ (Figure 6).

Gene expression analysis

After differentiation, mRNA expression of pancreatic development transcription factors and beta cell specific gene such as *PDX1*, *NGN3*, *Glut2* and insulin were detected. Expressions of the mentioned genes led to *in vitro* production of functional IPCs. Results represent three separate experiments (Figure 7A and B).

Insulin secretion after glucose challenge test

In respect of insulin secretion, a significant increase in insulin secretion from 0.91 ± 0.04 μIu/mL (2.8 mmol/L glucose) to to 8.34 ± 0.45 μIu/mL (16.7 mmol/L glucose) was recorded ($P < 0.05$). The insulin secretion was detected in undifferentiated *WJ-MSCs* (0.3 ± 0.03 μIu/mL) which was not changed in glucose challenge test.

DISCUSSION

Type 1 diabetes mellitus is caused by an autoimmune destruction of pancreatic beta cells. Although the insulin therapy remains the routine treatment for diabetes, but whole pancrease organ transplant or transplantation of pancreatic islets of Langerhans provides a cure for this disorder[2,3]. Various types of stem cells such as ESCs, induced pleuripotent stem cells (IPs) and mesenchymal stromal cells have been differentiated into IPCs by genetic modification and/or modification in culture conditions[7,8,11,12]. Using of ESCs has several limitations such as ethical problem, immune rejection, and risk of tumorigenesis.

To overcome these problems, human IPS is ideal for personalized therapy. However, epigenetic changes, and chromosomal instability during reprogramming remain the obstacle[6] .

Generation of IPCs from MSCs from a variety of tissues such as bone marrow, umbilical cord, and adipose tissue represents an alternative therapy. MSCs can also be used as a cellular vehicle for the expression of human insulin[6,13-15].

The MSC numbers in bone marrow and umbilical cord blood are low and require multiple *ex vivo* expansion. Extra-embryonic tissue such as fetal membrane and umbilical cord Wharton's jelly has the stemness phenotype, immunoprivileged properties, and faster proliferation than adult MSCs and is considered as unlimited source for tissue engineering and regenerative medicine[13]. The WJ-MSCs express HLA-G6 isoform, the unique ability which is important in immune modulation. Therefore, these cells are particularly suitable for cell based therapy. These tissues are normally discarded

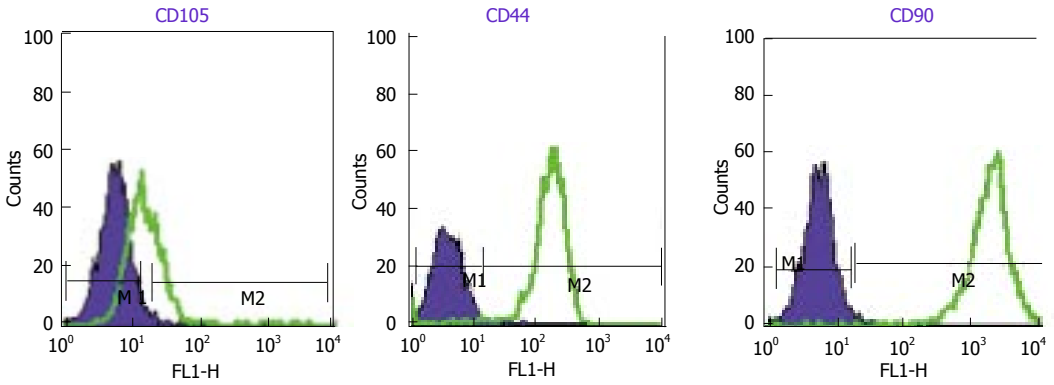

Figure 2 Flow cytometry histogram of Wharton's jelly derived mesenchymal stromal cells (CD90, CD105, CD44). WJ-MSCs are positive for these markers. Dark Blue lines indicate background fluorescence obtained with isotype control. WJ-MSCs: Wharton's jelly derived mesenchymal stromal cells.

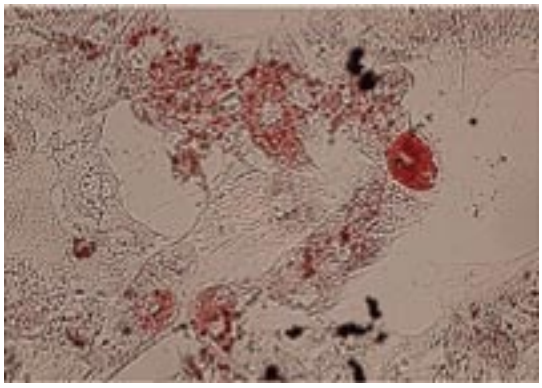

Figure 3 Fat droplets confirmed the adipogenic potential of Wharton's jelly derived mesenchymal stromal cells (Oil Red O staining × 400).

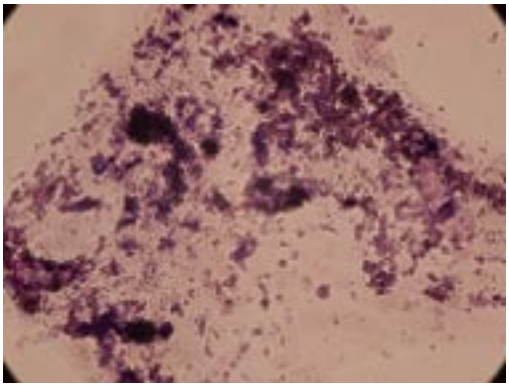

Figure 4 Calcium deposition confirmed the osteogenic potential of Wharton's jelly derived mesenchymal stromal cells (Alizarin Red staining × 100).

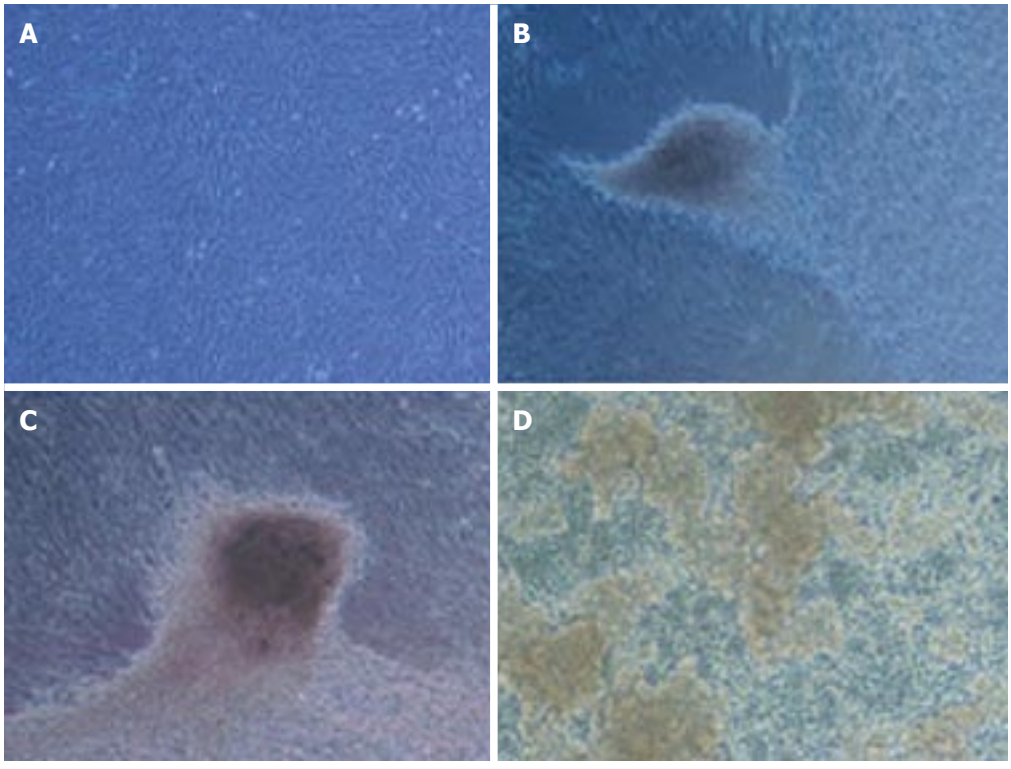

Figure 5 Morphological differentiation of insulin producing clusters from Wharton's jelly derived mesenchymal stromal cells in different stages (A-D × 100). A: WJ-MSCs were typically an adherent spindle shape; B and C: The cells gradually formed clusters in the medium 14 d after differentiation; D: At the end of final stage clusters were floated in the medium. WJ-MSCs: Wharton's jelly derived mesenchymal stromal cells.

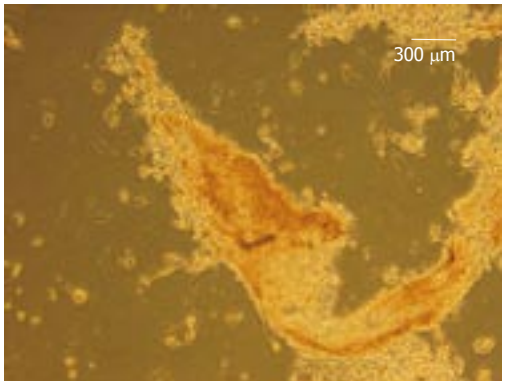

Figure 6 Dithizone staining. The differentiated cells stained as red color (× 100).

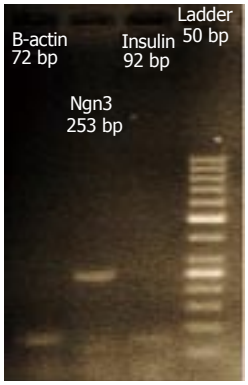

Figure 7 Expression of *Ngn3*, Insulin, *PDX1*, *Oct4* and Nanog transcription factors were confirmed by electrophoresis.

Table 2 Comparison between different methods of islet like cells clusters differentiation from different stem cell sources

Stem cell sources	Differentiation protocol	Efficiency (generation of insulin producing cell)	Ref.
Placenta-derived Mesenchymal stem cells	(α-MEM + 1% BSA + 1 × ITS + 0.3 mmol/L taurine) 3 d (α-MEM + 1.5% BSA + 1 × ITS + 3 mmol/L taurine + GLP-1 + nicotinamide) 7 d (α-MEM + 1.5% BSA + 1 × ITS + 3 mmol/L taurine + GLP-1 + nicotinamide) 10 d	65%-70% ILCs (represents 20%-25% beta-cells per islet)	Kadam *et al*[31]
Human embryonic stem cells	(DMEM-F12, 20% SR, 2 mmol/L GlutaMAX, 1% NEAA and 0.1 mmol β-mercaptoethanol) 7 d (DMEM-F12, 1% ITS, 2 mmol/L GlutaMAX, 5 μg/mL Fibronectin) 7 d (DMEM-F12, 1% N2, 2% B27, 2 mmol/L GlutaMAX, 10 ng/mL bFGF) 7 d (DMEM-F12, 1% N2, 2% B27, 2 mmol/L GlutaMAX, and 10 mmol/L nicotinamide) for 7-9 d	61.7% ± 9.5% insulin positive cells	Wei *et al*[32]
Human embryonic stem cells	(DMEM-F12, 100 ng/mL activin A, 1 μmol/L wortmannin, 1% N2, 1% B27) 4 d (IMDM/F12, 2 μmol/L retinoic acid, 20 ng/mL FGF7, 50 ng/mL Noggin, 0.25 μmol/L KAAD-cyclopamine, 1% B27) 4 d (DMEM, 50 ng/mL EGF, 1% ITS, 1% N2) 5 d (DMEM-F12, 1% ITS, 10 ng/mL bFGF, 10 mM nicotinamide, 50 ng/mL exendin-4) 7-9 d	41.6% ± 11.8% insulin positive cells	Wei *et al*[32]
hESC lines YT1 and YT2	(RPMI1640, 0.2% FBS, 0.56 N2, 0.56 B27, 100 ng/mL activin A, 1 mmol/L wortmannin) 4 d (RPMI1640, 0.5% FBS, 0.5% ITS, 0.56 B27, 2 mM retinoic acid, 20 ng/mL FGF-7, 50 ng/mL Noggin) 4 d (DMEM, 0.5% FBS, 1% ITS, 16 N2, 50 ng/mL EGF) 5 d (DMEM/F12, 1% ITS, 10 ng/mL bFGF, 10 mmol/L nicotinamide, 50 ng/mL exendin-4, 10 ng/mL BMP4) until maturation	17.1% insulin positive cells	Hua *et al*[33]
Human embryonic stem cells	(MCDB-LG, 100 ng/mL GDF8, 2.5 mmol/L MCX-928, 100 ng/mL) 1 d (MCDB-LG, 100 ng/mL GDF8) 2-4 d (MCDB-LG, 50 ng/mL FGF7) 2 d (MCDB-HG, 50 ng/mL FGF7, 20 ng/mL ActivinA, 0.25 μmol/L SANT-1, 2 μmol/L Retinoic Acid, 200 nmol/L LDN193189) 4 d (MCDB-HG, 0.25 μmol/L SANT-1, 200 nmol/L LDN193189, 500 nmol/L TBP, 100 nmol/L CYP26A inhibitor) 3 d (MCDB-HG, 200 nmol/L LDN193189, 1 μmol/L ALK5i, 100 nmol/L CYP26A inhibitor) 3 d (MCDB-HG, 200 nmol/L LDN193189, 1 μmol/L ALK5i) 3 d (MCDB-HG, 200 nmol/L LDN193189, 1 μmol/L ALK5i, 100 nmol/L VitaminA) 7-14 d	6%-10% insulin positive cells	Bruin *et al*[34]
Human embryonic stem cells	(EB formation) 2 d (DMEM-F12, KSR, ActivinA, Retinoic acid) 6 d (DMEM-F12, KSR, bFGF, Noggin) 12 d (DMEM-F12, N2, B27, Laminin, bFGF, Nicotinamid, GLP-1) 12 d (CMRL1066, Nicotinamid, ITS, Zn2SO4, Glutamax, HEPES, KSR, GLP-1, Exendin1, HGF) 10 d	24.5% insulin producing cell	Bose *et al*[35]

Bone marrow mesenchymal stem cells	(Human BMSCs were transfected with adenovirus carrying PDX1 or VEGF) 2 d	50% of cells was differentiated to beta cell	Milanesi *et al*[36]
Human bone marrow-derived stem cells	(RPMI 1640, 5.5 mmol/L glucose, 5% FCS, 10 mmol/L nicotinamide, 10 nmol/L exendin 4) 5 d	20% insulin producing cell	Tang *et al*[37]
Human bone marrow-derived mesenchymal stem cells	(DMEM, 0.5 mmol/L β-mercaptoethanol) 2 d (DMEM, 1% non-essential amino acids, 20 ng/mL bFGF, 20 ng/mL EGF, 2% B27, 2 mmol/L L-glutamine) 8 d (DMEM, 10 ng/mL betacellulin, 10 ng/mL activin-A, 2% B27, 10 mmol/L nicotinamide) 8 d	5% insulin producing cell	Gabr *et al*[38]
Human labia minor dermis-derived fibroblasts	(DMEM/F12, ITS) 7 d (DMEM, 10% FBS, 100 U/mL penicillin/streptomycin, ITS, 10 mmol/L nicotinamide) 7 d	50% insulin positive cell 2×10^6 cells generated around 400–600 ICAs	Kim *et al*[39]
Human adipose tissue derived adult stem cells	(DMEM/F12, 17.5 mmol/L glucose, 1% BSA, 1 × ITS, 4 nmol/L activin A, 1 mmol/L sodium butyrate, 50 mmol/L 2-mercaptoethanol, 2 ng/mL FGF) 2 d (DMEM/F12, 17.5 mmol/L glucose, 1% BSA, ITS, 0.3 mmol/L Taurine) 2 d (DMEM/F12, 17.5 mmol/L glucose, 1.5% BSA, ITS, 3 mmol/L Taurine, 100 nmol/L GLP-1, 1 mmol/L nicotinamide and 1× non-essential amino acids) 12-14 d		Chandra *et al*[40]
Human Adult Fibroblast-Like Limbal Stem Cells	(RPMI-1640, 100 ng/mL Activin A) 2-3 d (RPMI-1640, 2% FBS, 50 ng/mL bFGF) 3-4 d (DMEM, 10% FBS, 1% B27, 2% N2, 1 mmol/L nicotinamide) 3-4 d (DMEM, 10% FBS, 1% B27, 2% N2, 1 mmol/L nicotinamide, 50 ng/mL exendin-4) 3-4 d (RPMI-1640, 100 ng/mL Activin A) 2-3 d (RPMI-1640, 2% FBS, 50 ng/mL FGF 10 μmol/L) 3-4 d (DMEM, 1% B27, 50 ng/mL FGF 10.2 μmol/L retinoic acid) 3-4 d (DMEM, 1% B27, 50 ng/mL exendin-4) 3-4 d	70% to 77% insulin producing cell	Criscimanna *et al*[41]
Dental pulp stem cells	(DMEM-KO, 1% BSA, 1x ITS, 4 nmol/L activin A, 1 mM sodium butyrate, 50 μmol/L 2-mercaptoethanol) 2 d (DMEM-KO, 1% BSA, ITS, 0.3 mmol/L taurine) 2 d (DMEM-KO, 1.5% BSA, ITS, 3 mmol/L taurine, 100 nmol/L GLP-1, 1 mmol/L nicotinamide, 1x non-essential amino acids) 5 d	2×10^6 (Number of cells generated from around 156 ± 23 ILCs)	Govindasamy *et al*[42]
Stem cells from human exfoliated deciduous teeth (SHED)	(KO-DMEM, 1% BSA, 1 × ITS, 4 nmol/L activinA, 1 mmol/L sodium butyrate) 2 d (KO-DMEM, 1% BSA, ITS, 0.3 mmol/L taurine) 1 d	231 ± 21 number of generated ILCs from initial cell seeding density 2×10^6	Kanafi *et al*[43]
Dental pulp stem cells	(KO-DMEM, 1.5% BSA, ITS, 3 mmol/L taurine, 100 nmol/L glucagon-like peptide-1, 1 mmol/L nicotinamide) 7 d	112 ± 2 number of generated ILCs from initial cell seeding density 2×10^6	Kanafi *et al*[43]

MEM: Minimum Essential Medium Eagle; BSA: Bovine serum albumin; ITS: Insulin transferin selenium; ILCs: Islet like clusters; GlutaMAX: Glutamin; NEAA: Non-essential amino acids; DMEM: Dulbecco's Modified Eagle Medium; bFGF: Basic Fibroblast growth factor; FGF7: Fibroblast growth factor 7; Cyclopamine-KAAD: A Smoothened (Smo) antagonist; EGF: Epidermal growth factor; BMP4: Bone morphogenetic protein 4; MCDP: Name of medium; LG: Low glucose; HG: High glucose; SANT-1: (4-Benzyl-piperazin-1-yl)-(3,5-dimethyl-1-phenyl-1H-pyrazol-4-ylmethylene)-amine; HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; KSR: Name of medium for embryonic stem cell culture; GLP-1: Glucagon-like peptide-1; HGF: Hepatocyte growth factor; KO-DMEM: Knockout DMEM.

after birth and using them is not associated with ethical problem[16,17].

In this study, WJ-MSCs were isolated and their differentiation into adipocytes, and osteocytes confirmed their multilineage potentials. The retinoic acid we used to start the differentiation process. RA is an essential molecule for dorsal pancreas development in mouse. The effects of RA are achieved through RA binding and activation of retinoic acid receptors such as RARα, RARβ, and RARγ. Over expression of RARβ was detected in early stage of pancreases differentiation and absence of RARβ impaired the terminal differentiation of α and β-cells[18,19]. In vitamin-A deficiency, pancreatic islet function was Impaired. RA directly induces Pdx1 expression in ESCs and Pdx1 is an important transcription factor in the early development of pancreatic progenitors and bud expansion[20,21]. The retinoic acid response element is located at upstream of the transcription start site of Pdx1. Retinoie Acid Receptor (RARβ) expression is depended on epigenetic regulation[18,19]. Hyper methylation of the RARβ2 promoter was reported in pancreatic cancer, and diabetes. Therefore, it is postulated that the epigenetic silencing of RARβ, combined with vitamin A deficiency, may play a role in pathogenesis of diabetes[18,19]. Glucose is considered as a growth factor for β-cells replication both *in vitro* and *in*

vivo[22]. Subsequently, using of EGF was associated with proinsulin biosynthesis and 3H-thymidine incorporation in experimental model[23,24]. Nicotinamide was used during the second stage of differentiation. Various published protocols reported this substance as an effective inducer in pancreatic differentiation. Nicotinamide preserve islet viability through poly-ADP-ribose polymerase[25].

Glucagon-like peptide 1 (GLP-1) and its long acting mimetic exendin-4 are usually used for treatment of type 2 diabetes. Exendin 4 simultaneously stimulates beta cell secretory capacity as well as maintains insulin stores by translational control of proinsulin biosynthesis. Exendin-4 can also stimulate b-cell replication in human islets from young donors[26-28].

In our study, at the end of *in vitro* differentiation protocols, pancreatic endocrine genes and insulin were expressed. However, the insulin secretion after glucose challenge test was very low. As mentioned previously, incomplete beta cell phenotype and consequently poor insulin release in response to glucose challenge test might explain this problem[29,30].

In the literature, various sources of mesenchymal stem were differentiated into insulin secreting cells with different efficacy[31-43] (Table 2). However, the insulin secretion capacity of the cells was variable. The insulin challenge test was done with different protocols; the concentration of insulin was measured with different assays and reference range. Therefore, the comparison of data was not easily performed.

In our experience, differentiation of WJ-MSCs to form IPCs needs further optimization for clinical practice. To overcome this problem, addition of growth factors, extracellular matrix and/or culturing the cells in three dimensional environments are suggestive.

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COMMENTS

Background

Different types of stem cells such as embryonic stem cells (ESCs), induced pleuripotent stem cells and mesenchymal stromal cells have been differentiated into insulin producing cells by modification in cell culture conditions and addition of small molecules. Umbilical cord Wharton's jelly derived stromal cells has the stemness phenotype, with faster proliferation than adult mesenchymal stromal cells (MSCs) and is considered as a good source for generation of insulin producing cells.

Research frontiers

In this research, insulin producing clusters (IPC) was differentiated from Wharton's jelly MSCs (WJ-MSCs) without any genetic manipulation.

Innovations and breakthroughs

The IPCs was differentiated from WJ-MSCs by changing the cell culture medium and growth factors without genetic manipulation. However, the insulin release in response to glucose challenge test was not sufficient. Similar studies used various sources of mesenchymal stem and differentiated into insulin secreting cells with different efficacy and variable insulin secretion capacity. The insulin challenge test was done with different protocols; the concentration of insulin was measured with different assays and reference range. Therefore, the comparison of data was not easily performed.

Applications

Differentiation of WJ-MSCs to form IPCs needs further optimization for clinical practice.

Peer-review

The authors have performed a good study, the manuscript is interesting.

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CASE REPORT

Perifollicular granulomas with IgG4 plasmacytosis: A case report and review of literature

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Author contributions: Liang L and Chen L designed the report, collected the patient's clinical data and wrote the paper; Zhou J collected the patient's clinical data and revised the manuscript.

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Informed consent statement: This study was exempted by the Committee for Protection of Human Subjects.

Conflict-of-interest statement: None.

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Abstract

Perifollicular granuloma is a unique histologic feature and whether it is associated with immunoglobulin G4 (IgG4)-related disease is controversial. We report a case of a 38-year-old man who presented with worsening left eye pain, proptosis, tearing, gritty sensation, blurred vision and multiple lymphadenopathy. An axillary lymph node resection showed reactive follicular and interfollicular lymph node hyperplasia, and increased eosinophils and plasma cells (at least 80% of IgG^+ plasma cells were positive for IgG4). A distinct feature was the presence of multifocal, perifollicular histiocytic granulomas, which formed a wreath around the entire follicles. The human herpes virus 8 was not detected by immunohistochemistry. In addition, an extensive panel of special stains, immunohistochemistry, and flow cytometry was negative for lymphoma, fungal, or mycobacterial infection. The findings were suggestive of IgG4-related sclerosing disease-associated lymphadenopathy. Further laboratory testing showed a significant increase of serum immunoglobulin E (> 23000 IU/mL) and slight increase of total IgG, but normal serum IgG4. Even though perifollicular granuloma is a nonspecific histopathologic feature and can be seen in other diseases, such as nodular lymphocyte predominant Hodgkin lymphoma, IgG4-related lymphadenopathy should be listed in the differential diagnoses of benign reactive lymph nodes, especially when perifollicular granuloma and plasmacytosis coexist.

Key words: Immunoglobulin G4-related disease; Lymphadenopathy; Plasma cells; Granuloma

Core tip: We report a case of a 38-year-old man who

presented with worsening left eye pain and multiple lymphadenopathy. An axillary lymph node resection showed increased eosinophils and plasma cells (at least 80% of immunoglobulin (Ig)G$^+$ plasma cells were positive for IgG4 and the presence of multifocal, perifollicular histiocytic granulomas, which formed a wreath around the entire follicles. An extensive workup was negative for lymphoma, fungal, or mycobacterial infection. The findings were suggestive of IgG4-related sclerosing disease-associated lymphadenopathy. Thus, IgG4-related lymphadenopathy should be listed in the differential diagnoses of benign reactive lymph nodes, especially when perifollicular granuloma and plasmacytosis coexist.

Liang L, Zhou J, Chen L. Perifollicular granulomas with IgG4 plasmacytosis: A case report and review of literature. *World J Clin Cases* 2015; 3(7): 650-654 Available from: URL: http://www.wjgnet.com/2307-8960/full/v3/i7/650.htm DOI: http://dx.doi.org/10.12998/wjcc.v3.i7.650

INTRODUCTION

Immunoglobulin G4 (IgG4)-related disease is a recently recognized fibro-inflammatory condition that can involve multiple organs and cause tumor-like enlargement[1,2]. It is characterized by a lymphoplasmacytic infiltrate enriched in IgG4-positive plasma cells, while elevated serum concentrations of IgG4 are found in 60% to 70% of patients[3]. IgG4-related disease has a male predilection (male to female ratio 8:1)[4].

According to the consensus statement from a multinational, multidisciplinary group of experts, the major histopathological features to make the diagnosis of IgG4-related disease include a dense lymphoplasmacytic infiltrate, plasma cells, storiform fibrosis, and obliterative phlebitis[5]. However, these features are usually uncommonly seen in certain organs, such as lymph nodes.

We describe a case of a 38-year-old man with swelling of soft tissue surround the eye and multiple lymphadenopathy and an axillary lymph node resection showed reactive follicular and interfollicular lymph node hyperplasia, increased eosinophils and plasma cells (at least 80% of immunoglobulin IgG$^+$ plasma cells were positive for IgG4) and multifocal, perifollicular histiocytic granulomas, which formed a wreath around the entire follicles. Review of literature also found our findings may add to the knowledge of IgG4-related disease.

CASE REPORT

Clinical history

We report a case of a 38-year-old man who presented with worsening left eye pain, proptosis, tearing, gritty sensation, and blurred vision. Magnetic resonance imaging of orbits confirmed enlargement of the left medial rectus, superior oblique and inferior rectus muscle, and enhancing soft tissue signal encasing the left optic nerve sheath. Computed tomography scan of chest and abdomen showed multiple lymphadenopathy. Clinically, lymphoma was suspected. Patient's rheumatology work up was negative, including anti-neutrophil cytoplasmic antibodies, anti-double stranded DNA, anti-nuclear antibodies, anti-Smith, anti-ribonucleoprotein, anti-complement C3 and C4. Complete blood count shown mild eosinophilia, otherwise, it was unremarkable.

Microscopic and immunohistochemical features

An axillary lymph node resection showed reactive follicular and interfollicular lymph node hyperplasia, and increased eosinophils and plasma cells (at least 80% of IgG$^+$ plasma cells were positive for IgG4). A distinct feature was the presence of multifocal, perifollicular histiocytic granulomas, which formed a wreath around the entire follicles (Figure 1A). Increased plasma cells were marked by CD138 immunohistochemical stain (Figure 1B). IgG4$^+$ plasma cells are markedly increased (Figure 1D), compared with total IgG stain (Figure 1C). EBER (EBV encoded small RNA by *in situ* hybridization) was negative. The human herpes virus 8 was not detected by immunohistochemistry. In addition, an extensive panel of special stains, immunohistochemistry, and flow cytometry was negative for lymphoma, fungal, or mycobacterial infection. The findings were suggestive of IgG4-related sclerosing disease-associated lymphadenopathy.

Follow-up

Further laboratory testing showed a significant increase of serum IgE (> 23000 IU/mL) and slight increase of total IgG (1802 mg/dL), but normal serum IgG4 (27 mg/dL). The patient was started on prednisone and methotrexate with reduction in proptosis and in the size of orbital mass by computerized tomography (CT) scan. While patient was maintained with methotrexate and tapering on steroid, he was noted to have left eye redness and itching. Rituximab was added and methotrexate was discontinued. The patient's symptom subsided.

DISCUSSION

According to the consensus statement from a multinational, multidisciplinary group of experts, the major histopathological features to make the diagnosis of IgG4-related disease include a dense lymphoplasmacytic infiltrate, plasma cells, storiform fibrosis, and obliterative phlebitis[5]. However, these features are usually not seen in certain organs, such as lymph nodes. Fibrosis and obliterative phlebitis are usually not present in lymph nodes.

Lymph nodes in IgG4-related disease may show variable histopathologic features. Cheuk *et al*[4] divided it into five different categories, including multicentric

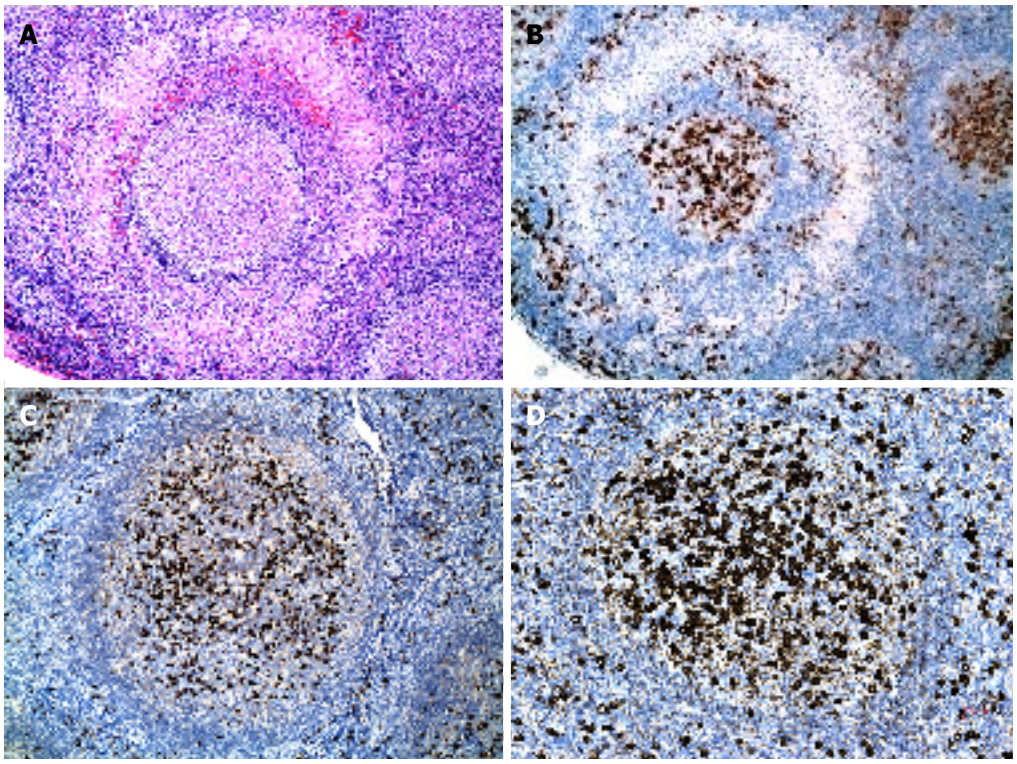

Figure 1 Perifollicular histiocytic granulomas that formed a wreath around the entire follicle. A: Hematoxylin and eosin stain; B: CD138 immunohistochemical stain highlights the plasma cells; C: Immunoglobulin G (IgG) immunohistochemical stain; D: IgG4 immunohistochemical stain demonstrated that more than 80% of IgG^+ cells were positive for IgG4.

Castleman disease-like (type Ⅰ), follicular hyperplasia (type Ⅱ), interfollicular expansion (type Ⅲ), progressive transformation of germinal centers (type Ⅳ), and inflammatory pseudotumor-like (type Ⅴ). Nevertheless, an increase in $IgG4^+$ plasma cells with an IgG4/IgG plasma cell ratio exceeding 0.4, and/or an absolute number of $IgG4^+$ plasma cells of more than 50/high-power field (hpf) are the currently accepted cutoff for IgG4-related disease. However, presence of $IgG4^+$ plasma cells in isolated reactive lymphadenopathy is not exclusively specific for IgG4-related disease[6]. Martinez *et al*[6] reported seven of the 55 solitary reactive lymph nodes with increased IgG4/IgG plasma cell ratio of more than 0.4, and six of them showed more than 50 $IgG4^+$ plasma cells per high power field, but none of these patients had history of IgG4-related disease. On the other hand, Uehara *et al*[7] reported that presence of fibrosis in lymph nodes, together with increased IgG4 ratio and other features of IgG4-related disease, may suggest the diagnosis of IgG4-related lymphadenopathy.

Even though epithelioid cell granulomas is usually not considered a feature of IgG4-related disease at extranodal sites, it has been described in lymph nodes. Siddiqi *et al*[8] described seven cases with perifollicular granuloma in a concentric or crescent-like arrangement encircling lymphoid follicles and associated with a marked elevation of intra-germinal center $IgG4^+$ plasma cells. However, the specificity of these findings were debated by Cheuk *et al*[4]. Grimm *et al*[9] reported histiocytic proliferation in 11 of 29 cases of lymphadenopathy with increased IgG4 plasma cells, and a prominent ringing of follicles by epithelioid histiocytes in 3 patients (Table 1). In addition, Takahashi *et al*[10] reported a case of IgG4-related lymphadenopathy with prominent granulomatous inflammation, most likely due to reactivation of Epstein-Barr virus. Takeuchi *et al*[11] performed Epstein-Barr virus (EBV)-encoded RNA (EBER) in situ hybridization and identified EBER-positive cells in 18 of 31 cases (58%) of IgG4-related lymphadenopathy, significantly higher rate than non-IgG4-related reactive lymphoid hyperplasia. However, EBER was negative in our case, and either negative or rarely positive in the two cases with EBER performed in Grimm group's study (Table 1). Further study is needed to determine whether there is a causal relationship.

IgG4-related disease is a great mimicker. One of the differential diagnoses is multicentric Castleman's disease. However, $IgG4^+/IgG^+$ plasma cell ratio is usually less than 0.4 in multicentric Castleman disease. Furthermore, elevated serum levels of interleukin-6 and vascular endothelial growth factor favor the diagnosis of multicentric Castleman's disease[12]. Rosai-Dorfman disease can also show increased IgG4-positive plasma cells, as well as other autoimmune diseases including rheumatoid lymphadenopathy, are also in the differential diagnosis[13,14]. Moreover, bacterial, viral, fungal and parasitic infections have to be carefully ruled out. In our case, special stains and cultures were performed and the results were negative. The patient

Table 1 Perifollicular granulomatous inflammation and immunoglobulin G4-related disease

Case	Ref.	Age (yr)	Gender	Location	IgG4/IgG ratio	Eosinophils	Fibrosis	EBER
1	Siddiqi *et al*[8]	47	M	Cervical	0.7	Mild	Marked	NA
2	Siddiqi *et al*[8]	63	M	Axillary	0.7	None	None	NA
3	Siddiqi *et al*[8]	50	F	Cervical	0.5	Minimal	Mild	NA
4	Siddiqi *et al*[8]	34	M	Cervical	0.6	None	None	NA
5	Siddiqi *et al*[8]	12	M	Cervical	0.7	Mild	None	NA
6	Siddiqi *et al*[8]	58	M	Unknown	0.7	None	None	NA
7	Siddiqi *et al*[8]	83	M	Axillary	0.7	Mild	Mild	NA
8	Grimm *et al*[9]	47	M	Cervical	> 0.4	NA	NA	NA
9	Grimm *et al*[9]	58	F	NA	> 0.4	NA	NA	Negative
10	Grimm *et al*[9]	83	M	Axillary	> 0.4	NA	Present	Rarely positive
11	Current case	38	M	Axillary	> 0.8	Increased	None	NA

NA: Not available; EBER: EBV encoded small RNA by *in situ* hybridization; M: Male; F: Female.

recovered quickly after immunosuppressive therapy, which was not consistent with infectious lymphadenitis. Other diseases with perifollicular granuloma have been reported, including reactive lymph nodes of unknown etiology, progressive transformation of germinal centers, and nodular lymphocyte predominance Hodgkin lymphoma[4]. However, in our case, morphologic features, immunohistochemistry and flow cytometry results were not consistent with lymphoma.

In our case, the pathologic evaluation is based on lymph node resection specimen. Even though biopsy of the orbital lesion was considered in initial evaluation, it wasn't performed based on the decision of multidisciplinary team, because this invasive procedures carried a significant risk. In addition, our patient had a normal serum level of IgG4. It is a not uncommon finding and is seen in up to 40% of patients with IgG4-related disease[3]. Our patient was treated with prednisone, methotrexate and Rituximab. His eye symptoms subsided dramatically and a post-treatment imaging study showed shrinking of the orbital mass. Thus, clinical presentations and histopathologic findings in this case supported of a diagnosis of IgG4-related disease.

According to the most recently published International consensus guidance statement, the first line treatment of active IgG4-related disease are glucocorticoids, but a combination of glucocorticoids and a steroid-sparing immunosuppressive agent (*e.g.*, azathioprine, methotrexate or mycophenolate) may be beneficial for some patients[15]. In addition, Rituximab has been reported to be an effective drug to treat patients with IgG4-related ophthalmopathy, especially those intolerant of steroid or with recurrent or refractory disease[16].

In summary, we described a case of IgG4-related disease with distinct features of perifollicular granulomas and literature reviews. This may expand our knowledge of pathologic findings of IgG4-related disease in a lymph node, although clinical history, lab results and radiological findings must be taken into consideration when making the diagnosis.

COMMENTS

Case characteristics

A 38-year-old man who presented with worsening left eye pain, proptosis, tearing, gritty sensation and blurred vision.

Clinical diagnosis

Multiple lymphadenopathy was identified.

Differential diagnosis

Lymphoma, Castleman's disease, fungal or mycobacterial infection, autoimmune disorders, *etc*.

Laboratory diagnosis

Serum immunoglobulin E (> 23000 IU/mL) and slight increase of total immunoglobulin G (IgG), but normal serum IgG4. An extensive panel of special stains, immunohistochemistry, and flow cytometry was negative for lymphoma, fungal, or mycobacterial infection.

Imaging diagnosis

Magnetic resonance imaging of orbits showed enlargement of the left medial rectus, superior oblique and inferior rectus muscle, and enhancing soft tissue signal encasing the left optic nerve sheath. Computed tomography scan of chest and abdomen showed multiple lymphadenopathy.

Pathological diagnosis

An axillary lymph node resection showed reactive follicular and interfollicular lymph node hyperplasia, and increased eosinophils and plasma cells (at least 80% of IgG^+ plasma cells were positive for IgG4). A distinct feature was the presence of multifocal, perifollicular histiocytic granulomas, which formed a wreath around the entire follicles.

Treatment

The patient was started on prednisone and methotrexate with reduction in proptosis and in the size of orbital mass by computerized tomography scan. While patient was maintained with methotrexate and tapering on steroid, he was noted to have left eye redness and itching. Rituximab was added and methotrexate was discontinued. The patient's symptom subsided.

Related reports

Perifollicular granuloma is a unique histologic feature and whether it is associated with IgG4-related disease is controversial. Very few cases have been reported in the English literature.

Term explanation

IgG4-related disease is a recently recognized fibro-inflammatory condition that can involve multiple organs and cause tumor-like enlargement, which is characterized

by a lymphoplasmacytic infiltrate enriched in IgG4-positive plasma cells.

Experiences and lessons

IgG4-related lymphadenopathy should be listed in the differential diagnoses of benign reactive lymph nodes, especially when perifollicular granuloma and plasmacytosis coexist.

Peer-review

The authors have performed a good study, the manuscript is interesting.

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CASE REPORT

Reversible postural orthostatic tachycardia syndrome

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Author contributions: Abdulla A made the diagnosis, performed the test to confirm the diagnosis and finalised the writing; Rajeevan T performed literature search and preliminary writing.

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Abstract

Postural orthostatic tachycardia syndrome (POTS) is a relatively rare syndrome recognised since 1940. It is a heterogenous condition with orthostatic intolerance due to dysautonomia and is characterised by rise in heart rate above 30 bpm from base line or to more than 120 bpm within 5-10 min of standing with or without change in blood pressure which returns to base line on resuming supine position. This condition present with various disabling symptoms such as light headedness, near syncope, fatigue, nausea, vomiting, tremor, palpitations and mental clouding, *etc*. However there are no identifiable signs on clinical examination and patients are often diagnosed to have anxiety disorder. The condition predominantly affects young female between the ages of 15-50 but is rarely described in older people. We describe an older patient who developed POTS which recovered over 12 mo. Recognising this condition is important as there are treatment options available to alleviate the disabling symptoms.

Key words: Postural; Orthostatic; Tachycardia; Dysautonomia; Hypotension; Postural tachycardia syndrome; Older person

Core tip: This is a short report and literature review on postural orthostatic tachycardia syndrome (POTS). POTS commonly affects younger patients and is rarely reversible. Here we describe an older patient who presented with disabling POTS which was reversed. Although rare, it is now being recognised in older people and increasing awareness among geriatricians is important as early diagnosis and treatment may alleviate the disabling symptoms. Reviewing the literature we argue whether hypotension should be considered as a feature of POTS.

Abdulla A, Rajeevan T. Reversible postural orthostatic tachycardia syndrome. *World J Clin Cases* 2015; 3(7): 655-660 Available from: URL: http://www.wjgnet.com/2307-8960/full/v3/i7/655.htm DOI: http://dx.doi.org/10.12998/wjcc.v3.i7.655

INTRODUCTION

Postural orthostatic tachycardia syndrome (POTS) is an orthostatic intolerance due to dysautonomia. This heterogeneous group of syndromes is characterised by rise in heart rate above 30 bpm from base line or to more than 120 bpm within 5-10 min of standing with or without change in blood pressure. This condition present with various disabling symptoms such as light headedness, near syncope, fatigue, nausea, vomiting, tremor, palpitations and mental clouding, *etc*. Various pathophysiological mechanisms have been recognised. It commonly affects younger patients and is rarely reversible. However it has been recognised in older people too. Recognising this condition is important as there are treatment options available to alleviate the disabling symptoms.

CASE REPORT

A 70-year-old woman presented with an 8 mo history of dizziness on standing and unsteadiness on her feet. More recently she experienced a fall and several near falls. She complained of nausea resistant to antiemetics and weight loss. She felt generally weak and had become dependent for all her activities of daily living. She had a brief hospital admission 10 mo prior to the current presentation and was investigated for the persistent nausea and vomiting. Upper gastrointestinal endoscopy, barium swallow and computed tomography scan of abdomen, pelvis and chest showed no identifiable pathology.

She had past medical history of polymyalgia rheumatica, hypertension and anxiety. She was on maintenance dose of prednisolone 1 mg, ramipril 2.5 mg and diazepam at night. She lived with her husband, previously independent for her activities of daily living, was a non smoker and consumed alcohol occasionally.

On assessment she appeared anxious and was lethargic. Her heart rate was 80 bpm regular. Blood pressure was 179/85 mmHg sitting and 119/73 mmHg on standing associated with rise in heart rate to120 bpm. She complained of dizziness on standing. Systemic examination including cardiovascular and neurological examinations was unremarkable.

Routine blood tests including thyroid function tests and random cortisol were normal. Twenty-four hours urine collections on 4 consecutive days for 5-hydroxyindoleacetic acid (5HIAA), cortisol, dopamine, epinephrine, norepinephrine and sodium were sent. 5HIAA excretion was elevated on 2 occasions to 49 and 99 micromol/d (normal 10-42) as was excretion of epinephrine to 190 nmol/d (normal 0-144). Elevation of catecholamines was possibly related to hyperadrenergic condition and significance of 5HIAA elevation was unexplained as it returned to normal in subsequent assays. Twelve lead electrocardiogram (ECG) and 24 h tape showed sinus rhythm. An echocardiogram showed signs of left ventricular hypertrophy. She underwent tilt table test which showed a rise in heart rate above 30 bpm from baseline associated with fall in blood pressure (Figure 1).

She was commenced on propranolol 10 mg tds and fludrocortisone at a starting dose of 50 mcg daily which was increased to 150 mcg. She responded remarkably well; her postural tachycardia resolved with improvement in symptoms of dizziness and nausea. She gradually regained her mobility and was able to resume her personal activities.

All the endocrine tests were repeated after 2 mo of her recovery of symptoms. All her baseline endocrine tests were normal at this point.

On follow up at 4 mo her blood pressure was 150/70 mmHg. She was slowly weaned off fludrocortisone with no recurrence of symptoms. Head up tilt test was repeated at 15 mo (Figure 2) which showed complete recovery and no change in heart rate or blood pressure with postural change.

DISCUSSION

POTS is a relatively rare syndrome recognised since 1940[1,2]. It is most often seen in women of child bearing age (between the ages of 15 and 50), nevertheless it may appear at any age. This is rarely described in older people and pathophysiology can be significantly different in older group of people. The mechanism depends on underlying pathology. It is not always easy to identify the mechanism therefore treatment can be difficult.

Standing results in approximately 0.5 to 1.0 L of blood pooling in the lower extremities and splanchnic circulation. A normal hemodynamic response to postural change requires normal function of the cardiovascular and autonomic nervous systems. An increase in sympathetic outflow, increases peripheral vascular resistance, venous return, and cardiac output, limiting the decrease in blood pressure. Normal compensatory mechanisms result in a decrease in systolic blood pressure (5 to 10 mmHg), an increase in diastolic blood pressure (5 to 10 mmHg), and an increase in pulse rate (10 to 25 beats per minute). Orthostatic hypotension is defined as a decrease in systolic blood pressure of 20 mmHg or a decrease in diastolic blood pressure of 10 mmHg within three minutes of standing compared with blood pressure from the sitting or supine position.

POTS is an orthostatic intolerance due to dysautonomia and is divided into primary and secondary types. It is important to identify the type to facilitate treatment. By definition the primary form occurs in the absence of an underlying condition. In contrast the secondary form of POTS is due to chronic conditions such as diabetes mellitus, sarcoidosis, connective tissue disorders, *etc*[3]. The condition runs a remitting and relapsing course.

This heterogeneous group of syndromes is characterised by rise in heart rate above 30 bpm from base line or to more than 120 bpm within 5-10 min of standing with or without change in blood pressure. In

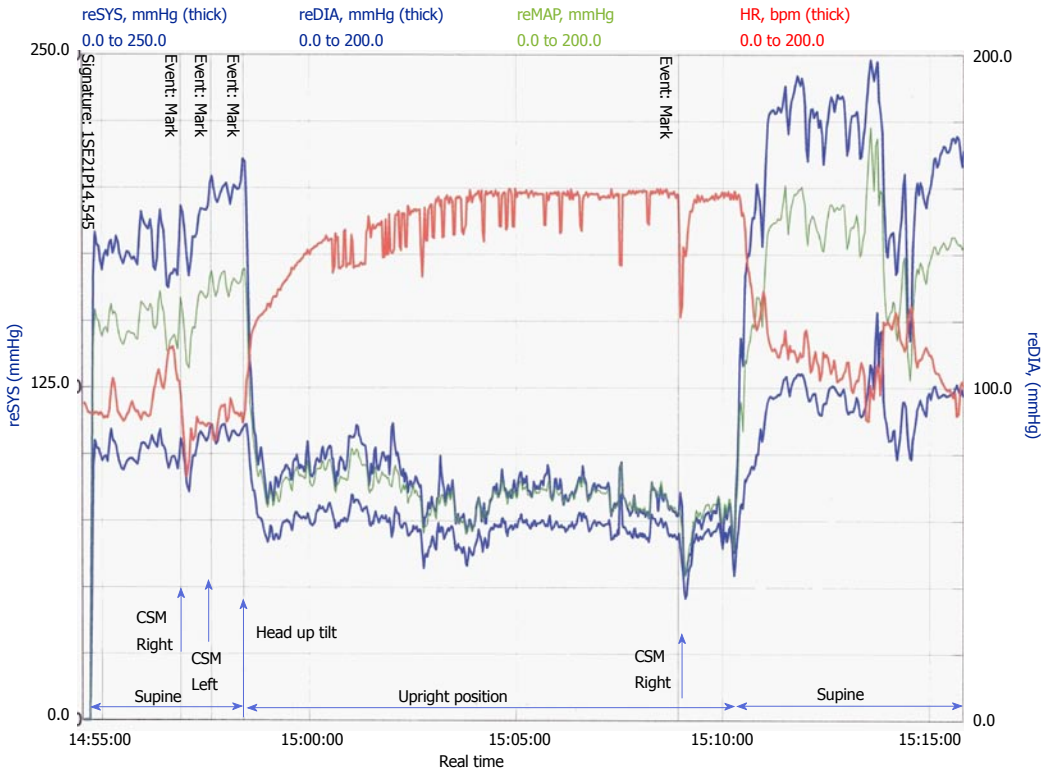

Figure 1 The significant increase in heart rate on head-up tilt (red line) associated with drop in systolic and diastolic blood pressure (in blue). Note the return of both heart rate and blood pressure on returning to supine position. MAP: Mean arterial pressure; reSYS (right Y-axis): Systolic blood pressure; reDIAS (left Y-axis): Diastolic blood pressure; HR: Heart rate; CSM: Carotid sinus massage.

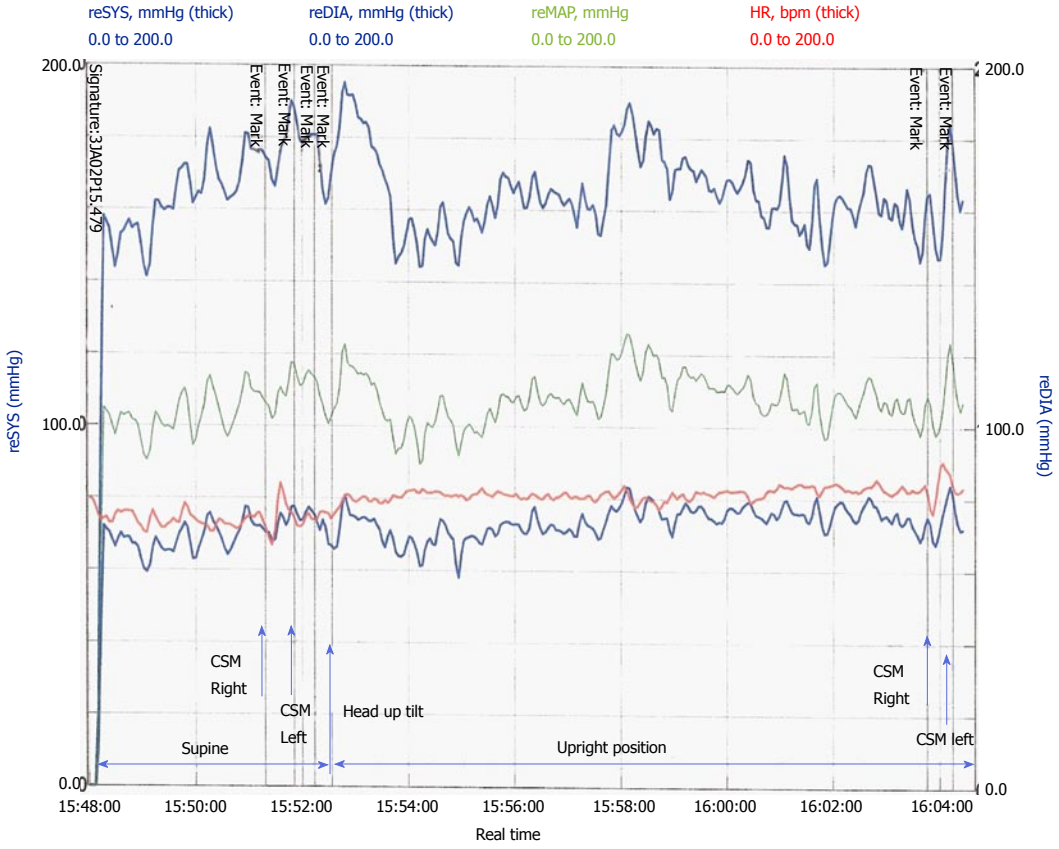

Figure 2 An essentially normal response with minimal change in heart rate on head-up tilt (red line) and non-significant alteration in blood pressure (in blue). CSM: Carotid sinus massage; reSYS (right Y-axis): Systolic blood pressure.

Table 1 Differentiating partial dysautonomic and hyperadrenergic forms

	Partial dysautonomia	Hyperadrenergic form
Frequency	Most common primary form	Less common primary form
Mechanism	Inability of peripheral vessels to constrict	Tachycardia due to elevated catecholamine
Onset	Abrupt	Gradual
Pathophysiology	Autoimmune mediated	Familial–single point mutation
Investigation	Serum acetylcholine receptor antibodies	Standing catecholamine level

fact some authors require no change in blood pressure on standing as a pre-requisite for the diagnosis of POTS but this is controversial[2,4-7]. Many of the mechanisms described in POTS such as vasodilatation, blunted response to angiotensin Ⅱ, low blood volume and red cell volume, abnormal vascular structure and venous capacitance can potentially drop the blood pressure. The drop in blood pressure due to above mechanisms may be compensated to a certain degree in POTS patients by disproportionately elevated heart rate though at times the compensation may be incomplete.

Pathophysiology

The various pathophysiological mechanisms involved in POTS are: (1) high level of standing norepinephrine level (due to reduced norepinephrine transporter expression resulting increased systemic norepinephrine spill over); (2) presence of ganglionic acetylcholine receptor antibodies; (3) alpha 1 adrenergic receptor denervation or hyposensitivity; (4) beta adrenergic super sensitivity; (5) peripheral autonomic denervation with preserved cardiac and cerebral innervations; (6) Partial renal sympathetic denervation leading to reduced renin/Aldosterone[8]; (7) increased angiotensin Ⅱ level with blunted responsiveness of receptors to angiotensin Ⅱ[9]; (8) Low blood volume and Red cell volume; (9) Abnormal vascular structure with impaired venous capacitance; and (10) Increased capillary permeability.

Not all of these mechanisms present in any one patient and treatment should be tailored accordingly. Symptoms are most likely due to cerebral hypoperfusion[10].

Types of POTS

As mentioned earlier POTS patients present with varying clinical features depending on the underlying pathology. Unifying feature is orthostatic intolerance which improves on lying or sitting down. The primary form is not associated with or caused by other chronic disorders. Here the onset is usually abrupt particularly when it occurs following viral illness, immunisation, pregnancy or surgery. An exception is the developmental form which occurs following a period of rapid growth[3]. In this case it runs a slow progress to reach a peak within 2 years.

Two major types of primary forms are identified. They are partial dysautonomic and hyperadrenergic forms (Table 1). Partial dysautonomic form is due to peripheral autonomic neuropathy which results in excessive pooling of blood in blood vessels of lower limbs and mesenteric circulation with the reflex tachycardia. Antibodies to ganglionic acetylcholine receptors are often found in patients with post viral autonomic neuropathy[3,11]. In contrast the hyperadrenergic form is usually a familial condition where there is rise in norepinephrine level on standing which causes the orthostatic tachycardia and orthostatic intolerance[12]. The rise in norepinephrine is due to reduced clearance secondary to poorly functioning reuptake transporter protein[6]. These patients suffer from profuse sweating, anxiety, tremulousness, tachycardia and hypertension[12,13].

Secondary forms are mainly due to chronic disorders such as diabetes mellitus, joint hyper mobility syndrome, sarcoidosis, systemic lupus syndrome, heavy metal poisoning and chemotherapies which affect the nervous system.

Symptoms

Various symptoms are described such as light headedness, near syncope, fatigue, nausea, vomiting, tremor, palpitations, mental clouding, *etc*. Symptoms can be brought on minimal exertion or activities such as eating, showering and walking short distance[2,5,14]. Both symptoms and tachycardia resolve with sitting or lying down. Patients are often diagnosed to have anxiety disorder[1,5].

Investigations

Head up tilt (HUT) is the investigation of choice although some studies suggest that standing haemodynamics is more specific[2,15]. It is important to rule out other conditions which cause tachycardia such as phaeochromocytoma, carcinoid, thyrotoxicosis, cardiac arrhythmia, *etc*. Tachycardia in these conditions is not related to change in posture.

Important investigations are blood tests which should include full blood count, renal function, thyroid function, calcium level, glucose, catecholamines on standing from supine position. Twenty-four hours urine collection for 5HIAA, catecholamines, sodium level are relevant investigations in POTS to rule out other causes of tachycardia and aim treatment options[12]. Routinely an ECG should be performed and further investigations such as 24 h tape and echocardiogram are carried out if indicated. The fact that this syndrome is not often recognised by clinicians, it leads to unnecessary investigations before the diagnosis is made especially in older people.

Management

Conservative: Review of medications which can aggravate POTS and appropriately stopping these medications; these include: (1) drugs that enhance vasodilatation-alpha adrenoreceptor blockers, angiotensin converting enzyme inhibitor (ACEI), calcium channel blockers and nitrates; (2) drugs that enhance tachycardia-beta adrenoreceptor stimulants, tricyclic antidepressants; and (3) drugs that worsens volume depletion-diuretics and ACEI.

Exercise - Aerobic exercise and lower limb resistance training will help pumping the blood. The intensity and duration of exercise should be built up gradually and also depend on patient's age.

Avoid salt and fluid depletion - Increasing salt and fluid take have great impact on reducing severity of symptoms. Fluid intake of 2 L/d and salt intake of 3-5 g/d is recommended. Performing 24 h urinary collection for urinary sodium level would help to identify the patients who would benefit from salt supplements. Studies show that patients with urinary excretion < 124 mmol/d is an indicator of good response to salt treatment[16].

Pharmacological treatment: Vasoconstrictors: Fludrocortisone is the most commonly used drug in orthostatic intolerance. Its action is mediated by improving peripheral sensitivity of alpha adrenoceptors, fluid and salt retention. Midodrine is an alpha-1 adrenoreceptor agonist not only increases the peripheral vascular resistance but also helps orthostatic intolerance by having an effect on heart rate. Other vasoconstrictors used with variable results are: (1) methylphenidate - increases vasoconstriction by increasing catecholamine release and inhibiting monoamine oxidase; (2) erythropoietin: increases the sensitivity of angiotensin II; (3) clonidine is a central sympatholytic and increases peripheral vascular resistance; and (4) octreotide: somatostatin analogue is potent vasoconstrictor.

Heart rate limiting drugs: Beta blockers are the main group of drugs and among them propranolol is favoured by clinicians. There are limited studies with regards to the dosage at which it is effective in treating POTS. Moderate dose of propranolol (20 mg) not only reduces heart rate but also improves symptoms, whereas higher dose (80 mg) is effective in reducing heart rate but does not improve symptoms. In fact it has been reported to worsen symptoms[17]. Other drugs which can reduce heart rate and alleviate symptoms are selective serotonin reuptake inhibitors (SSRI) and selective noradrenalin reuptake inhibitors. SSRI have been used for cardiogenic syncope and orthostatic hypotension. Serotonin plays and important role in central control of heart rate.

Ivabradine has effect on reducing the heart rate and symptom control.

Volume expanders: As mentioned earlier fludrocortisone is a mineralocorticoid and enhances the fluid and salt retention. Erythropoietin stimulates red cell production and increase the red cell mass and blood volume. Treatment with Erythropoietin is reserved for people with refractory symptoms in spite of other medications. Cost and administration by subcutaneous injection are the limiting factors for its use. Desmopressin increases the reabsorption of fluid from kidney, but its use in POTS has not been adequately studied.

Other medications: Pyridostigmine is an acetylcholine esterase inhibitor and is a very promising drug particularly for POTS following viral illness and POTS secondary to autoimmune process and paraneoplastic syndrome.

In conclusion, POTS is a condition characterised by orthostatic intolerance with excessive increase in heart rate due to disturbances in autonomic control. Patients with this condition suffer from numerous disabling symptoms with no specific abnormalities on clinical examination. Head up tilt test is the choice of investigation. Identifying the subtypes is the key to achieve successful management.

Our patient is a rare case of POTS in older people with full recovery.

Her symptoms were severe enough to warrant hospital admission. Medications were commenced once diagnosis was established with HUT and she made good recovery with her mobility and other symptoms.

COMMENTS

Case characteristics

A 70-year-old woman presented with dizziness and unsteadiness on feet.

Clinical diagnosis

Heart rate increased to more than 30 bpm from baseline on standing associated with fall in blood pressure.

Differential diagnosis

Postural hypotension and other conditions which causes tachycardia such as pheochromocytoma, thyrotoxicosis carcinoid syndrome and cardiac arrhythmia.

Laboratory diagnosis

Head up tilt test showed rise in heart rate from 80 to 120 bpm on standing with drop in blood pressure.

Imaging diagnosis

Chest X-ray and computerised tomography of abdomen, pelvis and chest were normal.

Treatment

The patient was treated with fludrocortisone and propranolol.

Related reports

Postural orthostatic tachycardia syndrome (POTS) is described predominantly in young females and to our knowledge this is the first case reported in older person.

Term explanation

Head up tilt test is the tilt-table test involves placing a patient on a flat table with a foot support, then tilting the table upward for a period of time to observe

changes in blood pressure and heart rate.

Experiences and lessons

POTS is a rare disabling condition; but is treatable when diagnosis is established which needs high index of suspicion.

Peer-review

This is an interesting case report.

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CASE REPORT

Arteriovenous malformation of the vestibulocochlear nerve

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Author contributions: Tucker A and Tsuji M contributed to conception and design; Tucker A contributed to acquisition of data; Tucker A, Tsuji M and Yamada Y contributed to analysis and interpretation of data; Tucker A contributed to drafting the article; Tucker A and Yamada Y contributed to critically revising the article; all authors reviewed submitted version of the article; Tucker A approved the final version of the manuscript on behalf of all authors.

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Abstract

We describe a rare case of an arteriovenous malformation (AVM) embedded in the vestibulocochlear nerve presenting with subarachnoid hemorrhage (SAH) treated by microsurgical elimination of the main feeding artery and partial nidus volume reduction with no permanent deficits. This 70-year-old woman was incidentally diagnosed 4 years previously with two small unruptured tandem aneurysms (ANs) on the right anterior inferior cerebral artery feeding a small right cerebellopontine angle AVM. The patient was followed conservatively until she developed sudden headache, nausea and vomiting and presented to our outpatient clinic after several days. Magnetic resonance imaging demonstrated findings suggestive of early subacute SAH in the quadrigeminal cistern. A microsurgical flow reduction technique *via* clipping between the two ANs and partial electrocoagulation of the nidus buried within the eighth cranial nerve provided radiographical devascularization of the ANs with residual AVM shunt flow and no major deficits during the 2.5 year follow-up. This is only the second report of an auditory nerve AVM. In the event of recurrence, reoperation or application of alternative therapies may be considered.

Key words: Arteriovenous malformation; Flow reduction; Microsurgery; Subarachnoid hemorrhage; Vestibulocochlear nerve

Core tip: Arteriovenous malformations (AVMs) originating within or impinging on cranial nerves are extremely rare, and there is an increased risk of hemorrhage in

AVMs associated with aneurysms. The authors describe the second report of a patient with a vestibulocochlear nerve AVM who presented with subarachnoid hemorrhage. A discussion of the delicate diagnostic and therapeutic implications is presented.

Tucker A, Tsuji M, Yamada Y, Hanabusa K, Ukita T, Miyake H, Ohmura T. Arteriovenous malformation of the vestibulocochlear nerve. *World J Clin Cases* 2015; 3(7): 661-670 Available from: URL: http://www.wjgnet.com/2307-8960/full/v3/i7/661.htm DOI: http://dx.doi.org/10.12998/wjcc.v3.i7.661

INTRODUCTION

Arteriovenous malformations (AVMs) originating within or impinging on cranial nerves are extremely rare, most of which have been identified in the the optic chiasma[1-6] or trigeminal nerve[7-24]. Posterior fossa AVMs account for only 7%-15% of all intracranial AVMs, however these lesions tend to involve greater morbidity and mortality and recent studies have shown an independent association between infratentorial AVM location and hemorrhagic presentation[25]. Moreover, there is an increased risk of hemorrhage in AVMs accompanied by aneurysms (ANs)[26].

Based on a search of the available literature, the authors found only one report of an intrinsic auditory nerve AVM[27], however the patient suffered a total hearing deficit. Herein we describe a rare case of an AVM embedded in the vestibulocochlear nerve presenting with subarachnoid hemorrhage (SAH) treated initially by microsurgical elimination of the main feeding artery and partial nidus volume reduction with no permanent deficits.

CASE REPORT

This 70-year-old right-handed woman with an unremarkable past medical history was incidentally diagnosed 4 years prior with two small tandem ANs on the right anterior inferior cerebral artery (AICA) feeding a small right cerebellopontine angle AVM. The patient was referred to our hospital for further evaluation and treatment and despite informed consent regarding the potential risks of subsequent hemorrhage and recommendations for microsurgery, Gamma Knife stereotactic radiosurgery, or endovascular embolization, or other interventions, she and her family chose conservative outpatient observation.

Digital subtraction angiography (DSA) was recommended for primary evaluation, however based on the invasiveness, potential complications, and inconvenience, she declined this modality in favor of three-dimensional computed tomography angiography (3D-CTA) and magnetic resonance angiography (MRA) for initial diagnostic evaluation followed by biannual and thereafter annual serial magnetic resonance imaging (MRI) (Figure 1). Dedicated informed consent was routinely offered to the patient and family including an explanation of the cumulative bleeding risks and recommendations for aggressive surgical or alternative treatment options. However the patient declined treatment and was followed on an outpatient basis until she presented to our outpatient clinic with a complaint of severe headache, nausea and vomiting of several days duration. The neurological exam was unremarkable with no apparent cranial nerve deficits. Head computed tomography (CT) did not demonstrate any evidence of bleeding, however, high intensity signals only in the quadrigeminal cistern on T1-weighted MRI were observed, suggesting early subacute SAH (Figure 2). Fluid attenuated inversion recovery imaging was not performed. MRA source imaging and magnetic resonance cisternography demonstrated a small (11.4 mm × 4.2 mm) relatively compact nidus located in the cerebellopontine angle around the cisternal portion of the facial and vestibulocochlear nerves (cranial nerves Ⅶ and Ⅷ, respectively) with extension into the internal auditory canal (Figure 3). On the arterial phase of DSA the right AICA was identified as the predominant feeder with drainage into the right petrosal vein, superior petrosal sinus, and cavernous sinus (Figure 4). Based on the lack of any significant cranial nerve deficits, the occurrence of either minor AVM leakage, hypertensive venous bleeding, or subarachnoid hemorrhage from AN rupture was postulated. Because of the symptomatic nature of this Spetzler-Martin grade Ⅲ AVM (S1V1E1) with two feeding artery aneurysms, and after a comprehensive explanation of the risks and benefits of the various treatment options, including the risks of microsurgical neurological sequelae, the persistent delayed risk of bleeding following radiation therapy, and the possible risk of recurrence or other complications following endovascular embolization, the patient and family agreed to a recommendation of microsurgical treatment.

Surgical procedure

The patient was placed in the right lateral oblique position and a right retrosigmoid craniotomy was performed. After exposing the right lateral cistern and cerebellopontine angle, the right facial and vestibulocochlear nerves (cranial nerves Ⅶ and Ⅷ, respectively) were identified followed by confirmation of the right lower cranial nerves (cranial nerves Ⅸ, Ⅹ, and Ⅺ), the right AICA, proximal basilar-AICA bifurcation, and surrounding trigeminal and abducens nerves (cranial nerves Ⅴ and Ⅵ, respectively). The right AICA was determined to be the predominant feeder supplying a small peripheral nidus buried within the right cranial nerve Ⅷ at the meatus of the internal auditory canal with venous drainage into an engorged red petrosal vein (Figure 5A and B). Close inspection revealed a mid-sized fusiform AN at the proximal AICA

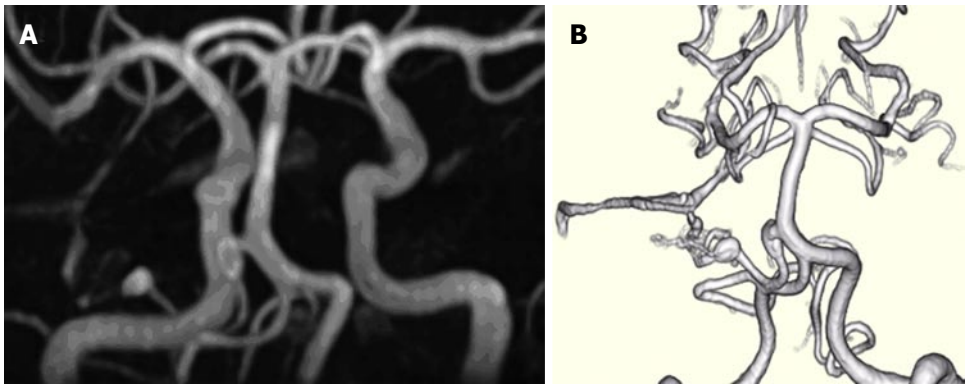

Figure 1 Three-dimensional time-of-flight magnetic resonance angiography. A: Three-dimensional time-of-flight magnetic resonance angiography (3D-TOF-MRA) four years prior to admission; B: Volume rendering 3D-TOF-MRA study 1 year prior to admission, demonstrating two unruptured prenidal feeder artery aneurysms, one 8 mm × 3 mm fusiform proximal anterior inferior cerebral artery (AICA) aneurysm near the takeoff of the basilar artery and one 4.5 mm × 3 mm elliptical distal pedicle AICA aneurysm located at the entrance to the main inflow area of a small inconspicuous vascular nidus.

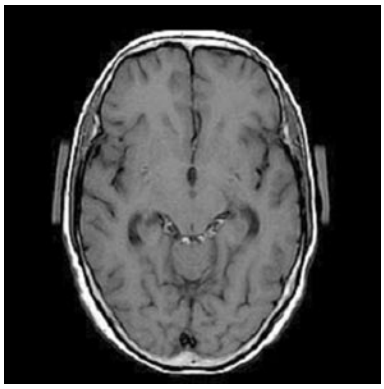

Figure 2 On admission, this 70-year-old woman presented with a severe headache, nausea and vomiting of several days duration. T1-weighted magnetic resonance imaging demonstrated high intensity signals only in the quadrigeminal cistern, suggestive of early subacute subarachnoid hemorrhage.

near the takeoff from the basilar artery and a small distal pedicle feeding artery AN at the inflow area of the nidus. Xanthochromic cerebrospinal fluid was observed with a slight predominance near the proximal AICA AN, however there was no clear evidence of a rupture point from either of the ANs, nidus, or venous drainers. Based on the preoperative imaging studies, the nidus was suspected to extend into the auditory canal, and complete resection was not attempted because of the anticipated high risk of auditory nerve injury. Instead a feeder clip was applied to the AICA which demonstrated normalization of color from red to blue in the right petrosal vein. However, intraoperative microvascular doppler ultrasonography and indocyanine green video angiography confirmed the presence of persistent flow within the AVM despite temporary clipping, suggesting the existence of collateral supply from the external carotid artery system or other sources. Definitive clipping between the proximal and distal AICA ANs followed by partial electrocoagulation of both the nidus and the predominant entry area to the nidus were performed (Figure 5C) with the intention of alleviating hemodynamic stress on the remaining nidus and proximal AN. Figure 6 shows an overall intraoperative schematic illustration. Although auditory brainstem response monitoring remained normal throughout all procedures, preventative intravenous corticosteroids were administered postoperatively.

Postoperative course

The patient had an uneventful postoperative course with follow-up clinical and head CT examinations every 2 to 3 mo for a period of 2.5 years. Biannual follow-up outpatient MRI and MRA source imaging during that period showed clip artifact at the entrance to the internal auditory canal with apparent disappearance of proximal and distal right AICA aneurysms, and a prominent right superior petrosal sinus, indicative of residual shunt flow (Figures 7 and 8 compare preoperative and postoperative changes). The patient experienced a transient right hearing deficit, diplopia, and vertigo which almost completely resolved on discharge. More extensive diagnostic imaging and treatment options including Gamma Knife radiosurgery were under consideration pending the onset of radiological regrowth or symptomatic recurrence.

DISCUSSION

Intrinsic AVMs limited exclusively to the cranial nerves are extremely uncommon although there have been many reports of AVM rupture resulting in damage to nearby cranial nerves or indirect feeder compression of cranial nerves with associated symptoms manifesting in various conditions such as optic nerve apoplexy[1-6], oculomotor nerve ophthalmoplegia[28], trigeminal neuralgia[7,8,10-18,20,21,23,24], hemifacial spasm[29,30], glossopharyngeal neuralgia[31], and hypoglossal nerve paresis[32]. Several rare reports of AVMs originating from within the trochlear nerve[33] and trigeminal nerve[9,19,22] have also been documented. Maher *et al*[19] proposed an embryological basis for explaining the occurrence of AVMs arising in cranial nerves such as the trigeminal nerve, whereby normally migrating fetal axons carry

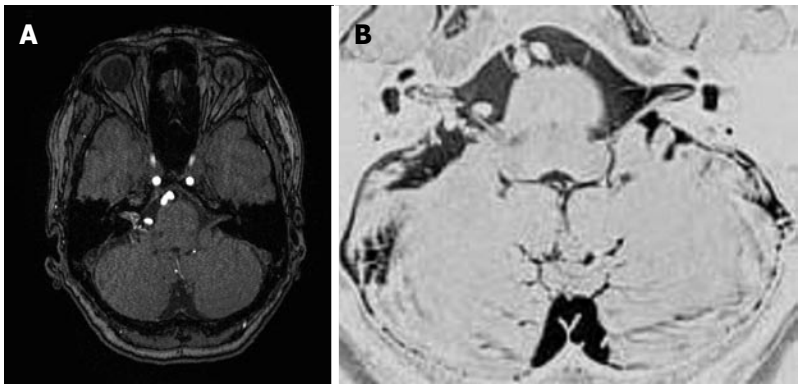

Figure 3 Magnetic resonance angiographic source imaging (A) and magnetic resonance cisternography (B) reveal a small (11.4 mm × 4.2 mm) relatively compact nidus located in the cerebellopontine angle at the cisternal portion of the cranial nerves VII and VIII with extension into the internal auditory canal.

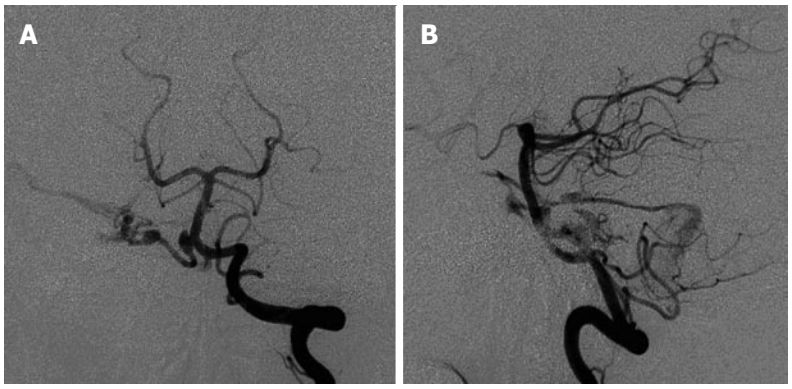

Figure 4 Anterioposterior (A) and lateral (B) views of cerebral angiogram for this patient showing the right anterior inferior cerebral artery as the predominant feeder supplying a small nidus with drainage into the right petrosal vein, superior petrosal sinus, and cavernous sinus [Spetzler-Martin grade III (S1V1E1)].

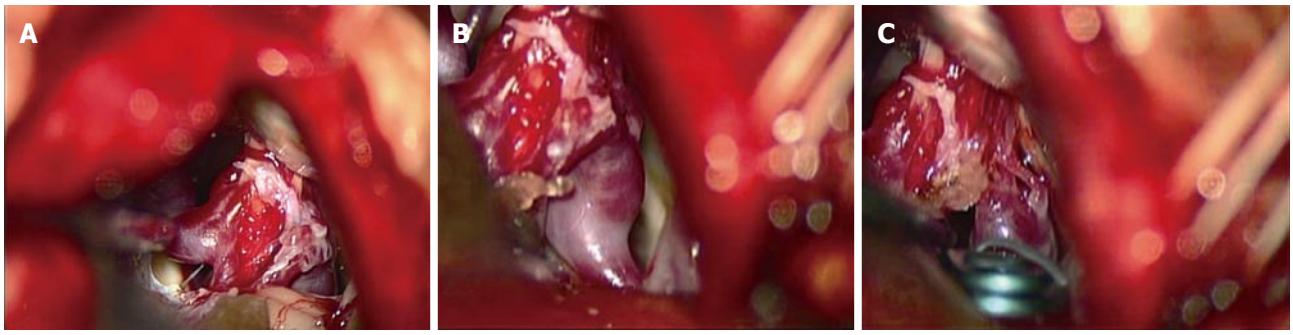

Figure 5 Intraoperative microscopic view. A: Intraoperative microscopic view showing a right retrosigmoid craniotomy in the a right lateral oblique position which exposed a small nidus buried within the right cranial nerve VIII at the meatus of the internal auditory canal; B: View showing distal right anterior inferior cerebral artery feeding aneurysm, nidus-VIII cranial nerve complex, and draining petrous vein; C: Definitive clip application proximal to the aneurysm and partial electrocoagulation of the nidus and the inflow area just proximal to the nidus was performed.

precursor AVM cells toward an ectopic cranial nuclei location during the 5th to 6th weeks of embryogenesis.

To date, there has been only one case report of an AVM located within the auditory nerve bundle which presented with SAH yet this case involved complete sensorineuronal hearing loss[27]. Because of the severe functional deficit from onset in that patient, total removal of both the nidus and nerve remnant were performed. Interestingly, in many of the cases of intrinsic trigeminal or trochlear AVMs as well as in our case, the patients presented with symptoms unrelated to cranial nerve dysfunction from direct AVM rupture[19,33], or symptoms were not caused by the nidus itself but by neural compression from surrounding vessels such as the feeding superior cerebellar artery[17,18,20,22] or impingement from other vessels at the trigeminal nerve

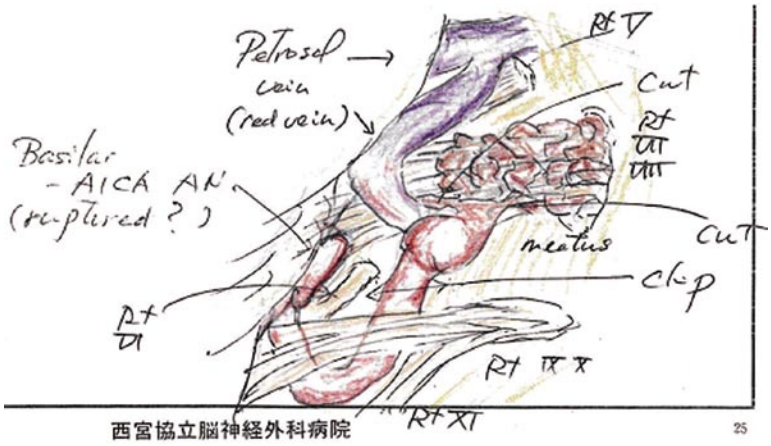

Figure 6 Intraoperative schematic illustration depicting anatomical structures and definitive clipping between the proximal and distal feeding anterior inferior cerebral artery aneurysms and partial electrocoagulation of both the nidus and the main inflow area to the nidus.

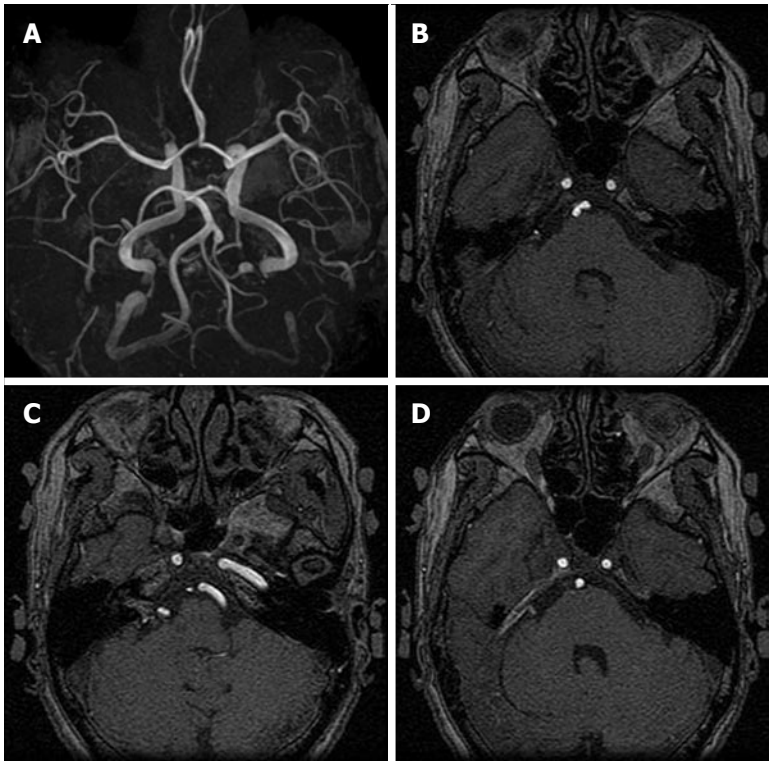

Figure 7 Preoperative three-dimensional time-of-flight magnetic resonance angiography showing proximal and distal right anterior inferior cerebral artery aneurysms (A), and magnetic resonance angiographic source imaging showing proximal right anterior inferior cerebral artery aneurysm (B), distal right anterior inferior cerebral artery aneurysm with relatively strong internal auditory canal nidus signal intensity (C), and right superior petrosal sinus venous shunt (D).

root entry zone[15,16]. In a study by Sumioka *et al*[22] a review of over 600 cases of trigeminal neuralgia were identified to be caused by vascular compression while only a small minority showed no evidence of vascular involvement. In these non-vascular cases, symptoms of trigeminal neuralgia were postulated to be derived from either the twisting or "tilting" by mechanical compressive forces, thickened arachnoid, or the direct effects of embedded AVMs.

In addition, many of the cases of chiasmal or optic nerve apoplexy due to intrinsic AVMs (including cavernous malformations) were typically diagnostically labeled as angiographically occult and only after performing high-resolution MRI were these cryptic micro-AVMs identified[3]. MRI has also been considered to offer a considerable advantage over standard angiography both for reducing complications and procedural related morbidity as well as providing detailed delineation of neurovascular structural relationships crucial for making treatment decisions during the preoperative and postoperative periods[34]. Moreover, in a recent report of the largest surgical series of radiological and clinical follow-up of AVM recurrence after surgery, MRI with and without contrast administration was

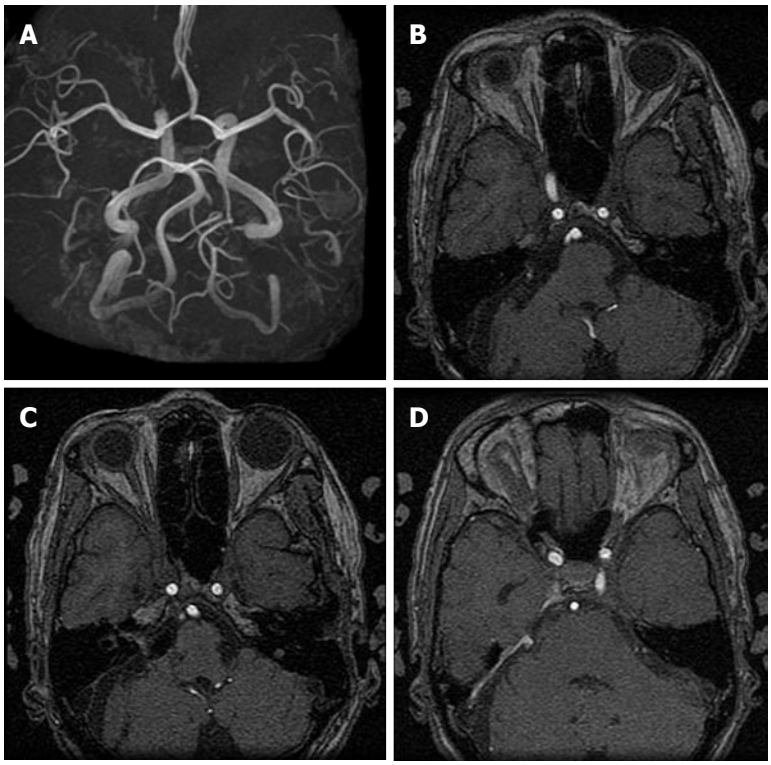

Figure 8 Postoperative three-dimensional time-of-flight magnetic resonance angiography (3D-TOF-MRA) and magnetic resonance angiographic source imaging showing anterior inferior cerebral artery (AICA) stump with disappearance of proximal and distal right AICA aneurysms (A, B), decreased intensity of right AICA and seemingly disappearance of proximal aneurysm, clip artifact at the entrance to the internal auditory canal and decreased intensity of intracanicular nidus (C), and persistence of prominent right superior petrosal sinus venous shunt (D).

recommended as the initial method for monitoring recurrence, with subsequent performance of digital subtraction angiography only in the event of suspected recurrence[35].

Likewise, a review of the recent literature seems to indicate a more palliative, minimally invasive trend for the treatment of cranial nerve AVMs. Sumioka *et al*[22] have suggested that the goal of treating intrinsic AVMs of the trigeminal nerve is generally symptomatic treatment, with prevention of rebleeding and preservation of cranial nerve function. Although treatment of cranial nerve cavernous malformations has been shown to be relatively successful with a lack of major associated complications to the surrounding neurovascular structures, direct microsurgical AVM resection in and around the optic nerve as well as other cranial nerves has typically been associated with significant damage to the nerve itself as well as surrounding microvasculature structures[9,18,22,23]. Indeed Sasagawa *et al*[5] have suggested that curative surgical treatment of optic nerve AVMs is considered nearly impossible, while other reports have shown that microsurgery can be used diagnostically as well as theraputically *via* limited surgical treatment such as optic nerve decompression or by subtotal operation for prevention of AVM growth or rebleeding[1]. As an example, an 8-year-old boy who presented with optic nerve apoplexy was found to have an AVM of the optic nerve and chiasm, but was treated by a diagnostic craniotomy alone without resection[5]. In another case of trigeminal neuralgia caused by an intrinsic trigeminal nerve AVM, complete resection of the AVM was judged to be too risky and only partial microsurgical nidus coagulation, transposition of the offending superior cerebellar artery, and subsequent Gamma Knife radiosurgery was performed for eventual complete radiological obliteration[22].

In our case the choice of treatment took into consideration both the angioarchitectural and clinical features as well as patient preference for a less aggressive option involving minimal risk. Due to the presence of symptomatic SAH presumed to be related to AN rupture and based on the high risk of nerve injury from complete AVM extirpation, a more palliative flow reduction strategy of feeder artery electrocoagulation at the inflow area of the nidus, effective feeder AN trapping, and partial electrocoagulation of the nidus was performed. Although intraoperative findings showed a slight predominance of xanthochromic cerebrospinal fluid near the proximal AICA AN, which was suggestive of having a higher likelihood of being the causative source of bleeding, because of the risk of damaging the abundant small perforators in the basilar-AICA takeoff area, a more distal obliteration point closer to the main inflow region of the AVM was chosen. Despite the seemingly unconventional nature of this method, in principle, we preliminarily followed standard microsurgical AVM

treatment protocol including coagulation of the major superficial feeding artery at a location as close as possible to the point of feeder entry into the nidus[34]. And according to current recommendations for treatment of coexisting intracranial ANs, we attempted to diminish the hemodynamic stress by obliteration of high flow shunting, a variation on a technique which has been shown to result in either regression or disappearance of the prenidal AN[34,36]. However, by placing a clip distal to the proximal AN, there could be a risk of subsequent increased hemodynamic stress and hemorrhage for this aneurysm, especially due to limited distal outflow. Careful follow-up diagnostic studies and consideration of endovascular or other treatment modalities would be recommended. Finally, the observation of a temporary postoperative hearing disturbance may possibly be explained by intraoperative manipulation of the cochlear nerve. However, the possibility of vascular compromise with compensatory extracarotid flow could explain the recovery in hearing, yet this mechanism would be expected to occur over a longer period of time.

Classically there has been caution regarding the potential increased risks of bleeding from incomplete, partial, or subtotal AVM resection[8], as well as recent concerns of bleeding after palliative embolization[34]. In contrast, embolization designed to reduce flow and decrease risk of rupture has been indicated in various conditions, including treatment of associated ANs and large inoperable AVMs, or "inoperable" vascular lesions with recurrent hemorrhage, or locations in eloquent regions, where multimodality approaches of preoperative embolization combined with microsurgical extirpation have been considered. However, there is no evidence in support of such empirical methods and ultimately treatment must be individualized[34,37]. Recently, the Barrow vascular group has found moderate success for treatment of vertebrobasilar aneurysms *via* low-flow bypass and flow reduction *via* complete or partial basilar artery vessel occlusion or distal vertebral artery occlusion[38]. Although our procedure as well as the angioarchitecture of the lesions differed in many ways from the technique used in that study, both lesions resided near critical neurovascular structures and in principle, both involved application to some degree of a procedure designed to reduce flow to the inflow vessel of the aneurysm. Another study from the same institution achieved satisfactory long-term outcome using a technique of subtotal nidus resection for effective devascularization of glomus spinal AVMs (lesions known to have similar angioarchitectural features as found in intracranial AVMs)[39], a technique similar in some ways to our technique of partial nidus electrocoagulation. And a recent pooled analysis of treatment outcomes for spinal glomus AVMs has shown that hemorrhagic risk can be significantly reduced by partial endovascular treatment[40]. In this sense, although the ultimate goal of surgical treatment is AN elimination and AVM extripation, care can be tailored according to the individual lesion, whereby a less invasive, more palliative treatment option can be employed as the initial preemptive strategy.

Radiation therapy as a primary treatment for asymptomatic brain AVMs or as an adjunctive therapy for recurrent AVMs has been advocated for treatment of lesions smaller than 3 cm in diameter and in deep or eloquent brain areas depending on size, angioarchitecture, and other factors, greater than 1- to 3-year obliteration rates range from 60% to 90%[34,41,42]. Moreover, radiosurgery for brainstem AVMs can attain up to 88% complete obliteration[43] and other reports claim complete obliteration rates in approximately two thirds of patients with a 1.7% annual hemorrhage rate for the first 3 years[44]. However, well known drawbacks include a 2.5% to 10% annual hemorrhage risk until complete obliteration, the potential for delayed hemorrhage after angiographically proven obliteration, and depending on location, there is a reported risk of clinically significant complications directly from radiosurgery for AVMs in excess of 3% to 6%, especially in cases of brainstem and other deep seated AVMs[34,43-45]. Radiosurgery for AVMs of the optic nerve and surrounding areas have been reported to cause optic neuropathy and injury to nearby neurovascular structures and consequently recommendations for maximal doses have been limited to less than 8 Gy[46]. And a study of Gamma Knife surgery for pituitary adenomas reported a 4.1% incidence of new visual dysfunction[47]. However, stereotactic radiosurgery for trigeminal neuroma presenting with trigeminal neuralgia has been shown to achieve symptomatic control without additional deficits[48]. Finally, there have been recommendations for other alternative management strategies including endovascular embolization for preoperative reduction of arterial inflow, occlusion of deep feeders, and prenidal aneurysm treatment, however, embolization in general has been associated with relatively high rates of morbidity and mortality (0% to 27%), mostly because of inadvertent embolization of feeding arteries or draining veins in normal surrounding neurovascular structures or post-embolization bleeding due to changes in flow dynamics[34]. Recently, primary embolization treatment with Onyx has shown total AVM obliteration rates of approximately 50%[49].

Major limitations of our study include a relatively brief 2.5 year follow-up in a single case report. Furthermore, we did not perform confirmatory follow-up DSA for verifying persistent patency, degree of alternative feeders, or other evidence of recurrence, however, this was a well informed decision in respect of the patient's ongoing preference for less invasive imaging modalities such as MRI which as mentioned, has been shown to be useful for therapeutic decision making during both the preoperative and postoperative periods as well as for identification of cryptic angiographically occult AVMs. However, as in our case, follow-up MRI showed diminished AICA and nidal flow, with persistence of venous shunt flow. Indeed, mere MRI or MRA confirm-

ation may only show relative flow reduction or stasis in flow and may not accurately represent actual residual or persistent vascular supply. In addition, clip artifact on MRI may prevent accurate assessment, while other testing such as 3D-CTA or DSA, may provide a more definitive understanding of the angioarchitecture. Standard follow-up DSA and application of additional therapy were offered to the patient, but the patient declined and ultimately application of such modalities were considered to be implemented on an as-needed basis in the event of suspected development of recurrence. We surmised that either thrombosis or diminished flow resulted in apparent radiological devascularization of the ANs and no associated clinical recurrence, however, the persistence of AVM shunt flow on follow-up MRI indicates that there is a continued risk of bleeding, although perhaps much less risk than without feeder artery and AN clipping. Furthermore, persistent external carotid artery or other feeding sources, or the potential for added changes in flow dynamics from our treatment could lead to subsequent AVM regrowth or bleeding, bleeding from the proximal basilar-AICA takeoff AN, or other *de novo* AN development. Currently the patient is symptom free and continues to express no interest in invasive testing or further treatment with Gamma Knife radiosurgery or other modalities.

ACKNOWLEDGMENTS

The authors deeply thank Mrs Kumiko Enomoto for providing administrative, technical, and material support.

COMMENTS

Case characteristics

A 70-year-old woman with a history of two incidental aneurysms (ANs) on the right anterior inferior cerebral artery (AICA) feeding a small right cerebellopontine angle arteriovenous malformation (AVM) presented with sudden headache, nausea and vomiting.

Clinical diagnosis

The neurological exam was unremarkable with no apparent cranial nerve deficits.

Differential diagnosis

Although admission head computed tomography did not demonstrate any evidence of bleeding, T1-weighted magnetic resonance imaging showed high intensity signals only in the quadrigeminal cistern, suggesting early subacute subarachnoid hemorrhage, and based on the lack of any significant cranial nerve deficits, the occurrence of either minor AVM leakage, hypertensive venous bleeding, or subarachnoid hemorrhage (SAH) from AN rupture was postulated.

Intraoperative diagnosis

The AICA was determined to be the predominant feeder supplying a small peripheral nidus buried within the right cranial nerve VIII (vestibulocochlear nerve) at the meatus of the internal auditory canal with a mid-sized fusiform AN at the proximal AICA near the takeoff from the basilar artery and a small distal pedicle feeding artery AN at the inflow area of the nidus, and although xanthochromic cerebrospinal fluid was observed, there was no clear evidence of a rupture point from either of the ANs, nidus, or venous drainers.

Treatment

The patient was treated initially by microsurgical elimination of the main feeding artery via clipping between the proximal and distal AICA ANs, followed by partial electrocoagulation of both the nidus and the predominant entry area to the nidus with the intention of alleviating hemodynamic stress on the remaining nidus and proximal AN.

Related reports

The authors describe the second report of a patient with a vestibulocochlear nerve AVM who presented with subarachnoid hemorrhage. Curative treatment of AVMs arising within delicate cranial nerve structures is difficult and fraught with a paucity of surgical evidence.

Term explanation

Flow reduction is an indirect less definitive treatment typically performed by proximal occlusion of the parent artery designed to alleviate hemodynamic stress on distally located ANs or AVMs and other potential hemorrhagic lesions.

Experiences and lessons

After dedicated informed consent, the patient in this case report chose less invasive testing and flow reduction treatment which resulted in no symptoms or permanent deficits throughout the 2.5 year follow-up, however despite radiographical devascularization of the ANs, residual AVM shunt flow and the potential for added changes in flow dynamics could lead to subsequent AVM or AN regrowth or bleeding.

Peer-review

The authors report on a rare case of an arteriovenous malformation of the vestibulocochlear nerve. The case was managed with microsurgery with only transient deficits noted.

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CASE REPORT

Conservative management of type 2 gallbladder perforation in a child

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Abstract

Gallbladder perforation (GBP) is a rare but serious complication of cholecystitis and needs to be managed promptly. Acalculus cholecystitis leading to GBP is frequently associated with enteric fever and found in critically ill patients, and a surgical approach is not always feasible in such patients. Use of percutaneous tube cholecystostomy (PTC) in such patients is a known entity but it is usually followed by interval cholecystectomy. Here we report a case of perforated gallbladder in a child managed conservatively and successfully with PTC as the definitive treatment wherein cholecystectomy was avoided. The functionality of the gallbladder was confirmed by a Tc99m-HIDA scan.

Key words: Spontaneous; Gallbladder; Perforation; Percutaneous tube cholecystostomy

Core tip: Percutaneous cholecystostomy for selected patients with gallbladder perforation or distended gallbladder with symptoms is a good technique to tide over the acute crisis and may even avert the need for cholecystectomy.

Dikshit V, Gupta R, Kothari P, Gupta A, Kamble R, Kesan K. Conservative management of type 2 gallbladder perforation in a child. *World J Clin Cases* 2015; 3(7): 671-674 Available from: URL: http://www.wjgnet.com/2307-8960/full/v3/i7/671.htm DOI: http://dx.doi.org/10.12998/wjcc.v3.i7.671

INTRODUCTION

Gallbladder perforation (GBP) is rare in children and is seen as a complication of cholecystitis. Gallbladder stone disease is the most frequent cause of acute cholecystitis and acalculus cholecystitis is seen in only 5%-10% of cases[1]. The majority of the reported cases of GBP are associated with enteric fever. High level of

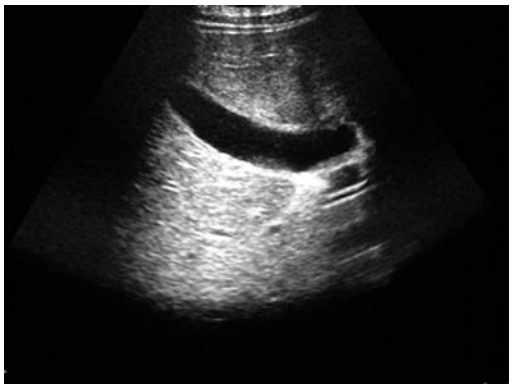

Figure 1 Over-distended gallbladder.

suspicion, early diagnosis and prompt management are of paramount importance in dealing with this entity.

CASE REPORT

A 12-year-old male patient admitted on the medical side was referred to us with complaints of pain in the abdomen for 12 d. The pain was dull aching in nature, more in the right hypochondrium. He had a history of 7-8 episodes of vomiting, gastric in nature with mild fever and headache.

The child was febrile and had tachycardia. There was no icterus or features of liver failure. The abdomen was distended and there was guarding in the right hypochondrium without any rigidity. The liver was palpable 2 cm below the costal margin and the gallbladder was felt as a firm, globular and tender mass.

Complete haemogram, bilirubin, liver enzymes, prothrombin time and renal function tests, were within normal limits. Widal test and blood culture were negative. An erect X-ray of the abdomen was suggestive of gaseous distention of bowel loops. Ultrasonography (USG) on admission was suggestive of a hugely distended gallbladder with echogenic sludge within and mild hepatosplenomegaly. The patient was being treated conservatively and a follow-up USG was done 2 d later for persistence of pain. This time sonography revealed an over-distended (13 cm) gallbladder with perforation at the tip with a narrow tract and small volume of sealed-off collection with duodenal wall thickening and edema (Figure 1).

With these findings the patient was transferred to us and underwent a contrast-enhanced computed tomography (CT) scan of the abdomen which confirmed the USG findings (8.5 mm sized rent seen in the gallbladder wall with mild peri-gallbladder collection 4.2 cm × 2.3 cm × 2.2 cm suspected of sealed-off gallbladder perforation. No free fluid was noted in the abdomen) (Figure 2). USG guided percutaneous cholecystostomy using an 8-Fr pigtail catheter was done and around 150 mL of bile was drained (Figure 3).

Bile culture showed Enterobacter species sensitive to piperacillin and amikacin. The patient received intravenous piperacillin at 250 mg/kg per day for 10 d and amikacin at 15 mg/kg per day for 5 d. Daily bile output through the cholecystostomy tube was around 120 to 140 mL. There was improvement in the patients' general condition with resolution of fever and abdominal pain.

One week after the procedure bile culture showed no growth and liver function tests (LFTs) were normal. Tube cholecystogram showed free passage of contrast into the bowels (Figure 4), hence, intermittent clamping of the pigtail was started with monitoring for fever, pain and change in LFTs. Clamping trial after 4 d was successful and the pigtail was removed on post procedure day 13.

The patient was discharged symptom free and a Tc-99m Mebrofinin (HIDA) scan done 1 mo later showed morphologically normal liver with normal hepatic function and hepatobilliary drainage (Figure 5). At follow-up after 1 year of the episode, the patient remained to be asymptomatic.

DISCUSSION

GBP is a rare complication of acute cholecystitis (2%-11%)[1,2] and is more often seen in patients having critical illness like severe trauma, burns and cardiovascular surgeries. Compared with adult population GBP is even rarer in children and is mainly due to acalculus cholecystitis, trauma, enteric fever, gallbladder wall necrosis due to sepsis or sometimes it may occur spontaneously[3,4].

The most common part of the gallbladder to perforate is the fundus followed by the body, with the reason being attributed to poor blood supply[1]. The majority of the cases of cholecystitis followed by perforation are seen in gallbladder stone disease where the cystic duct often gets occluded, leading to retention of secretions and rise in the intraluminal pressure. Acalculus cholecystitis is seen in 5%-10% of patients with acute cholecystitis[1] and may lead to perforation as seen in our case.

GBP may be traumatic, iatrogenic or idiopathic (spontaneous) and was classified by Niemeier[5] into 3 types. Type 1 (acute) is associated with generalized biliary peritonitis; type 2 (subacute) consists of localized fluid collection at the site of perforation, pericholecystic abscess and localized peritonitis; and type 3 (chronic) has an internal or external fistula formation.

Most recent studies have cited highest rates of type 2 GBP and the same was the case in our patient.

Historically GBP has been associated with a high mortality rate which ranges from 11% to 26%[6] and great care must be taken to diagnose the condition as early as possible. Generally GBP mimics bowel perforation and many cases are diagnosed intraoperatively. Modalities useful for this condition are USG and CT scan, with the latter being more sensitive[1]. Abdominal X-ray may not always show free gas in the

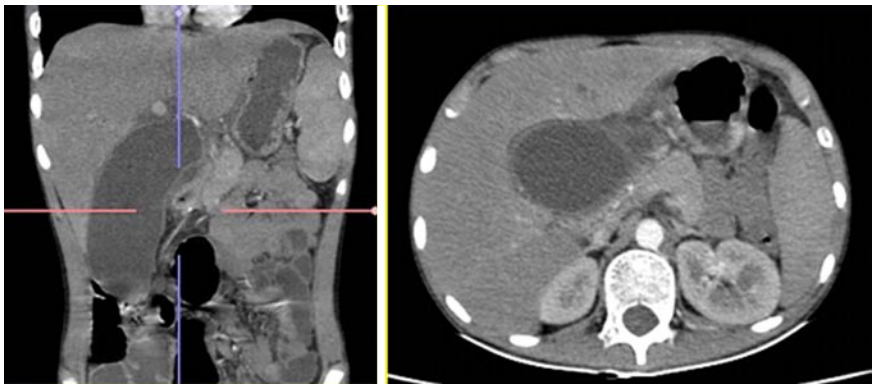

Figure 2 Computed tomography images.

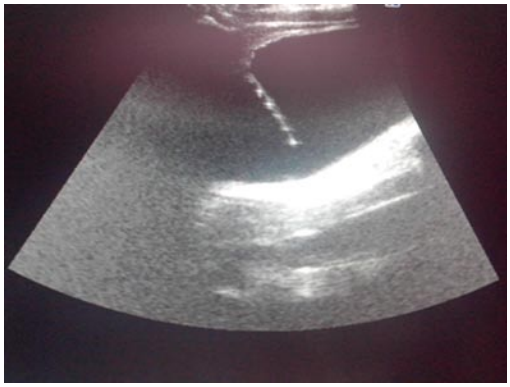

Figure 3 Percutaneous cholecystostomy.

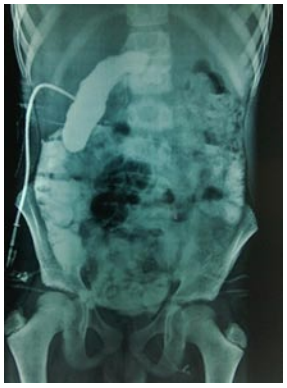

Figure 4 Tube cholecystogram.

peritoneum. HIDA scan, retrograde cholangiography and peritoneal lavage may also be used[7].

Reported complications include bile peritonitis, intrahepatic abscess formation (possible mechanisms include direct extension, subcapsular extension and hematogenous dissemination *via* the portal vein), subhepatic abscess formation, pelvic abscess formation, pneumonia, pancreatitis and acute renal failure[8].

Once diagnosed GBP mandates early intervention, and cholecystectomy with peritoneal lavage is considered sufficient. Laproscopic approach may also be used[2]. In our patient USG and CT scan showed a sealed-off

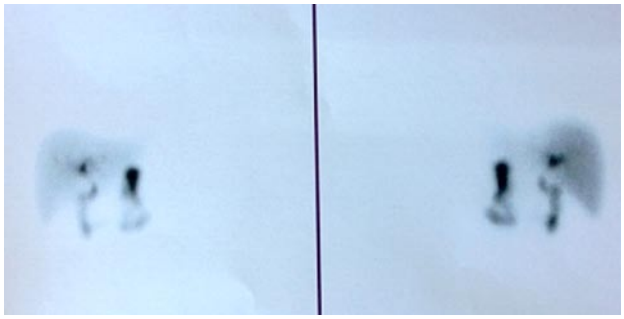

Figure 5 HIDA scan.

perforation without any free fluid in the peritoneum but a distended gallbladder which was persistent even on post prandial scans. Hence, a tube cholecystostomy was done under USG guidance by which the patient improved dramatically and a cholecystectomy was avoided. In many reports tube cholecystostomy is followed by interval cholecystectomy, but in our case we successfully avoided the procedure, thus preserving the gallbladder. Similar management in children has been reported by Mirza B[4] and Alghamdi[9]. The patient also underwent a Tc99m-HIDA scan to confirm the integrity and functionality of the biliary outflow tract, which did well at one-year follow-up.

It should also be mentioned that one must be very vigilant regarding the complications of GBP like persistent bile leak, persistent peritonitis, gallbladder necrosis, *etc.*[4] as they may warrant surgical exploration if not responding to percutaneous cholecystostomy.

In conclusion, we would like to highlight that GBP is a rare condition but demands a high level of suspicion, early diagnosis and prompt management. Using a percutaneous tube cholecystostomy in selected cases may help in avoiding cholecystectomy.

COMMENTS

Case characteristics

The patient was a 12-year-old boy who came with pain in the abdomen, fever and vomiting. He had abdominal distention and guarding in the right hypochondrium.

Clinical diagnosis
The initial suspicion was bowel perforation.

Differential diagnosis
Gallbladder perforation, even though rare, was kept as a differential diagnosis due to guarding localized to right hypochondrium.

Laboratory diagnosis
Complete hemogram, bilirubin, liver enzymes, prothrombin time and renal function tests, were within normal limits. Widal test and blood culture were negative.

Imaging diagnosis
An erect X-ray of the abdomen was unremarkable except gaseous distention of bowel loops. Ultrasonography revealed a perforated gallbladder which was confirmed by a computed tomography scan.

Treatment
As the gallbladder remained persistently over distended and the patient continued to have fever, a tube cholecystostomy was performed which led to resolution of the symptoms. Tube cholecystogram showed free flow of bile into the bowel without any leak.

Related reports
Similar technique of tiding over the acute phase of cholecystitis has been used before but is usually followed by interval cholecystectomy. In the case authors performed a HIDA scan at one month interval to confirm the functionality of the gallbladder and the billiary outflow system and found it satisfactory, thus cholecystectomy was successfully avoided.

Experiences and lessons
Gallbladder perforation is a rare but serious complication of cholecystitis and needs to be managed promptly. Percutaneous cholecystostomy is a good technique to tide over such crisis and can even be used as the definitive treatment, thus avoiding cholecystectomy altogether.

Peer-review
This is an interesting case history.

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