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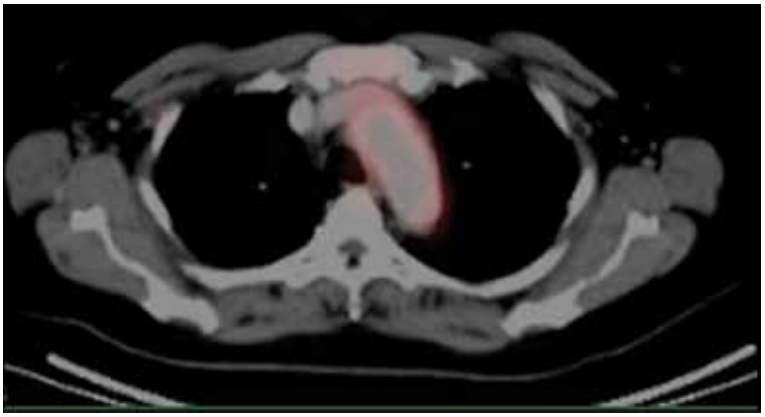

A nonproductive cough that would give most people a headache but not this patient: What is your diagnosis?

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EDITORIAL

Obesity: is evolution to blame?

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Fighting the obesity epidemic has become an important target for many health programs in industrialised countries, but attempts to maintain persistent clinically significant weight loss by lifestyle interventions, behavioural therapy or medical treatment have not been very successful so far. Current research is mainly focused on unravelling the medical consequences of obesity. It aims to understand how excessive caloric intake and the resulting increased fat mass cause insulin resistance and other features of the metabolic syndrome. However, is studying the consequences of obesity the best choice to control the worldwide obesity problem? Based on our traditional medical thinking founded by Hippocrates, treatment of medical disorders should rely on an understanding of their underlying cause in addition to fighting their consequences. Would more knowledge on the cause of obesity, beyond the concept of excessive caloric intake and reduced energy expenditure, help us to treat our obese patients?

In this issue, Hanno Pijl puts this challenge into a fascinating evolutionary perspective and proposes that we should explore evolution to understand the current obesity epidemic.[1] He explains how a very early climate change enabled us to shift from carbohydrates to fish- and meat-based diets, in turn stimulating encephalisation. The resulting greater cognitive abilities stimulated access to high quality food even further, while seasonal food insecurity spurred the evolution of thrifty genes. The current rapid change in our habitat, driven by technology, exposes most of us to unlimited availability of calories, in particular in the form of refined sugars and saturated fat. Combined with a decreased necessity for physical activity, obesity almost seems a logical consequence. Still, not every adult is obese.

CHANGE IN MACRONUTRIENT INTAKE

Recently our understanding of the way by which the change in macronutrient intake affects body weight has increased significantly. Both in rodents and humans, a diet rich in saturated fat and sucrose (HF/HS) compared with a high fat (HF)- or high sucrose (HS)-only diet, affects appetite control by increasing the drive to eat.[2,3] Moreover, HF/HS has more potent negative effects on glucose metabolism compared with HF or HS alone, irrespective of fat mass.[4] This could be one explanation why the change in the composition of our daily food might promote insulin resistance and obesity. But what could be the underlying mechanism of the effect of an HF/HS diet on food intake and metabolism? The answer to this important question probably lies in our brain. The brain, especially the hypothalamus, is responsible for orchestrating our energy metabolism. Peripheral metabolic signals inform our brain on the actual energy status. The hypothalamus reacts by integrating signals for eating behaviour, anterior pituitary function, as well as the sympathetic and parasympathetic outflow to insulin-sensitive tissues including the pancreas. HF/HS diets induce a state of relative insensitivity to these peripheral signals,[5] resulting in a hungry mediobasal hypothalamus reflected by elevated orexigenic signals, such as neuropeptide Y, a reduced insulin response and insulin resistance. Reducing the insulin response and inducing a state of insulin resistance reduces energy uptake in insulin-sensitive tissues and facilitates energy loss. Is this a way our body tries to get rid of the surplus energy? Perhaps, but then again storage of energy surplus in adipose tissue guarantees survival in times of food shortage. One might speculate that a threshold for optimal weight is present within each person. Trespassing this threshold will inevitably result in attempts to reduce further energy storage and to promote energy loss. From an evolutionary point of view, such weight boundaries make sense because both under- and overweight hamper fertility and mobility, putting us at risk to get caught by predators. Pijl proposes that insulin resistance may serve yet another purpose, i.e., to protect the brain from glucose deprivation. Although this would make sense in a lean fasting individual, insulin resistance in obese subjects is most explicit in the postprandial state when glucose

deprivation is least expected. However, an increase in free fatty acids (FFA) is present in both conditions, possibly reflecting a signal involved in insulin resistance. Despite a possible mechanism on how present-day HF/HS diets interfere with caloric intake and metabolic health, a clear hypothesis on why it is beneficial for survival to promote energy intake in the presence of HF/HS food is lacking at present.

As discussed by Pijl, fat intake has shifted from unsaturated to saturated fat. Is this shift an additional risk for health and body weight homeostasis? Studies in rodents have shown that unsaturated fatty acids, but not saturated fatty acids, have an anorexigenic action by stimulating pro-opiomelanocortin (POMC) gene expression in the hypothalamus.[6] In addition saturated fatty acids have a well-established negative effect on insulin signalling[7] besides a pro-inflammatory potential.[8] Intake of saturated fat in combination with refined sugars (HF/HS) would thus induce a state of excessive caloric intake, insulin resistance and inflammation. It follows that many palatable foods are bad news for metabolic health. Food programs in schools should incorporate this knowledge, e.g., by excluding HF/HS snacks from the assortment.

CHANGE IN ENERGY EXPENDITURE

As pointed out by Pijl, the industrial revolution made our lives much easier as physical fitness was no longer required to guarantee availability of food. Energy expenditure related to physical activity on average accounts for 30 to 50% of our daily energy expenditure. Increasing energy expenditure by performing regular physical activity sports will promote a zero energy balance. Current guidelines advocate 30 minutes of physical activity daily. If a man with a stable weight of 70 kg briskly walks for 30 minutes, seven days a week, his physical activity-induced increase in energy expenditure corresponds to 135 kcal x 7 = 945 kcal/week or 49,140 kcal/year. When he refrains from this daily walk without adjusting his diet by minus 50,000 kcal yearly, he will gain approximately 6 kg every year. This simple example illustrates how much a small change in energy expenditure affects body weight. Still, the question why some subjects do not adjust their caloric intake while reducing energy expenditure remains unanswered and suggests that an unbalanced hypothalamic control of eating behaviour may be a major pathogenetic factor.

GENES

Although a high percentage of adults is overweight and obese, the majority of adults fall within the optimal BMI range. These lean adults, living together with their obese peers in an obesogenic environment, deserve more scientific attention. What protects these adults from becoming obese? Moreover, why do not all obese subjects become diabetic despite the presence of excessive amounts of adipose tissue? Explaining differences between individuals always involves the issue of genetic susceptibility as well as epigenetic factors. As Pijl points out, most genetic variants established in populations with DM2 involve genes encoding for proteins involved in normal β-cell function. While polymorphisms in these genes may explain in part why an obese insulin-resistant subject would become hyperglycaemic, the obese phenotype remains largely unexplained. Monogenetic causes of obesity are present (5 to 7%) in the minority of the obese population.[9] Most of these mutated genes, such as those in the melanocortin 4 receptor, encode for proteins that are expressed in the hypothalamus and involved in appetite control. Genes undoubtedly play an important role in obese persons without these mutations, but until now their exact role and contribution remain unknown. A recent study in 250,000 individuals confirmed 14 known obesity susceptibility loci and identified 18 new ones, but the combined effect on BMI of these loci was only modest and accounted for only 6 to 11% of the genetic variation in BMI.[10] As a consequence, whether the thrifty genes hypothesis can explain the 21st century's prevalence of obesity remains speculative at this stage.

TREATMENT OPTIONS?

The most logical treatment of obesity is to reduce caloric intake and increase energy expenditure. Since abandoning the Western lifestyle is illusive and manipulating appetite control has proven to be extremely difficult, reducing energy intake by a combination of decreasing the physical ability to consume large quantities of food and reducing the uptake of calories seems to be the most promising strategy. Indeed, bariatric surgery has proven to be the sole effective therapy in the long term, especially when restrictive and malabsorptive surgery is applied.[11] Increasing energy expenditure by implementing more physical activity in daily life is another utopistic view on how to treat obesity. Increasing energy expenditure by designing agents which are able to uncouple energy need from energy production theoretically would be an interesting option. Finally, replacement of saturated fat by unsaturated fat by manipulating food (including meat) through genetic techniques or by adding metabolically active compounds might be a fruitful strategy.

In summary, our environment has changed dramatically resulting in the continuous availability of high caloric food as well as a reduction in daily energy expenditure.

For a growing percentage of children and adults, this environment promotes obesity and a metabolically unhealthy state. The reason why we do not adapt to our current environment as would be expected from an evolutionary point of view could be because it changed so fast that our genes couldn't keep up with it. Until we have adapted to our new environment, rigorous and rather crude interventions such as bariatric surgery seem to be the only way of reducing obesity-related morbidity and mortality.

REFERENCES

1. Pijl H. Obesity: evolution of a symptom of affluence. How food has shaped our existence. Neth J Med. 2011; 69:159-66.
2. la Fleur SE, Vanderschuren LJ, Luijendijk MC, Kloeze BM, Tiesjema B, Adan RA. A reciprocal interaction between food-motivated behavior and diet-induced obesity. Int J Obes (Lond). 2007 Aug;31(8):1286-94.
3. Giesen JC, Havermans RC, Douven A, Tekelenburg M, Jansen A. Will work for snack food: the association of BMI and snack reinforcement. Obesity. 2010 May;18(5):966-70.
4. la Fleur SE, Luijendijk MC, van Rozen AJ, Kalsbeek A, Adan RA. A free-choice high-fat high-sugar diet induces glucose intolerance and insulin unresponsiveness to a glucose load not explained by obesity. Int J Obes (Lond). In press 2011.
5. la Fleur SE, van Rozen AJ, Luijendijk MC, Groeneweg F, Adan RA. A free-choice high-fat high-sugar diet induces changes in arcuate neuropeptide expression that support hyperphagia. Int J Obes (Lond). 2010 Mar;34(3):537-46.
6. Schwinkendorf DR, Tsatsos NG, Gosnell BA, Mashek DG. Effects of central administration of distinct fatty acids on hypothalamic neuropeptide expression and energy metabolism. Int J Obes (Lond). 2010 Aug 17.
7. Shulman GI. Cellular mechanisms of insulin resistance. J Clin Invest. 2000 Jul;106(2):171-6.
8. Iyer A, Fairlie DP, Prins JB, Hammock BD, Brown L. Inflammatory lipid mediators in adipocyte function and obesity. Nat Rev Endocrinol. 2010 Feb;6(2):71-82.
9. O'Rahilly S, Farooqi IS. Human obesity as a heritable disorder of the central control of energy balance. Int J Obes (Lond). 2008 Dec;32(7):S55-61.
10. Speliotes F. Association analyses of 249,796 individuals reveal 18 new loci associated with body mass index. Nat Genet. 2010;42:937-48.
11. Sjöström L, Narbro K, Sjöström CD, Karason K, Larsson B, Wedel H, et al. Swedish Obese Subjects Study. Effects of bariatric surgery on mortality in Swedish obese subjects. N Engl J Med. 2007 Aug 23;357(8):741-52.

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REVIEW

Obesity: evolution of a symptom of affluence.

How food has shaped our existence

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ABSTRACT

This paper delineates the evolutionary background of the unprecedented epidemic of obesity that has evolved over the last century. Some two million years ago, a change of climate in the habitat of our primate ancestors triggered dietary adaptations which allowed our brain to grow. A shift from principally carbohydrate-based to fish- and meat-based eating habits provided sufficient fuel and building blocks to facilitate encephalisation. Insulin resistance may have evolved simultaneously as a means to avert the danger of hypoglycaemia to the brain (in view of the reduction of carbohydrate intake). Ensuing cognitive capacities enabled the control of fire and the manufacturing of tools, which increased energy yield from food even further and eased the defence against predators. The latter development relieved the selective pressure to maintain an upper level of bodyweight (driven by predation of overweight individuals). Since then, random mutations allowing bodyweight to increase spread in the human gene pool by genetic drift. Also, (seasonal) food insecurity in hunter-gatherer societies spurred the evolution of thrifty genes to maximise nutrient intake and energy storage when food was available. The agricultural and industrial revolutions rapidly changed our habitat: virtually unlimited stocks of (refined) foodstuffs and mechanical substitutes of physical efforts push up energy balance, particularly in those of us who are still adapted to former environmental conditions: i.e. who carry thrifty genes and lack (genetic) protection against weight gain. Intrauterine epigenetic mechanisms potentially reinforce the impact of these genes on the propensity to grow obese.

"Thus, from the war of nature, from famine and death, the most exalted object which we are capable of conceiving, namely, the production of the higher animals, directly follows." (Charles Darwin in: On the Origin of Species, 1859)

KEYWORDS

History, insulin resistance, predation release, thrifty genes, type 2 diabetes mellitus

INTRODUCTION

Currently, the World Health Organisation (WHO) estimates more than one billion people worldwide to be overweight, of whom at least 300 million are obese.[1] This is particularly worrisome, because obesity increases the risk of various chronic diseases, i.e. cardiovascular disease, type 2 diabetes mellitus and certain forms of cancer.[1] Therefore, the WHO and other non-governmental organisations have called for global action to prevent further escalation and reduce the number of obese people.[2]

It is of primary importance for the prevention and treatment of any disease to understand its root cause. Although hominid artifacts supposedly depicting obese humans date back as far as 500,000 years,[3] the current epidemic has evolved over the last one hundred. Why is that? Clearly, the industrial revolution plays a major role: motorised labour and transportation, in concert with the availability of virtually unlimited amounts of food in (Western) societies marked by technical and socioeconomic progress, push energy balance upward in many individuals. In fact, given these developments, the appropriate question is not why so many people are obese, but why so many of us appear to escape this physical fate.

This paper delineates the biological and social underpinnings of the obesity epidemic from an evolutionary point of view. Climate change and ensuing dietary adaptations profoundly influenced the development of our brain, which probably permitted two recent events triggering the current epidemic of metabolic disease to occur: the agricultural and industrial revolutions. Although the technical breakthroughs that facilitated

these socioeconomic upheavals doubtlessly contribute to the bodyweight increase of contemporary homo sapiens, it is very important for the development of prevention and treatment strategies to bear in mind that biological features of our species also play their part.

THE EARLY DAYS: EVENTS FACILITATING BRAIN GROWTH

The story begins some four to six million years ago. Our primate ancestors lived in the woods of Eastern Africa (Tanzania, Kenya, Ethiopia). Their diet primarily comprised leaves, roots, fruits and nuts at the time.[4] Thus, the main macronutrient we consumed was carbohydrate. Approximately two million years back, the climate in Eastern Africa changed profoundly (it became dryer and colder), which had a major impact on our habitat. Forests disappeared and were replaced by arid grasslands, inhabited by herbivore game (and large predators).[5] These ecological changes provided an excellent opportunity for our hominin ancestors with sufficient capability to exploit animal resources. Archaeological and anthropological evidence strongly suggests that we ultimately moved to these grasslands and coastlines, attracted by much higher quality food[6,7]: from then on proteins and unsaturated fatty acids (from game and fish) were abundant in our diet, comprising some 50 to 60% of intake on a percentage of total calories basis.[7,8] This dietary change in turn allowed a crucial event in our evolutionary history to occur: the growth of our brain. There are at least two reasons why this particular change of food habits was essential for our brain to be able to grow: first, unsaturated fatty acids are essential building blocks of neural tissue. Approximately 50 to 60% of the human adult brain is made up of lipids,[9] of which nearly one third are polyunsaturated, primarily arachidonic acid and docosahexaenoic acid.[10] Second, our brain is extremely expensive in terms of energy costs: it consumes >20% of total resting expenditure.[11,12] Energy yield from far more nutrient dense fish and meat is much higher than from (structural) plant components (e.g. bark, mature leaves).[13] The growth of our brain was probably essential for our intellectual development. In due course we learned how to control fire (first evidence dates back some 800,000 years.[14] Heating of food improved energy yield even further, as it clearly facilitates digestion.[15]

Notably, these biological and cultural developments merely *allowed* the brain to grow; they do not explain *why* it did. Various theories address the latter issue. For example, it has been proposed that intellectual development increased survival among hominids exploiting the complicated nutritional niche of hunting and gathering. It requires tools, social strategies and memory to effectively catch prey in open grasslands and forage for ripe fruits and nuts in continuously changing seasons and environments. Chance changes in brain morphology, intensifying neuronal number and connectivity (thereby promoting intelligence), may therefore have conferred a survival advantage on our hominin ancestors.[16] In addition, the use of tools and social interactions *per se* may have spurred neural development.[17] One other possibility I would like to put forward here is that we needed our intellect to escape from predators in open grasslands. Our species does not have powerful physical tools at its disposal to ward off life-threatening attacks by predators. Climbing trees or quickly getting away in an underground refuge are alternative means to escape which do not particularly fit with our physique either. Attacks by predators probably posed a major threat to our ancestors until technical (stone tools, fire control) and social (coordinated defence strategies) developments quite significantly facilitated survival.[18,19] Conceivably, growing intellectual capacity, as a function of neural connectivity, was a prerequisite for the emergence of these adaptations which clearly conferred a survival advantage. Therefore, hominids with larger brains harbouring extensive neural networks (i.e. greater intellectual capacity) may have survived more often than those with smaller brains.

TEN THOUSAND YEARS BACK: THE AGRICULTURAL REVOLUTION

Perhaps also as a result of intellectual progress, our ancestors became adventurous and migrated out of Africa for the first time some 1.8 million years ago (the precise timing of this event is hotly debated by the way). The majority of migrants moving away during this first out-of-Africa exodus ended up in Asia. Their offspring finally became extinct only recently (100,000 years back).[20] A second exodus, approximately 800,000 years ago, primarily landed in Europe, bringing forth (among others) the Neanderthal species which died out some 20 to 30 thousand years back.[20] Finally, during the third and last exodus which populated the world as it is today, people accidentally passed by the fertile grounds of the land of the Euphrates and Tigris rivers, including significant territory of modern-day countries such as Egypt, Iraq, Syria, Jordan, Lebanon, Israel, Iran and Turkey. The geophysical and climate characteristics of this region pre-eminently enabled the natural occurrence of the wild progenitors of the Neolithic founder crops (cereals, legumes and flax) and four of the five most important domesticated animals (cows, goats, sheep, and pigs). Because of these favourable environmental conditions, the so-called Fertile Crescent became the birthplace of modern agriculture and stock-breeding some 10,000 years ago.[21] Independent

development of agriculture occurred somewhat later in China, the African Sahel region, New Guinea and several areas in the Americas.[21]

The advent of agriculture profoundly affected the composition of our diet. As outlined above, hunter-gatherers thrived on a mix of carbohydrates, proteins and (unsaturated) fatty acids for millions of years. It is important to note that there has not been one universal diet consumed by all hunter-gatherer communities. Rather, as suggested by studies of contemporary hunter-gatherer tribes[22] and commonsense, the availability of food depended on geographic locale and climate conditions. Humans evolved as veritable omnivores, although it seems likely that >50% of hunter-gatherer subsistence comprised animal food.[8] However, various types of food cannot have been consumed on a regular basis before the advent of agriculture and animal husbandry. Agriculture in essence reintroduced carbohydrate as the principal macronutrient. Agricultural produce primarily contains carbohydrate; it partially supplanted hunter-gatherer protein and (unsaturated) fatty acid in our diet. Moreover, animal husbandry introduced dairy and promotes the consumption of saturated instead of unsaturated fat. The latter is for two reasons. First, cattle meat partially replaced fish in our diet and fish is an important source of unsaturated fatty acids. Second, the dominant fatty acids in *adipocytes* of wild mammals are saturated, whereas *muscle and other tissues* primarily contain polyunsaturated (PUFA) or monounsaturated fatty acids (MUFA).[23] Because subcutaneous and abdominal adipose stores are depleted during most of the year in wild animals, PUFA and MUFA constitute most of their total carcass fat.[23] The advent of animal domestication and stock breeding attenuated the (seasonal) depletion of (saturated) fat stores by year round feeding of stored plant foods. Therefore, cattle harbour much more saturated fat when domesticated than in the wild. Also, it became feasible to slaughter animals at peak body fat percentage.

How did these recent dietary changes affect our health? Almost all evidence indicates that it deteriorated. Average adult height declined substantially after the advent of agriculture.[24] Moreover, studies of bones and teeth show that the advent of agriculture coincides with a higher incidence of osteoporosis, rickets, caries and various other mineral and vitamin-deficiency disorders.[25,26] Finally, undisputed evidence indicates that the size of our brain is currently shrinking for the first time in our evolutionary history (perhaps because of a lack of unsaturated fatty acids for build up and maintenance), albeit in parallel with the decline of bodyweight and height.[27]

So how is it that agriculture turned out to be so successful? Agrarian societies rapidly conquered the world, while hunter-gatherers vanished either by defeat or by voluntary adoption of farming as a way of life.[21] Agriculture enabled us to settle down at a fixed spot: we were no longer dependent on local (un)availability of food forcing us to move on to other areas to hunt and gather. This allows the number of offspring to increase, because mothers are no longer obliged to carry their children around in continuous search of foraging areas (it is feasible to simultaneously carry one or perhaps two children at the very most). Building more robust accommodation and fenced villages facilitated defense against predators and hostile congeners. Predation and violence were major threats in hunter-gatherer times, although earlier social developments had significantly abated the danger of violent death (see above).[18,19] Furthermore, agriculture allowed rapid evolution of knowledge-based societies: only a few members of the community could maintain food security for all, the rest had plenty of time to focus on innovation. In sharp contrast, hunter-gatherers were obliged to collectively forage for food during a considerable part of the day. As communities grew, (political) organisation substantially reinforced their capacity to withstand hostile threats and successfully embark on campaigns to expand territory.[21] Clearly, these social corollaries of agriculture provided powerful benefits which explain its rapid world-wide scattering.

Overweight and obesity were probably exceptional for thousands of years after the advent of agriculture, although famous statuettes of obese individuals such as the Venus of Willendorf date back even further. Also, Hippocrates recognised the dangers of overweight some 2400 years ago: 'It is very injurious to health to take in more food than the constitution will bear, when, at the same time one uses no exercise to carry off this excess.... For as aliment fills, and exercise empties the body, the result of an exact equipoise between them must be to leave the body in the same state they found it, that is, in perfect health.'[28] Obesity predominantly occurred among members of the upper social class, who had continuous access to food and usually performed intellectual duties not requiring physical activity.[28] In this context it is vital to bear in mind that food shortage as a result of failed harvest, particularly affecting the man in the street, was fairly common in pre-industrial societies.[29]

RECENT DEVELOPMENTS: THE INDUSTRIAL REVOLUTION

In the late 18th century, major technological developments in the United Kingdom foreshadowed worldwide socioeconomic and cultural changes which signify a third turning point in human history. Machine-based

manufacturing and farming, enabled by spectacular progress in the field of fuelling, dramatically increased (agricultural) production capacity. Moreover, it became far more feasible to store food safely for longer periods of time. For the first time in history, nutrients were available for all (in those parts of the world profiting from the developments). Also, motorised labour and transport pre-empted physical efforts. Childhood mortality declined significantly (for various reasons). The world's population grew almost sixfold since the early 1800's.[30]

As a corollary of these advancements, the industrial revolution had a major impact on human energy balance equations. Reliable data documenting calorie intake are scarce, in particular for food consumption during the 19th and early 20th century. However, commonsense tells us that average intake must have increased substantially. The Food and Agriculture Organization (FAO) of the United Nations reports a continuing increase of total daily calorie consumption by 20 to 25% across the world since 1960.[31] Data from the US confirm the substantial increase of per capita calorie intake over the last 30 years.[32] Perhaps even more important, dietary composition also changed considerably: cereals were highly refined by mechanised mills; refined sugars were introduced and consumed on an ever-growing scale; sodium intake increased dramatically, whereas potassium intake declined; micronutrient density declined whereas calorie density increased; fibre content fell substantially; and saturated fat replaced (poly)unsaturated (*table 1*) (for an excellent review see Cordain *et al.*[33]).

Table 1. *Major differences in food components of modern-day humans as compared with our hunter-gatherer ancestors*

	Contemporary *vs.* hunter-gatherer
Calorie intake	↑
Physical activity	↓ ↓
Dietary composition	
Total carbohydrates:	↑
refined carbohydrates	↑ ↑
fibres	↓ ↓
Total protein	↓
Total fat:	~
PUFA	↓ ↓
ω-6:ω-3	↑ ↑
SFA	↑ ↑
Micronutrients:	↓
sodium	↑ ↑
potassium	↓

PUFA = polyunsaturated fatty acids
SFA = saturated fatty acids

Concurrently, our environment was deliberately designed to minimise the requirement for physical activity. Although reliable methods to quantify total daily energy expenditure are only just emerging, the secular decline in physical activity is obvious. Motorised labour and transport have profoundly suppressed calorie needs.[34,35] Finally, advancements in heating technology and clothing effectively protect us against the nuisance of cold weather, which substantially diminishes the energy requirements for adaptive thermogenesis.[36,37]

The consequences of these lifestyle changes for our energy balance and health are easy to contain. Indeed, when contemporary hunter-gatherer societies adopt the 'Western' way of life, obesity, diabetes and atherosclerosis become commonplace.[38-40] Conversely, temporary reversal of westernisation (by living as hunter-gatherers in their traditional country for seven weeks) essentially cures type 2 diabetes in obese Australian Aborigines.[41] In fact, the obvious question is why so many people maintain metabolic health in the face of the current environmental 'challenges'. The answer to this question is not entirely clear. I will briefly address four important hypotheses trying to explain this enigma.

THE THRIFTY GENE HYPOTHESIS

In the early 1960s, James Van Gundia Neel, a pioneer in the study of human genetics, launched his 'thrifty gene hypothesis',[42] which still dominates thinking about the biological roots of obesity and diabetes. Neel was one of the first to recognise the important role of genes in the pathogenesis of these ailments. His hypothesis is founded on the basic premise that genes which are part of the (human) gene pool must have had survival benefits in evolutionary history. Neel specifically proposed that a genetically determined excessive insulin response to nutrient ingestion would minimise the loss of precious glucose in harsh times of food scarcity. Hyperinsulinaemia would effectively promote storage of ingested calories. Overalimentation in modern times would result in plasma insulin levels that elicit 'insulin antagonism in plasma' as proposed by Vallance-Owen and colleagues,[43] and thereby cause diabetes. As other laboratories could not confirm the existence of circulating insulin antagonists, the original physiological basis of the hypothesis collapsed, which led Neel to revisit his reasoning in regard to the mechanistic link between the obese diabetic genotype and phenotype. Complex adaptive genetic traits would compile multi-faceted endocrine systems designed to retain calories in times of famine.[44] The genes involved 'are very predominantly fine old genes with, of course, some allelic variation, honed by millennia of selection for harmonious

interactions and appropriate epigenetic relationships, the proper function of which is overwhelmed by extraneously imposed parameters of very recent origin'.[44]

Neel's genetic premise still holds. There is widespread consensus that genes determine the variation in bodyweight and body fat distribution in a given (social) environment for at least 50 to 70%.[45,46] Genes are also involved in the pathogenesis of type 2 diabetes mellitus (DM2), although all single nucleotide polymorphisms (SNP) known to be associated with DM2 to date add only marginally to risk prediction by conventional factors.[46,47] Monogenetic forms of either disease are well known, but complex genetic traits predispose to metabolic disorder in the vast majority of patients.[46] The mechanistic links between genotype and phenotype of both ailments remain largely unknown. However, it is remarkable that all monogenetic defects causing human obesity known to date disrupt hypothalamic circuits that control food intake.[48] Therefore, although it is often assumed that genetic factors underlying obesity affect metabolic rate or selective partitioning of excess calories into fat, current evidence suggests that genetic determinants of satiety and food intake are likely to be at least as important. The precise biological correlates of the majority of DM2 SNPs are not known, but many of them map close to genes expressed in the islets of Langerhans and/or are associated with β-cell dysfunction.[46,49] Inasmuch as the pathophysiology of DM2 is marked by dual defects of insulin secretion and action,[50] it is likely that the genes which predispose to DM2 (given the current affluent conditions) control the extent to which β-cell function can be maintained in the face of (also heritable, see below) insulin resistance.

Thus, the thrifty gene hypothesis proposes that those of us carrying a hereditary taint to efficiently harvest and/or store calories are the ones who run the greatest risk to grow obese in contemporary industrialised living climates. These genes conferred survival advantage in ancient times characterised by (seasonal) food insecurity. There is general consensus that genes play an important role in the pathogenesis of metabolic disease. Various alleles related to obesity are widespread among the population.[51] I also think that most evolutionary biologists still tend to agree with the conceptual underpinning of Neel's hypothesis. In keeping with his revised mechanistic explanation, obesity is caused by the concerted effects of multiple gene products in the vast majority of patients. However, in sharp contrast to Neel's original idea about the pathogenesis of DM2, mutations predisposing to this disease appear to hamper β-cell function. These alleles could probably spread in the gene pool, because there has never been selection pressure on β-cell capacity. Current environmental conditions (i.e. unlimited availability of food, particularly refined sugars) and (obesity associated) insulin resistance challenge β-cell function to an unprecedented extent, leading to failure in those of us with functional capacity in the lower range of the boundaries compatible with life.

THE PREDATION RELEASE HYPOTHESIS

I will just briefly summarise John Speakman's intriguing ideas explaining the epidemic of obesity in modern societies, because he elaborately outlined his novel hypothesis recently in an excellent paper.[19] The interested reader will find all relevant references in this paper. In essence, Speakman argues that there is insufficient evidence to support the notion that our ancestors have been exposed to perils of famine sufficiently severe for thrifty genes to propagate. Moreover, he puts forward that strong selection for thrifty genes would predict hunter-gatherers to grow fat in between epochs of famine, and various studies of contemporary hunter-gatherer societies do not report such weight gain. Finally, he asserts that any postulate involving thrifty genes as a root cause of obesity cannot explain the fact that so many people maintain normal bodyweight in the current environment, as such genes spread widely in the gene pool when given sufficient time to propagate. As an alternative, Speakman suggests that ancient genes controlled bodyweight within narrow limits, with mutations causing obesity selected against by the risk of predation. As mentioned earlier, predation posed a major threat to our hominid ancestors, and obese individuals must have been easy and attractive targets for obvious reasons (i.e. less mobile, more calories to consume). Some one million years ago, humans evolved social strategies to ward off predators. Furthermore, the control of fire and stone tools that could be used as weapons quite significantly facilitated the defence against lethal attacks. These developments relieved the selective pressure to maintain bodyweight below an upper setpoint. Since then, random mutations allowing bodyweight to increase were no longer removed from the gene pool and spread gradually through genetic drift. When food is available in virtually unlimited quantities and physical activity no longer required to meet the necessaries of life, bodyweight can grow unabatedly in those of us afflicted. The fact that the mutations spread through random drift rather than directed selection explains why so many people maintain a normal weight despite current environmental conditions.

FOETAL ORIGINS OF ADULT OBESITY

Barker and colleagues were the first to recognise that intrauterine conditions have a major impact on adult health.[52] Geographical studies demonstrated that contemporary rates of death from coronary heart disease

were closely associated with death rates among newborn babies in the past. Death among newborns was almost invariably attributed to low birth weight. The finding spurred scientific interest in the effects of the intrauterine environment on adult (metabolic) disease. Foetal and neonatal growth are marked by extraordinary plasticity, allowing intrinsic and environmental factors to impact on development so as to optimally adapt the offspring's phenotype to current environmental conditions. A huge body of evidence now supports the view that foetal nutrition shapes its metabolic phenotype through epigenetic modification of gene expression.[53] Intrauterine conditions affect gene expression through histone modification and methylation of DNA, which is heritable but does not bear on mutation of DNA itself (hence the term 'epigenetic').[54] It has now been firmly established that maternal overweight and elevated plasma levels of glucose and triglyceride levels are strongly predictive of foetal and neonatal fatness and body mass index of offspring at 8 years of age.[55] The precise epigenetic mechanisms involved are not known, but may relate to transcriptional modification of metabolic and behavioural gene pathways by in utero exposure to excess maternal lipids.[55] Conversely, and paradoxically, female (but not male) offspring of mothers exposed to famine during gestation in the Dutch 'Hongerwinter' are also obese at middle age.[56] The impact of foetal malnutrition on adult obesity was recently confirmed by a study among children whose mothers were undernourished during the Biafran civil war famine.[57]

The currently available data documenting the epigenetic origins of obesity allow for a model of its pathogenesis assuming the primacy of recent environmental changes. In particular, they imply that both parental obesity and nutritional deficits during gestation inheritably adapt foetal gene expression profiles so as to predispose the offspring to excessive weight gain. In this context, obesity does not necessarily involve genetic predisposition. Rather, environmental cues affecting food intake (e.g. aggressive advertising of foodstuffs) may induce parental metabolic changes, which alter gene expression profiles in their offspring so as to produce an inheritable trait predisposing to weight gain in subsequent generations. However, epigenetic mechanisms may obviously also cooperate with genetic traits to reinforce pathogenetic mechanisms underlying obesity.

THE CARNIVORE CONNECTION: PUTTING INSULIN RESISTANCE IN EVOLUTIONARY PERSPECTIVE

Insulin facilitates glucose and amino acid uptake in muscle and adipose tissue. It also promotes incorporation of fatty acids in adipose triglycerides. Conversely, it inhibits glucose and triglyceride production by the liver.[58,59] Thus, the postprandial rise of circulating insulin levels effectively clears ingested nutrients from the blood. Consequently, insulin resistance hampers postprandial disposal of glucose, (branched-chain) amino acids and fatty acids and promotes (postprandial) hepatic glucose output and triglyceride production. Therefore, insulin resistance is associated with a cluster of metabolic anomalies, including hyperglycaemia, hypertriglyceridaemia, low plasma HDL-cholesterol levels (directly linked with increased circulating VLDL-triglyceride levels), hypertension and abdominal obesity,[60] often referred to as the 'metabolic syndrome'. Essentially, insulin resistance hampers the use of glucose for fuel by peripheral tissues, saving it for the brain to combust. It provides even more glucose to the brain by simultaneous promotion of endogenous glucose production (with circulating amino acids and glycerol as precursors of gluconeogenesis). In sync, it supplies other tissues with fatty acids as an alternative fuel. The pathogenesis of insulin resistance involves complex gene-environment interactions.[61] What evolutionary pressures have pushed the widespread dissipation of the genes involved?

As pointed out earlier, our ancestor's dietary composition switched from primarily carbohydrate based to protein rich some two million years ago in response to a climate change in Eastern Africa.[4,7,62] Our brain chiefly relies on glucose for its energy requirements and cerebral energy consumption at physical rest amounts to a striking 25% of total bodily expenditure.[11,12] Thus, the dietary change simultaneously allowed the brain to grow (by provision of unsaturated fatty acid and energy) and created a direct threat to brain health and survival: glucose deprivation. Seventeen years ago, Jeanette Brand Miller and Stephen Colagiuri proposed that insulin resistance developed to overcome this environmental threat.[62,63] It is quite conceivable that insulin resistance conferred a survival benefit particularly in winter when food was scarce for hunter-gatherers: effective partitioning of precious glucose towards the brain may have been critical for maintenance of brain health. In this context, the seasonal cycling of fat storage (hoarding in summer in preparation for winter time) that marks wild mammals,[33] probably including hominid hunter-gatherers, is of mechanistic interest: adipose tissue plays a major role in the pathogenesis of insulin resistance.[64]

The agricultural revolution reintroduced carbohydrates as the dominant macronutrient in our diet. Subsequent industrialisation made food continuously available to the majority of the population and catapulted the consumption of refined sugars. In these circumstances, insulin resistance is no longer an asset. In contrast, it elevates blood glucose levels and predisposes to DM2.

SUMMARY AND PERSPECTIVE

Three major events in our evolution presaged the current epidemic of obesity and type 2 diabetes. Approximately two million years ago geophysical and climate changes in Eastern Africa triggered dietary adaptations that allowed the growth of our brain. A shift from principally carbohydrate-based to protein- and unsaturated fatty acid-rich food provided sufficient fuel and building blocks to facilitate encephalisation. Insulin resistance may have evolved simultaneously as a means to avert the danger of hypoglycaemia to the brain. Also, thrifty genes maximised food intake and energy storage when available and technical and social progress relieved the selective pressure to maintain an upper level of body weight. Ensuing intellectual capacities enabled two very recent developments that shaped our society of today: the agricultural and industrial revolutions. These socioeconomic landslides changed environmental conditions so quickly that many of us are not yet physically adapted. Reintroduction of carbohydrate as the predominant macronutrient, availability of virtually unlimited stocks of refined foodstuffs and mechanical substitutes of physical efforts render those of us who are genetically designed to survive in harsh circumstances particularly susceptible to obesity and type 2 diabetes mellitus.

It is of critical importance for the design of preventive measures to bear in mind that we have built our society as it is for good reasons: our recent evolutionary history of seasonal food insecurity strongly drives our inclination to maximise food stocks and consume if food is available as well as our tenor to sit still (and spare energy) as soon as the circumstances allow us to do so. These biological assets are obviously meaningless and even hazardous today. Although obesity and insulin resistance diminish human fecundity,[65] it will probably take thousands if not millions of years of genetic drift to deplete the gene pool, inasmuch as evolutionary pressure to eliminate these traits will be relatively insignificant, because the adverse consequences generally arise well into reproductive age. Moreover, modern medical technology can assist obese patients to reproduce. In this respect, the currently evolving epidemic of childhood obesity may have quite different effects.

Darwin's lessons are as meaningful as ever. For any preventive or therapeutic strategy focussing on obesity and diabetes to be truly effective, it is imperative to consider the evolutionary underpinnings of the problem. In particular, we need to understand that our behaviour and metabolism are driven by strong evolutionary roots. In view of the biological power of these roots, I am convinced that simply informing the public about the dangers of our behaviour and the potential solutions will yield only marginal results. Rather, we have to think of reasonable ways to curb our instincts nolens volens, or accept that nature will probably take a very long time to help us overcome the current epidemic.

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REFERENCES

1. WHO Global Strategy on Diet Physical Activity and Health. http://www.who.int/dietphysicalactivity/publications/facts/obesity/en/index.html.
2. WHO. Global Alliance for the Prevention of Obesity and Related Chronic Disease. http://www.preventionalliance.net/.
3. Haslam D, Rigby N. A long look at obesity. Lancet. 2010;376(9735):85-6.
4. Gaulin SJC, Konner M. On the natural diet of primates, including humans. In: Wurtman RJ, Wurtman JJ, editors. Nutrition and the Brain. New York: Raven Press, 1977;1-86.
5. Reed KE. Early hominid evolution and ecological change through the African Plio-Pleistocene. J Hum Evol. 1997; 32(2-3):289-322.
6. Leonard WR, Robertson ML, Snodgrass JJ. Energetics and the evolution of brain size in early *Homo*. In: Roebroeks JWM, editor. Guts and Brains. An integrative approach to the hominin record. Leiden: Leiden University Press, 2007:29-46.
7. Broadhurst CL, Cunnane SC, Crawford MA. Rift Valley lake fish and shellfish provided brain-specific nutrition for early Homo. Br J Nutr. 1998;79(1):3-21.
8. Cordain L, Eaton SB, Miller JB, Mann N, Hill K. The paradoxical nature of hunter-gatherer diets: meat-based, yet non-atherogenic. Eur J Clin Nutr. 2002;56 Suppl 1:S42-S52.
9. Huang M-C, Brenna JT. On the relative efficacy of alinolenic acid and preformed docosahexanoic acid as substrates for tissue docohexanoate during perinatal development. In: Mostofsky DI, Yehuda SJr, editors. Fatty acids: physiological and behavioral functions. Totowa,NJ: Humana Press, 2001:99-113.
10. Carlson BA, Kingston JD. Docosahexaenoic acid, the aquatic diet, and hominin encephalization: difficulties in establishing evolutionary links. Am J Hum Biol. 2007;19(1):132-41.
11. Leonard WR, Snodgrass JJ, Robertson ML. Effects of brain evolution on human nutrition and metabolism. Annu Rev Nutr. 2007;27:311-27.
12. Cunnane SC, Crawford MA. Survival of the fattest: fat babies were the key to evolution of the large human brain. Comp Biochem Physiol A Mol Integr Physiol. 2003;136(1):17-26.
13. Cordain L, Watkins BA, Mann NJ. Fatty acid composition and energy density of foods available to African hominids. Evolutionary implications for human brain development. World Rev Nutr Diet. 2001;90:144-61.
14. Goren-Inbar N, Alperson N, Kislev ME, Simchoni O, Melamed Y, Ben-Nun A, et al. Evidence of hominin control of fire at Gesher Benot Ya'aqov, Israel. Science. 2004;304(5671):725-7.
15. Carmody RN, Wrangham RW. The energetic significance of cooking. J Hum Evol. 2009;57(4):379-91.
16. McDonald K. Ecological hypotheses for human brain evolution: evidence for skill and learning processes in the ethnographic literature on hunting. In: Roebroeks JWM, editor. Guts and Brains. An integrative approach to the hominin record. Leiden: Leiden University Press, 2007:107-32.
17. Coward F, Gamble C. Big brains, small worlds: material culture and the evolution of the mind. Philos Trans R Soc Lond B Biol Sci. 2008;363(1499):1969-79.

18. Guthrie RD. *Haak and Steek*-The tool that allowed hominins to colonize the African savanna and to flourish there. In: Roebroeks JWM, editor. Guts and Brains. An integrative approach to the hominin record. Leiden: Leiden University Press, 2007:133-64.

19. Speakman JR. A nonadaptive scenario explaining the genetic predisposition to obesity: the 'predation release' hypothesis. Cell Metab. 2007;6(1):5-12.

20. Stringer C. Human evolution: Out of Ethiopia. Nature. 2003;423(6941):692-3, 695.

21. Diamond J. Guns Germs and Steel. The fates of human societies. New York: W.W.Norton & Company Ltd, 1999.

22. Cordain L, Miller JB, Eaton SB, Mann N, Holt SH, Speth JD. Plant-animal subsistence ratios and macronutrient energy estimations in worldwide hunter-gatherer diets. Am J Clin Nutr. 2000; 71(3):682-92.

23. Cordain L, Watkins BA, Florant GL, Kelher M, Rogers L, Li Y. Fatty acid analysis of wild ruminant tissues: evolutionary implications for reducing diet-related chronic disease. Eur J Clin Nutr. 2002;56(3):181-91.

24. Cohen MN. Health and the rise of civilisation. New Haven, Conn: Yale University Press, 1989.

25. Cohen MN. The significance of long-term changes in human diet and food economy. In: Harris M, Ross EB, editors. Food and Evolution: Toward a Theory of Human Food Habits. Philadelphia, PA: Temple University Press, 1987: 261-83.

26. Cassidy CM. Nutrition and health in agriculturalists and hunter-gatherers: a case study of two prehistoric populations. In: Jerome NW, Kandel RF, Pelto GH, editors. Nutritional Anthropology: Contemporary Approaches to Diet and Culture. Pleasantville, NY: Redgrave Publishing Co, 1980:117-45.

27. Ruff CB, Trinkaus E, Holliday TW. Body mass and encephalization in Pleistocene Homo. Nature. 1997;387(6629):173-6.

28. Haslam D. Obesity: a medical history. Obes Rev. 2007; 8 Suppl 1:31-6.

29. O'Grada C. Markets and famines in pre-industrial Europe. Journal of Interdisciplinary History. 2005;36:143-66.

30. Maddison A. The World Economy. Historical Statistics. Paris: OECD, 2003.

31. Food and Agricultural Organisation EaSDD. World Agriculture: towards 2015/2030. Summary Report. 2002.

32. Popkin BM, Gordon-Larsen P. The nutrition transition: worldwide obesity dynamics and their determinants. Int J Obes Relat Metab Disord. 2004;28 Suppl 3:S2-S9.

33. Cordain L, Eaton SB, Sebastian A, Mann N, Lindeberg S, Watkins BA, et al. Origins and evolution of the Western diet: health implications for the 21st century. Am J Clin Nutr. 2005;81(2):341-54.

34. O'Keefe JH, Vogel R, Lavie CJ, Cordain L. Achieving Hunter-gatherer Fitness in the 21(st) Century: Back to the Future. Am J Med. 2010;123(12):1082-6.

35. Haslam DW, James WP. Obesity. Lancet. 2005;366(9492):1197-209.

36. van Marken Lichtenbelt WD, Vanhommerig JW, Smulders NM, Drossaerts JM, Kemerink GJ, Bouvy ND, et al. Cold-activated brown adipose tissue in healthy men. N Engl J Med. 2009;360(15):1500-8.

37. Cypess AM, Lehman S, Williams G, Tal I, Rodman D, Goldfine AB, et al. Identification and importance of brown adipose tissue in adult humans. N Engl J Med. 2009;360(15):1509-17.

38. Diamond J. The double puzzle of diabetes. Nature. 2003;423(6940):599-602.

39. Daniel M, Rowley KG, McDermott R, Mylvaganam A, O'Dea K. Diabetes incidence in an Australian aboriginal population. An 8-year follow-up study. Diabetes Care. 1999;22(12):1993-8.

40. Ebbesson SO, Schraer CD, Risica PM, Adler AI, Ebbesson L, Mayer AM, et al. Diabetes and impaired glucose tolerance in three Alaskan Eskimo populations. The Alaska-Siberia Project. Diabetes Care. 1998;21(4):563-9.

41. O'Dea K. Marked improvement in carbohydrate and lipid metabolism in diabetic Australian aborigines after temporary reversion to traditional lifestyle. Diabetes. 1984;33(6):596-603.

42. Neel JV. Diabetes mellitus: a 'thrifty' genotype rendered detrimental by 'progress'? Am J Hum Genet. 1962;14:353-62.

43. Vallence-Owen J, Lilley MD. Insulin antagonism in the plasma of obese diabetic and prediabetics. Lancet. 1961;1(7181):806-7.

44. Neel JV, Weder AB, Julius S. Type II diabetes, essential hypertension, and obesity as 'syndromes of impaired genetic homeostasis': the 'thrifty genotype' hypothesis enters the 21st century. Perspect Biol Med. 1998;42(1):44-74.

45. Maes HH, Neale MC, Eaves LJ. Genetic and environmental factors in relative body weight and human adiposity. Behav Genet. 1997;27(4):325-51.

46. O'Rahilly S. Human genetics illuminates the paths to metabolic disease. Nature. 2009;462(7271):307-14.

47. McCarthy MI. Genomics, type 2 diabetes, and obesity. N Engl J Med. 2010;363(24):2339-50.

48. O'Rahilly S, Farooqi IS. Genetics of obesity. Philos Trans R Soc Lond B Biol Sci. 2006;361(1471):1095-105.

49. Staiger H, Machicao F, Fritsche A, Haring HU. Pathomechanisms of type 2 diabetes genes. Endocr Rev. 2009;30(6):557-85.

50. Stumvoll M, Goldstein BJ, van Haeften TW. Type 2 diabetes: principles of pathogenesis and therapy. Lancet. 2005;365(9467):1333-46.

51. Walley AJ, Asher JE, Froguel P. The genetic contribution to non-syndromic human obesity. Nat Rev Genet. 2009;10(7):431-42.

52. Barker DJ, Winter PD, Osmond C, Margetts B, Simmonds SJ. Weight in infancy and death from ischaemic heart disease. Lancet. 1989;2(8663):577-80.

53. Jackson AA, Burdge GC, Lillycrop KA. Diet, nutrition and modulation of genomic expression in fetal origins of adult disease. World Rev Nutr Diet. 2010;101:56-72.

54. Jaenisch R, Bird A. Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. Nat Genet. 2003;33 Suppl:245-54.

55. Heerwagen MJ, Miller MR, Barbour LA, Friedman JE. Maternal obesity and fetal metabolic programming: a fertile epigenetic soil. Am J Physiol Regul Integr Comp Physiol. 2010;299(3):R711-R722.

56. Stein AD, Kahn HS, Rundle A, Zybert PA, van der Pal-de Bruin, Lumey LH. Anthropometric measures in middle age after exposure to famine during gestation: evidence from the Dutch famine. Am J Clin Nutr. 2007;85(3):869-76.

57. Hult M, Tornhammar P, Ueda P, Chima C, Bonamy AK, Ozumba B, et al. Hypertension, diabetes and overweight: looming legacies of the Biafran famine. PLoS One. 2010;5(10):e13582.

58. DeFronzo RA. Pathogenesis of type 2 diabetes mellitus. Med Clin North Am. 2004;88(4):787-835, ix.

59. Pereira S, Marliss EB, Morais JA, Chevalier S, Gougeon R. Insulin resistance of protein metabolism in type 2 diabetes. Diabetes. 2008;57(1):56-63.

60. Alberti KG, Eckel RH, Grundy SM, Zimmet PZ, Cleeman JI, Donato KA, et al. Harmonizing the metabolic syndrome: a joint interim statement of the International Diabetes Federation Task Force on Epidemiology and Prevention; National Heart, Lung, and Blood Institute; American Heart Association; World Heart Federation; International Atherosclerosis Society; and International Association for the Study of Obesity. Circulation. 2009;120(16):1640-45.

61. Lahiry P, Pollex RL, Hegele RA. Uncloaking the genetic determinants of metabolic syndrome. J Nutrigenet Nutrigenomics. 2008;1(3):118-25.

62. Colagiuri S, Brand MJ. The 'carnivore connection'--evolutionary aspects of insulin resistance. Eur J Clin Nutr. 2002;56 Suppl 1:S30-S35.

63. Miller JC, Colagiuri S. The carnivore connection: dietary carbohydrate in the evolution of NIDDM. Diabetologia. 1994;37(12):1280-6.

64. Galic S, Oakhill JS, Steinberg GR. Adipose tissue as an endocrine organ. Mol Cell Endocrinol. 2010;316(2):129-39.

65. Pasquali R, Patton L, Gambineri A. Obesity and infertility. Curr Opin Endocrinol Diabetes Obes. 2007;14(6):482-7.

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REVIEW

Gene expression profiling in acute myeloid leukaemia

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ABSTRACT

Acute myeloid leukaemia (AML) is a heterogeneous disease characterised by clonal malignant haematopoiesis with a differentiation arrest and excessive proliferation of leukaemic blasts. Over the past decades, the heterogeneity of AML has been illustrated by evolving classifications based on morphology (French-American-British classification (FAB classification), cytogenetic abnormalities (e.g. t(8;21), monosomies etc.), phenotype and/or molecular abnormalities (e.g. Fms-like tyrosine kinase 3 gene internal tandem duplication (*FLT3*-ITD), mutations in nucleophosmin 1 (*NPM1*) and the transcription factor CCAAT/enhancer binding protein α (*CEBPA*), etc.). The current World Health Organisation (WHO) 2008 classification has integrated these classification modalities. Clinically, dissection of AML into various subtypes allows better survival prediction, but has still limited impact on treatment strategies, with the exception of all-*trans* retinoic acid treatment for AML-M3 and no allogeneic haematopoietic cell transplantation in complete remission (CR1) for patients with normal karyotype bearing an *NPM1* mutation without *FLT3*-ITD. However, enhanced understanding of the molecular biology of AML will likely result in more 'tailor-made' therapies, for example by adding specific tyrosine kinase inhibitors to standard chemotherapy.

In this review, we summarise the variables currently used to classify AML. Specifically, the contribution of microarrays in classification, prognosis and understanding of pathobiology of AML is discussed.

KEYWORDS

Acute myeloid leukaemia, gene expression profiling, microarray, prognostic factors

INTRODUCTION

ACUTE MYELOID LEUKAEMIA

Acute myeloid leukaemia (AML) is defined as a clonal disorder caused by malignant transformation of a bone marrow-derived, self-renewing stem or progenitor cell, which demonstrates an enhanced proliferation as well as aberrant differentiation resulting in haematopoietic insufficiency (i.e. granulocytopenia, thrombocytopenia or anaemia).[1,2] The clinical signs and symptoms of AML are diverse and nonspecific, but they are usually directly caused by the leukaemic infiltration of the bone marrow, with resultant cytopenia.[2] AML is considered to be a heterogeneous group of disorders with variable underlying abnormalities and clinical behaviour, including responses to treatment. Therefore, classification of the disease is important and several classification systems exist to subdivide AML.

FAB classification

Historically, AMLs were divided into subtypes based on the type of cell from which the leukaemia developed and the level of maturation (i.e. French-American-British (FAB) classification).[1-3] In addition, cytogenetic analysis of leukaemic blasts has resulted in the identification of non-random clonal chromosomal aberrations, of which some have been correlated to specific FAB subtypes (e.g. t(15;17) with AML-M3).

WHO classification

Nowadays, the World Health Organization (WHO) provides a classification system in which morphology, cytogenetics, molecular genetics, and immunological markers are incorporated and interrelated.[4] Recently, for the first time, specific gene mutations (i.e. mutations in *CEBPA* and *NPM1*) have been included as 'provisional entities' in

the revised WHO 2008 classification for AML.[5] There is growing evidence that these two gene mutations represent primary genetic lesions (so-called class II mutations) that impair haematopoietic differentiation.[6] Mutations in the fms-related tyrosine kinase 3 (*FLT3*) gene (e.g. *FLT3*-ITD or *FLT3* kinase domain mutations) are considered class I mutations conferring a proliferation and/or survival advantage. AML with *FLT3* mutations is not considered a distinct entity, although determining the presence of such mutations is recommended because they have prognostic significance.[7]

Prognostic factors

A number of clinical and biological features that reflect the heterogeneity of AML are used to predict the likelihood that a patient will have a response to treatment or relapse. Adverse prognostic factors in AML include increasing age, a poor performance before treatment, unfavourable cytogenetic abnormalities and a high white blood cell count.[1,2,8-10] Furthermore, therapy-related AML or AML arising after a myelodysplastic or myeloproliferative syndrome is usually more resistant to standard treatment than *de novo* AML.[11,12]

Cytogenetics

Important predictors of disease outcome are the pre-treatment cytogenetic and molecular findings in AML blasts.[2,13-20] To date, in AML approximately 200 different structural and numerical aberrations have been described.[7,20] Cytogenetic findings permit patient risk to be categorised as favourable, intermediate or unfavourable, with very different cure rates.[2,3,13-15,18,20-25] Although there may be (subtle) differences in the criteria used to define these risk groups among different study groups, the presence of for instance t(8;21)(q22;q22), t(15;17)(q22;q21) and inv16(p13q22)/t(16;16)(p13;q22) is generally classified as favourable-risk AML (with leucocytes <20 x 10^9). On the other end of the spectrum is the unfavourable-risk group, which includes blasts showing e.g. monosomies of chromosome 5 or 7, deletion of the long arm of chromosomes 3, 5 and 7 and complex karyotypes. Of note, the monosomal karyotype, defined as non-core-binding factor (CBF) leukaemias with a karyotype with at least two autosomal monosomies or one single autosomal monosomy in the presence of one or more structural cytogenetic abnormalities, is considered to be a better predictor of (very) poor outcome than the traditionally defined complex karyotype.[26] The intermediate-risk group includes AMLs with a normal karyotype and AMLs which are not classified in the other two risk groups.

Molecular genetics

In recent years, the discovery of mutations in e.g. genes encoding *FLT3*, *NPM1* and *CEBPA* has shown to be of major importance (*table 1*). Nowadays, it is increasingly possible to distinguish subsets of patients with differing outcomes from the large cohort with a normal karyotype AML or miscellaneous cytogenetic abnormalities considered as intermediate-risk cytogenetics. The majority of *FLT3* receptor tyrosine kinase gene mutations are internal tandem duplications (ITD); less frequent are mutations involving the tyrosine kinase domain (TKD). Several groups have consistently reported that *FLT3*-ITD is a major independent adverse risk factor in AML.[27-31] The prognostic relevance of *FLT3*-TKD mutations, however, remains controversial.[7] *FLT3*-ITD has a prevalence of 20 to 25% in young adults and nearly 35% in the older adult population. The ratio of the *FLT3*-ITD and the wild-type *FLT3* (measured by polymerase chain reaction, PCR) varies from patient to patient, and this difference may have clinical implications. Thiede *et al.* found that patients with an allelic ratio (AR) above the median (0.78) had significantly shorter overall and disease-free survival, whereas survival in patients with ratios below 0.78 did not differ from those without *FLT3* aberrations.[27] *CEBPA*, a transcription factor involved in normal myelopoiesis, is mutated in ~10% of AML cases and predicts a relatively favourable outcome in paediatric

Table 1. *Recurrent molecular abnormalities in adult AML*

Gene mutation	Percentage of cases	Prognostic significance	Reference*
Fms-related tyrosine kinase 3 (FLT3), internal tandem duplication (ITD)	20-35	Unfavourable	27-31
CCAAT/enhancer binding protein alpha (CEBPA)	5-10	Favourable, when mutated on both alleles	32-37
Nucleophosmin (NPM1)	25-35	Favourable in absence of FLT3-ITD	34,35,38,39
Wilms tumour 1 (WT1)	10-13	Unfavourable?	40-42
RAS	~15	-	34
Cytosolic isocitrate dehydrogenase 1/2 (IDH1, IDH2)	10-25	In subsets unfavourable?	47-50
Tet oncogene family member 2 (TET2)	12-20	Unfavourable?	51-53
KIT	2-8	Unfavourable?	54-58
DNA (cytosine-5-)-methyltransferase 3 alpha (DNMT3A)	22	Unfavourable?	59
Protein tyrosine phosphatase, non-receptor type 11 (PTPN11)	<5	-	60
Runt related transcription factor 1 (RUNX1)	<5	-	60

***Due to space limitations, only a selected number are given for each abnormality.**

and adult AML, however, only when *CEBPA* is mutated on both alleles.[32-37] Approximately 50% of adult normal karyotype AMLs harbour an *NPM1* mutation, which leads to delocalisation of the NPM1 protein to the cytoplasm.[38] *NPM1* and *FLT3*-ITD commonly co-exist in normal karyotype AML suggesting that they may cooperate in generating the leukaemic phenotype. The presence of an *NPM1* mutation (in the absence of an *FLT3*-ITD mutation) is associated with better outcome in terms of higher complete response rates and increased long-term survival compared with patients lacking the mutation.[34,35,39] Consequently, it has been suggested that cytogenetically normal AML involving the genotype of mutant *NPM1* without *FLT3*-ITD should no longer be classified as intermediate-risk leukaemia but rather should be classified as favourable-risk leukaemia.[35] Furthermore, patients with mutant *NPM1* without *FLT3*-ITD may not benefit from related-donor transplantation as first-line treatment.[35] Mutations in the Wilms' tumour gene (*WT1*), present in ~10% of patients with normal karyotype AML, have been found to be associated with poor outcome, especially in combination with an *FLT3*-ITD.[40-43] RAS mutations, occurring in ~15% of cases, are suggested to be prognostically neutral.[34]

Recently, mutations in genes involved in metabolism have been discovered.[44,45] In AML, but also in low-grade gliomas and secondary glioblastoma multiforme (GBM), mutations in cytosolic isocitrate dehydrogenase 1 (*IDH1*) and its mitochondrial homolog *IDH2* have been identified. Both IDH1 and IDH2 are important enzymes in the citrate cycle (Krebs cycle). Two distinct alterations are caused by the tumour-derived mutations in *IDH1* or *IDH2*: loss of its normal catalytic activity in the production of α-ketoglutarate (α-KG) and gain of the catalytic activity to produce 2-hydroxygulatrate (2-HG). Consequently, less α-ketoglutarate is available for biological processes in which it functions as a co-factor. Remarkably, *IDH1/2* mutations, occurring in ~10 to 25% of AML cases,[47-50] were mutually exclusive with mutations in gene encoding the a-ketoglutarate-dependent enzyme tet oncogene family member 2 (*TET2*) (occurring in 12 to 20% of AML cases).[51-53] Loss-of-function mutations in *TET2* were associated with similar epigenetic defects as *IDH1/2* mutants. Interestingly, a shared proleukaemogenic effect between *TET2* mutations and mutations in *IDH1* and *IDH2* was suggested since α-ketoglutarate is a co-factor for *TET2* in the hydroxylation of 5-methylcytosine and thus effects the methylation process.[46]

In cytogenetically favourable core binding factor (CBF AML (i.e. AML with t(8;21) or inv(16)/t(16;16)), the presence of a mutation in the *KIT* receptor tyrosine kinase has been shown to have an unfavourable influence on outcome in retrospective studies.[54-58] Recently, highly recurrent mutations in the DNA methyltransferase gene DNMT3A have been discovered and were found to be independently associated with poor outcome in AML.[59] Other mutations as those involving protein tyrosine phosphatase, non-receptor type 11 (*PTPN11*) and runt-related transcription factor 1 (*RUNX1*) are relatively rare (i.e. <5% of cases), making their relevance to risk-stratified treatment approaches uncertain at the present time.[60]

Effect of over-expressed genes on outcome

Quantitative expression levels of several genes (e.g. Brain And Acute Leukaemia Cytoplasmic gene *BAALC*),[61-63] Ets-related gene (*ERG*),[64,65] Meningioma-1 gene (*MN1*),[66,67] and Ecotropic Viral Integration-1 gene (*EVI1*)[68-70] have been shown to carry prognostic information in patients with (normal karyotype) AML (*table 2*). Except for *EVI1*, the molecular basis of up-regulation of these genes remains, however, poorly understood. Recently, it was shown that expression levels of *ERG*, *BAALC* and *MN1* are strongly correlated, which suggests that their prognostic significance may be overlapping.[64] Several studies have evaluated the prognostic significance of expression of multidrug resistance (MDR) genes with varying conclusions.[71-74] Expression of factors that may relate to interaction of leukaemic cells with bone marrow microenvironment (e.g. vascular endothelial growth factor A (*VEGFA*), and chemokine (C-X-C motif) receptor 4 (CXCR4)) as well as *VEGFC* have also been found to impact on outcome.[75-79] Finally, high expression of $p16^{INK4A}$ was found as a prognostic parameter for overall survival in older patients with AML.[80]

Table 2. *Effect of quantitative expression levels of genes on outcome*

Gene overexpression	Percentage of cases*	Prognostic significance	Reference*
Brain and acute leukaemia cytoplasmic gene (*BAALC*)	~50	Unfavourable	61-63
Ets-related gene (*ERG*)	~25	Unfavourable	64,65
Meningioma-1 gene (*MN1*)	~25-50	Unfavourable	66,67
Ecotropic viral integration-1 gene (*EVI1*)	6-11	Unfavourable	68-70
Chemokine (C-X-C motif) receptor 4 (CXCR4)	~33	-	77,78
Vascular endothelial growth factor C (*VEGFC*)	~50	Unfavourable	79
Cyclin-dependent kinase inhibitor 2A (*CDKN2A*, $p16^{INK4A}$)	~75	Unfavourable	80

Due to space limitations, only a selected number are given for each abnormality. * in case of overexpression, the percentage is based on the cut-off used in the referenced papers. This may involve simple dichotomisation (e.g. *BAALC*), resulting in 50% of the cases by definition exhibiting overexpression. Of note, also continuous expression levels of *VEGFC* correlated with poor outcome.

GENE EXPRESSION PROFILING

Although an increasing number of prognostically relevant (cyto) genetic variables have been identified in AML, not all cases are currently classified adequately. To date, tremendous evidence exists that DNA microarray-based gene expression profiling adds an important new facet to the study of AML, e.g. in relation to classification opportunities. In the past decade, microarrays, together with the availability of the complete nucleotide sequence of the human genome, have made it possible to measure expression levels of thousands of different mRNA transcripts simultaneously.[81-84] There are several (potential) applications for gene expression profiling (GEP) studies. GEP studies are well suited to reveal characteristic patterns (signatures) of activation or silencing or both of multiple genes that may reflect underlying biology of disease subtypes. Subsequently, this may provide diagnostic/prognostic information, and potentially reveal novel molecular targets for therapeutic intervention.

Prediction of known classes: 'class prediction'

In an early landmark study in 1999, researchers described for the first time the power of GEP in leukaemias.[85] In that particular study, GEP profiles were used to distinguish AML samples from those with acute lymphoblastic leukaemia in an unsupervised approach. Of note, the grouping of cases according to similar gene expression profiles is known as clustering.[86,87] Clustering in an unsupervised approach is done in an unbiased way, i.e. without the use of external information such as patient baseline characteristics, mutations or cytogenetics. Class prediction refers to the possibility to predict leukaemia subtypes, as defined by their phenotypes and genotypes, with the use of GEP signatures. For instance, it was demonstrated that the prognostically favourable AML subtypes (i.e. t(8;21), t(15;17) and inv(16)) have distinctive GEP profiles which have consistently been found to be predictable with almost 100% accuracy using GEP.[85,88-96] Interestingly, paediatric AML GEP profiles could also be used to predict adult AML samples with identical cytogenetic abnormalities.[90] In addition, GEP profiles have a high accuracy to predict subgroups with rare translocations, as shown for the t(8;16)(p11;p13) with CBP and MOZ (monocytic leukemia zinc finger protein) re-arrangements.[97,98] Moreover, unsupervised clustering revealed that mutations in *CEBPA* and also *NPM1* correlated with gene expression signatures.[92,99] However, the accuracy of prediction for other cytogenetic AML subsets, such as those with abnormalities involving band 11q23, abnormalities involving 3q, -5/5q-, -7/7q- or t(9;22) was lower.[88,89,93] Similarly, the prediction accuracy for specific molecular subsets of patients such as those harbouring *FLT3*-ITD, *FLT3*-TKD and mutations in *KRAS* and *NRAS* genes was lower.[93,100]

Prediction of new AML subgroups: 'class discovery'

GEP studies also have the potential to uncover new subgroups in AML.[88,92,101] This procedure is representative of class discovery. For example, Valk and colleagues identified 16 subgroups in 285 AMLs, several of which lacked previously known denominators.[92] In addition, at least five other GEP studies revealed previously unrecognised heterogeneity within established paediatric as well as adult AML subtypes.[88,90,102,103] Recently, it was demonstrated that a subset of AML patients who did not harbour *CEBPA* mutations could be characterised by a GEP signature resembling that of AML patients with *CEBPA* mutations.[104] Interestingly, further experiments revealed that in these cases, *CEBPA* was epigenetically silenced, which indicates that the detection of a distinct gene expression subtype had indeed led to the discovery of a biologically meaningful subgroup.

From a clinical point of view, one of the most important challenges in AML is to enlarge insight into the pathobiology of AML in the elderly. In recent decades, survival of paediatric and adult AML patients has improved significantly, while survival of older AML patients (>60 years) has remained virtually unchanged over the past decades resulting from the combination of poor chemotherapeutic tolerance and inherent chemotherapy resistance compared with younger AML patients.[1,2,15] Moreover, AML in older patients shows a lower frequency of favourable core-binding chromosomal abnormalities and a higher incidence of complex aberrant karyotypes. Recently, two studies showed that older patients with AML show distinct GEP signatures compared with younger patients with AML.[80,105] The latter study described that, unlike healthy cells, AML-derived blasts show a down-regulation of $p16^{INK4A}$ mRNA with increasing age. Based on this observation it was hypothesised that suppression of defence mechanisms which protect older cells against cellular and DNA damage might facilitate oncogenesis in older individuals.[80,106]

So, GEP could help researchers to discover hidden heterogeneity within AML subtypes.

GEP and predicting outcome in AML

GEP has also been applied to derive prognostic signatures for AML that would identify subsets of patients with differing outcomes. In these studies treatment outcome or resistance were used to define a prognostic predictor.[107,108] Hierarchical clustering analysis in 93 patients with core-binding factor AML revealed the stratification of two clusters with significantly different survival.[102] In cytogenetically normal AML, Bullinger *et al.* were able to divide cytogenetically normal samples into two diverse prognostically relevant clusters using GEP.[88] Importantly, the prognostic impact of this signature was independently validated in another cohort of AML samples using a

different platform and a longer follow-up.[109] Of note, the prognostic effect of the signature was in part related to the occurrence of *FLT3*-ITD mutations, only 81 of 133 probes could be validated due to differences in platforms and the prediction accuracy of the classifier was overall modest, with approximately 60% of the patients having their outcome predicted correctly.[109,110] Recently, another study in cytogenetically normal karyotype AMLs revealed a gene signature of 86-probe sets correlating significantly with overall survival.[111] The prognostic effect of this classifier was independent of age, *FLT3*-ITD and *NPM1* mutation status. In paediatric AML, a GEP study in 54 AML patients revealed 36 probe sets to be associated with prognosis.[112] However, in an independent paediatric AML GEP study this prognostic signature could not be confirmed.[90]

Remarks and limitations

Gene expression analysis can be performed on microarray platforms with varying kinds of probes (cDNA, short-oligonucleotide, long-oligonucleotide, etc.), production and labelling method (microbeads, spotting, in situ polymerisation, etc.). Specificity is highest for DNA-oligonucleotide microarrays of 40-60-mer probe length as they have a lower risk of cross-hybridisation.[113] The widely-used Affymetrix microarrays rely on 25-mer *in situ* synthesised probes.[114] The interpretation of the fluorescence intensity signals requires sophisticated computational methods for data normalisation and classification,[115] because each study generates large datasets. GEP is a multistep procedure that can only be briefly outlined here. Initially, data pre-processing and quality control steps are performed for detection of array artefacts and the evaluation of the homogeneity of experimental groups. Furthermore, it is important to be aware of interstudy variations with regard to data normalisation, gene filtering and clustering procedures, which could influence the outcome of the analysis.[84,116] Notably, significant efforts have led to the establishment of proposed guidelines to describe the minimum information about a microarray experiment (MIAME) that is needed to enable the interpretation of the results of the experiment unambiguously and potentially to reproduce the experiment. This is particularly important information if microarray data are deposited in a public database, such as the Gene Expression Omnibus.[117,118]

GEP holds promise for developing molecular portraits of cancer subtypes with different clinical outcomes that could not be sub-classified or identified upon (initial) clinical presentation. One of the possible challenges in GEP studies is the (low) number of samples as compared with the number of genes tested, the so-called 'curse of dimensionality' (i.e. overfitting).[119] In addition, there may be small numbers of genes whose expression discriminate cancer subtypes but they may not be driving causes of cancer initiation/ progression and therefore provide little survival information. Another not surprising issue is that independent studies can identify different panels of genes with similar discriminatory specificity and power. Furthermore, the number of genes expected to be differentially expressed between two (or more) classes of interest within a single cancer subtype is probably small, and the differences in expression may not be large (enough) in relation to experimental noise.[120] We have introduced the concept of TSR profiling that might improve the performance of predictive profiles.[121] These transcriptional system regulators (TSRs) allowed one to characterise the expression profile of an individual microarray with just 50 TSR scores instead of using ten thousands of individual genes: i.e. a >500-fold reduction of complexity, thus avoiding the problem of overfitting. There is a second advantage of TSR profiling: i.e. when signals of multiple genes are added to calculate TSR scores the signal-to-noise ratio improves because noise cancels out. Further studies are needed to investigate whether TSR scores may be more reproducible input variables for prediction models than expression signals of selected individual genes.

Biology versus statistics

A pending question in GEP studies is whether large-fold changes in individual genes have more biological relevance than smaller but coordinated fold-changes in a set of genes (particularly along a single biological pathway). The assumption that (only) changes of more than twofold are significant is still surprisingly widespread.[122] This threshold is based on initial publications by the Stanford group who found, from concordance analyses, that a more than twofold variation was significant for a particular set of experiments.[123] This factor of two was subsequently referred to by others as a universal significance threshold, without realising its development. Moreover, in principle, the particular changes in gene expression between classes of samples may be less informative than the pathways they impact. Finally, it is important to realise that relative levels of mRNA expression do not necessarily reflect biological activity, as the latter may be highly dependent on other factors, such as posttranslational modifications.

Clinical application

Following the introduction of GEP in leukaemia research a decade ago by Golub and colleagues, various study groups worldwide have consistently shown that GEP can be used to predict molecularly defined subtypes of AML.[124-128] However, from a clinical point of view, several questions surround GEP in AML: e.g. can GEP improve current diagnostics and risk classification schemes in AML, or the ability to predict outcome in AML patients beyond that currently provided by well-established

prognostic variables such as age, presenting white blood cell count and the presence of cytogenetic or molecular (e.g. mutations) abnormalities? To be able to answer such questions properly at least two important prerequisites should be met. Firstly, appropriate validation of GEP results in independent (prospective) study cohorts is needed. Secondly, for successful subgroup discovery it is crucial to have access to sufficiently large series of cases representing the various subtypes of AML. It may be unlikely that gene expression arrays will be used to diagnose cytogenetic and molecular abnormalities in the clinical setting when direct diagnostic assays are available and are more cost-effective.[129] However, it is important to realise that the particular value of GEP-based classification lies in its comprehensiveness (i.e. the ability to measure tens of thousands of transcripts at one time) and its possibility to uncover (hidden) heterogeneity (e.g. related to differing outcome) within established cytogenetic and/or molecular subtypes of AML. However, the latter is highly dependent on the availability of high-quality samples and robustly annotated clinical data, which often have to be collected over many years. Ultimately, once intensively (prospectively) validated and standardised, measuring a panel of selected genes in combination with clinical (e.g. age, WBC count) and established variables (e.g. cytogenetics, and mutations) might be of importance in guiding doctors (therapeutic) decisions. Finally, from a cell biological point of view, particular efforts should be directed towards proper understanding of the biological mechanism and regulation of 'genes with prognostic significance'. This aspect will clearly need to be further studied, also in terms of targeted therapy development and testing.

Which cells to profile?

There is not only heterogeneity among AML patients, heterogeneity is also evident within the AML cells of one patient. AML is thought to be initiated and maintained by a few leukaemia-initiating cells (LICs) that have an enhanced self-renewal capacity, can engraft in nonobese diabetic/severe combined immunodeficient mice and are, nowadays, believed to be restricted to the $CD34^+/CD38^-$ or $CD34^+/CD38^+$ fraction.[130-134] However, there is evidence from mouse studies that mixed lineage leukaemia-associated human leukaemias can also arise from more progenitor cells.[135,136] Furthermore, a recent study suggested that for some *NPM1* mutated AMLs the LICs are also present in the $CD34^-$ fraction.[137] Most AML GEP studies, however, have been performed with the total AML mononuclear cell (MNC) fraction. Because cell lineage and differentiation stages might (theoretically) affect gene-expression based clustering, the differential expression of genes associated with the differentiation stage might obscure more basic gene expression information related to tumour initiation and maintenance. Consequently, profiling of more purified cell populations, instead of total MNC fractions, might enhance the possibilities of GEP in identifying novel prognostic markers or subgroup discovery.[138] However, this approach directly depends on the accepted definition of immunophenotypic markers of leukaemia-initiating cells. Finally, there is compelling emerging evidence that cell nonautonomous contributions to leukaemia play a pivotal role in disease maintenance and propagation (i.e. the microenvironment, the niche).[75]

CONCLUSIONS AND FUTURE PERSPECTIVES

Gene expression profiling using microarrays is currently the standard for analysing the transcriptome. However, profiling of e.g. microRNA (miRNA) levels, chromosomal copy number changes and epigenetic modifications have also played a pivotal role in enhanced molecular understanding of the (patho)biology of cancer, including AML. For example, similarly to mRNA profiling, miRNA profiling has revealed that specific subgroups of AML share distinctive miRNA signatures with prognostic significance.[139-142] Furthermore, methylation profiling of a large series of AML patients identified several clusters, of which some could not be explained by the enrichment of any currently known recurrent cytogenetic, molecular, or clinical features.[143] In recent times, next-generation sequencing (NGS) technologies have become available that enable gene expression analysis by direct shotgun sequencing of complementary DNA synthesised from RNA samples.[144-147] NGS technologies have an impressive range of applications, and are increasingly being developed. In contrast to microarrays, sequencing technologies do not depend on predefined sequences, thus allowing for detection of, for example, new splicing variants or single-nucleotide polymorphisms. Furthermore, it allows genome-wide profiling of epigenetic marks.[148] It is hypothesised that in the near future, NGS technologies could be used to obtain high-quality sequence data from a genome isolated from a single cell, which would be a substantial breakthrough, particularly for cancer genomics.[149] Once we know the genomic landscape of cancer more adequately, what should follow? While genome-wide characterisation of cancer subtypes will likely reveal significant clues about genes that play a role in cancer progression, it is important to follow-up on these clues by carrying out functional screens of altered genes. Functional screening would aim to identify those (somatic) alterations that are imperative in tumour initiation and progression. Furthermore, functionally relevant mutations must be distinguished from passenger

mutations (i.e. unimportant genetic changes caused by genomic instability of cancer cells). Finally, functional screening may establish candidate genes and their protein products for targeted therapy development or testing, as well as for diagnostic/prognostic assay development.

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REFERENCES

1. Estey E, Dohner H. Acute myeloid leukaemia. Lancet. 2006;368(9550):1894-907.
2. Lowenberg B, Downing JR, Burnett A. Acute myeloid leukemia. N Engl J Med. 1999;341(14):1051-62.
3. Bennett JM, Catovsky D, Daniel MT, Flandrin G, Galton DA, Gralnick HR, et al. Proposed revised criteria for the classification of acute myeloid leukemia. A report of the French-American-British Cooperative Group. Ann Intern Med. 1985;103(4):620-5.
4. Vardiman JW, Harris NL, Brunning RD. The World Health Organization (WHO) classification of the myeloid neoplasms. Blood. 2002;100(7):2292-302.
5. Vardiman JW, Thiele J, Arber DA, Brunning RD, Borowitz MJ, Porwit A, et al. The 2008 revision of the World Health Organization (WHO) classification of myeloid neoplasms and acute leukemia: rationale and important changes. Blood. 2009;114(5):937-51.
6. Kelly LM, Gilliland DG. Genetics of myeloid leukemias. Annu Rev Genomics Hum Genet. 2002;3:179-98.
7. Dohner H, Estey EH, Amadori S, Appelbaum FR, Buchner T, Burnett AK, et al. Diagnosis and management of acute myeloid leukemia in adults: recommendations from an international expert panel, on behalf of the European LeukemiaNet. Blood. 2010;115(3):453-74.
8. Lowenberg B, Griffin JD, Tallman MS. Acute myeloid leukemia and acute promyelocytic leukemia. Hematology Am Soc Hematol Educ Program. 2003;82-101.
9. Sorror ML, Maris MB, Storer B, Sandmaier BM, Diaconescu R, Flowers C, et al. Comparing morbidity and mortality of HLA-matched unrelated donor hematopoietic cell transplantation after nonmyeloablative and myeloablative conditioning: influence of pretransplantation comorbidities. Blood. 2004;104(4):961-8.
10. Stone RM, O'Donnell MR, Sekeres MA. Acute myeloid leukemia. Hematology Am Soc Hematol Educ Program. 2004;98-117.
11. Estey E, Thall P, Beran M, Kantarjian H, Pierce S, Keating M. Effect of diagnosis (refractory anemia with excess blasts, refractory anemia with excess blasts in transformation, or acute myeloid leukemia [AML]) on outcome of AML-type chemotherapy. Blood. 1997;90(8):2969-77.
12. Larson RA. Is secondary leukemia an independent poor prognostic factor in acute myeloid leukemia? Best Pract Res Clin Haematol. 2007;20(1):29-37.
13. Byrd JC, Mrozek K, Dodge RK, Carroll AJ, Edwards CG, Arthur DC, et al. Pretreatment cytogenetic abnormalities are predictive of induction success, cumulative incidence of relapse, and overall survival in adult patients with de novo acute myeloid leukemia: results from Cancer and Leukemia Group B (CALGB 8461). Blood. 2002;100(13):4325-36.
14. Grimwade D, Walker H, Oliver F, Wheatley K, Harrison C, Harrison G, et al. The importance of diagnostic cytogenetics on outcome in AML: analysis of 1,612 patients entered into the MRC AML 10 trial. The Medical Research Council Adult and Children's Leukaemia Working Parties. Blood. 1998;92(7):2322-33.
15. Kaspers GJ, Zwaan CM. Pediatric acute myeloid leukemia: towards high-quality cure of all patients. Haematologica. 2007;92(11):1519-32.
16. Keating MJ, Smith TL, Kantarjian H, Cork A, Walters R, Trujillo JM, et al. Cytogenetic pattern in acute myelogenous leukemia: a major reproducible determinant of outcome. Leukemia. 1988;2(7):403-12.
17. Mrozek K, Heinonen K, Bloomfield CD. Clinical importance of cytogenetics in acute myeloid leukaemia. Best Pract Res Clin Haematol. 2001;14(1):19-47.
18. Slovak ML, Kopecky KJ, Cassileth PA, Harrington DH, Theil KS, Mohamed A, et al. Karyotypic analysis predicts outcome of preremission and postremission therapy in adult acute myeloid leukemia: a Southwest Oncology Group/Eastern Cooperative Oncology Group Study. Blood. 2000;96(13):4075-83.
19. Yunis JJ, Brunning RD, Howe RB, Lobell M. High-resolution chromosomes as an independent prognostic indicator in adult acute nonlymphocytic leukemia. N Engl J Med. 1984;311(13):812-8.
20. Mrozek K, Heerema NA, Bloomfield CD. Cytogenetics in acute leukemia. Blood Rev. 2004;18(2):115-36.
21. Grimwade D, Walker H, Harrison G, Oliver F, Chatters S, Harrison CJ, et al. The predictive value of hierarchical cytogenetic classification in older adults with acute myeloid leukemia (AML): analysis of 1065 patients entered into the United Kingdom Medical Research Council AML11 trial. Blood. 2001;98(5):1312-20.
22. Leverger G, Bernheim A, Daniel MT, Flandrin G, Schaison G, Berger R. Cytogenetic study of 130 childhood acute nonlymphocytic leukemias. Med Pediatr Oncol. 1988;16(4):227-32.
23. Martinez-Climent JA, Lane NJ, Rubin CM, Morgan E, Johnstone HS, Mick R, et al. Clinical and prognostic significance of chromosomal abnormalities in childhood acute myeloid leukemia de novo. Leukemia. 1995;9(1):95-101.
24. Raimondi SC, Kalwinsky DK, Hayashi Y, Behm FG, Mirro J, Jr., Williams DL. Cytogenetics of childhood acute nonlymphocytic leukemia. Cancer Genet Cytogenet. 1989;40(1):13-27.
25. Raimondi SC, Chang MN, Ravindranath Y, Behm FG, Gresik MV, Steuber CP, et al. Chromosomal abnormalities in 478 children with acute myeloid leukemia: clinical characteristics and treatment outcome in a cooperative pediatric oncology group study-POG 8821. Blood. 1999;94(11):3707-16.
26. Breems DA, van Putten WL, de Greef GE, Zelderen-Bhola SL, Gerssen-Schoorl KB, Mellink CH, et al. Monosomal karyotype in acute myeloid leukemia: a better indicator of poor prognosis than a complex karyotype. J Clin Oncol. 2008;26(29):4791-7.
27. Thiede C, Steudel C, Mohr B, Schaich M, Schakel U, Platzbecker U, et al. Analysis of FLT3-activating mutations in 979 patients with acute myelogenous leukemia: association with FAB subtypes and identification of subgroups with poor prognosis. Blood. 2002;99(12):4326-35.
28. Schnittger S, Schoch C, Dugas M, Kern W, Staib P, Wuchter C, et al. Analysis of FLT3 length mutations in 1003 patients with acute myeloid leukemia: correlation to cytogenetics, FAB subtype, and prognosis in the AMLCG study and usefulness as a marker for the detection of minimal residual disease. Blood. 2002;100(1):59-66.
29. Kottaridis PD, Gale RE, Frew ME, Harrison G, Langabeer SE, Belton AA, et al. The presence of a FLT3 internal tandem duplication in patients with acute myeloid leukemia (AML. adds important prognostic information to cytogenetic risk group and response to the first cycle of chemotherapy: analysis of 854 patients from the United Kingdom Medical Research Council AML 10 and 12 trials. Blood. 2001;98(6):1752-9.
30. Kiyoi H, Towatari M, Yokota S, Hamaguchi M, Ohno R, Saito H, et al. Internal tandem duplication of the FLT3 gene is a novel modality of elongation mutation which causes constitutive activation of the product. Leukemia. 1998;12(9):1333-7.

31. Kiyoi H, Naoe T, Nakano Y, Yokota S, Minami S, Miyawaki S, et al. Prognostic implication of FLT3 and N-RAS gene mutations in acute myeloid leukemia. Blood. 1999;93(9):3074-80.

32. Dufour A, Schneider F, Metzeler KH, Hoster E, Schneider S, Zellmeier E, et al. Acute Myeloid Leukemia With Biallelic CEBPA Gene Mutations and Normal Karyotype Represents a Distinct Genetic Entity Associated With a Favorable Clinical Outcome. J Clin Oncol. 2010;28(4):570-7..

33. Marcucci G, Maharry K, Radmacher MD, Mrozek K, Vukosavljevic T, Paschka P, et al. Prognostic significance of, and gene and microRNA expression signatures associated with, CEBPA mutations in cytogenetically normal acute myeloid leukemia with high-risk molecular features: a Cancer and Leukemia Group B Study. J Clin Oncol. 2008;26(31):5078-87.

34. Mrozek K, Marcucci G, Paschka P, Whitman SP, Bloomfield CD. Clinical relevance of mutations and gene-expression changes in adult acute myeloid leukemia with normal cytogenetics: are we ready for a prognostically prioritized molecular classification? Blood. 2007;109(2):431-48.

35. Schlenk RF, Dohner K, Krauter J, Frohling S, Corbacioglu A, Bullinger L, et al. Mutations and treatment outcome in cytogenetically normal acute myeloid leukemia. N Engl J Med. 2008;358(18):1909-18.

36. Wouters BJ, Lowenberg B, Erpelinck-Verschueren CA, van Putten WL, Valk PJ, Delwel R. Double CEBPA mutations, but not single CEBPA mutations, define a subgroup of acute myeloid leukemia with a distinctive gene expression profile that is uniquely associated with a favorable outcome. Blood. 2009;113(13):3088-91.

37. Ho PA, Alonzo TA, Gerbing RB, Pollard J, Stirewalt DL, Hurwitz C, et al. Prevalence and prognostic implications of CEBPA mutations in pediatric acute myeloid leukemia (AML): a report from the Children's Oncology Group. Blood. 2009;113(26):6558-66.

38. Falini B, Mecucci C, Tiacci E, Alcalay M, Rosati R, Pasqualucci L, et al. Cytoplasmic nucleophosmin in acute myelogenous leukemia with a normal karyotype. N Engl J Med. 2005;352(3):254-66.

39. Gale RE, Green C, Allen C, Mead AJ, Burnett AK, Hills RK, et al. The impact of FLT3 internal tandem duplication mutant level, number, size, and interaction with NPM1 mutations in a large cohort of young adult patients with acute myeloid leukemia. Blood. 2008;111(5):2776-84.

40. Gaidzik VI, Schlenk RF, Moschny S, Becker A, Bullinger L, Corbacioglu A, et al. Prognostic impact of WT1 mutations in cytogenetically normal acute myeloid leukemia: a study of the German-Austrian AML Study Group. Blood. 2009;113(19):4505-11.

41. Paschka P, Marcucci G, Ruppert AS, Whitman SP, Mrozek K, Maharry K, et al. Wilms' tumor 1 gene mutations independently predict poor outcome in adults with cytogenetically normal acute myeloid leukemia: a cancer and leukemia group B study. J Clin Oncol. 2008;26(28):4595-602.

42. Virappane P, Gale R, Hills R, Kakkas I, Summers K, Stevens J, et al. Mutation of the Wilms' tumor 1 gene is a poor prognostic factor associated with chemotherapy resistance in normal karyotype acute myeloid leukemia: the United Kingdom Medical Research Council Adult Leukaemia Working Party. J Clin Oncol. 2008;26(33):5429-35.

43. Hollink IH, van den Heuvel-Eibrink MM, Zimmermann M, Balgobind BV, Arentsen-Peters ST, Alders M, et al. Clinical relevance of Wilms tumor 1 gene mutations in childhood acute myeloid leukemia. Blood. 2009;113(23):5951-60.

44. Mardis ER, Ding L, Dooling DJ, Larson DE, McLellan MD, Chen K, et al. Recurring mutations found by sequencing an acute myeloid leukemia genome. N Engl J Med. 2009;361(11):1058-66.

45. Parsons DW, Jones S, Zhang X, Lin JC, Leary RJ, Angenendt P, et al. An integrated genomic analysis of human glioblastoma multiforme. Science. 2008;321(5897):1807-12.

46. Figueroa ME, bdel-Wahab O, Lu C, Ward PS, Patel J, Shih A, et al. Leukemic IDH1 and IDH2 mutations result in a hypermethylation phenotype, disrupt TET2 function, and impair hematopoietic differentiation. Cancer Cell. 2010;18(6):553-67.

47. Abbas S, Lugthart S, Kavelaars FG, Schelen A, Koenders JE, Zeilemaker A, et al. Acquired mutations in the genes encoding IDH1 and IDH2 both are recurrent aberrations in acute myeloid leukemia: prevalence and prognostic value. Blood. 2010;116(12):2122-6.

48. Boissel N, Nibourel O, Renneville A, Gardin C, Reman O, Contentin N, et al. Prognostic impact of isocitrate dehydrogenase enzyme isoforms 1 and 2 mutations in acute myeloid leukemia: a study by the Acute Leukemia French Association group. J Clin Oncol. 2010;28(23):3717-23.

49. Marcucci G, Maharry K, Wu YZ, Radmacher MD, Mrozek K, Margeson D, et al. IDH1 and IDH2 gene mutations identify novel molecular subsets within de novo cytogenetically normal acute myeloid leukemia: a Cancer and Leukemia Group B study. J Clin Oncol. 2010;28(14):2348-55.

50. Paschka P, Schlenk RF, Gaidzik VI, Habdank M, Kronke J, Bullinger L, et al. IDH1 and IDH2 mutations are frequent genetic alterations in acute myeloid leukemia and confer adverse prognosis in cytogenetically normal acute myeloid leukemia with NPM1 mutation without FLT3 internal tandem duplication. J Clin Oncol. 2010;28(22):3636-43.

51. Abdel-Wahab O, Mullally A, Hedvat C, Garcia-Manero G, Patel J, Wadleigh M, et al. Genetic characterization of TET1, TET2, and TET3 alterations in myeloid malignancies. Blood. 2009;114(1):144-7.

52. Delhommeau F, Dupont S, Della Valle V, James C, Trannoy S, Masse A, et al. Mutation in TET2 in myeloid cancers. N Engl J Med. 2009;360(22):2289-301.

53. Nibourel O, Kosmider O, Cheok M, Boissel N, Renneville A, Philippe N, et al. Incidence and prognostic value of TET2 alterations in de novo acute myeloid leukemia achieving complete remission. Blood. 2010;116(7):1132-5.

54. Boissel N, Leroy H, Brethon B, Philippe N, de Botton S, Auvrignon A, et al. Incidence and prognostic impact of c-Kit, FLT3, and Ras gene mutations in core binding factor acute myeloid leukemia (CBF-AML). Leukemia. 2006;20(6):965-70.

55. Cairoli R, Beghini A, Grillo G, Nadali G, Elice F, Ripamonti CB, et al. Prognostic impact of c-KIT mutations in core binding factor leukemias: an Italian retrospective study. Blood. 2006;107(9):3463-8.

56. Care RS, Valk PJ, Goodeve AC, Abu-Duhier FM, Geertsma-Kleinekoort WM, Wilson GA, et al. Incidence and prognosis of c-KIT and FLT3 mutations in core binding factor (CBF) acute myeloid leukaemias. Br J Haematol. 2003;121(5):775-7.

57. Paschka P, Marcucci G, Ruppert AS, Mrozek K, Chen H, Kittles RA, et al. Adverse prognostic significance of KIT mutations in adult acute myeloid leukemia with inv(16) and t(8;21): a Cancer and Leukemia Group B Study. J Clin Oncol. 2006;24(24):3904-11.

58. Schnittger S, Kohl TM, Haferlach T, Kern W, Hiddemann W, Spiekermann K, et al. KIT-D816 mutations in AML1-ETO-positive AML are associated with impaired event-free and overall survival. Blood. 2006;107(5):1791-9.

59. Ley TJ, Ding L, Walter MJ, McLellan MD, Lamprecht T, Larson DE, et al. DNMT3A mutations in acute myeloid leukemia. N Engl J Med. 2010;363(25):2424-33.

60. Grimwade D, Hills RK. Independent prognostic factors for AML outcome. Hematology Am Soc Hematol Educ Program. 2009;385-95.

61. Baldus CD, Tanner SM, Ruppert AS, Whitman SP, Archer KJ, Marcucci G, et al. BAALC expression predicts clinical outcome of de novo acute myeloid leukemia patients with normal cytogenetics: a Cancer and Leukemia Group B Study. Blood. 2003;102(5):1613-8.

62. Baldus CD, Thiede C, Soucek S, Bloomfield CD, Thiel E, Ehninger G. BAALC expression and FLT3 internal tandem duplication mutations in acute myeloid leukemia patients with normal cytogenetics: prognostic implications. J Clin Oncol. 2006;24(5):790-7.

63. Langer C, Radmacher MD, Ruppert AS, Whitman SP, Paschka P, Mrozek K, et al. High BAALC expression associates with other molecular prognostic markers, poor outcome, and a distinct gene-expression signature in cytogenetically normal patients younger than 60 years with acute myeloid leukemia: a Cancer and Leukemia Group B (CALGB) study. Blood. 2008;111(11):5371-9.

64. Metzeler KH, Dufour A, Benthaus T, Hummel M, Sauerland MC, Heinecke A, et al. ERG expression is an independent prognostic factor and allows refined risk stratification in cytogenetically normal acute myeloid leukemia: a comprehensive analysis of ERG, MN1, and BAALC transcript levels using oligonucleotide microarrays. J Clin Oncol. 2009;27(30):5031-8.

65. Marcucci G, Baldus CD, Ruppert AS, Radmacher MD, Mrozek K, Whitman SP, et al. Overexpression of the ETS-related gene, ERG, predicts a worse outcome in acute myeloid leukemia with normal karyotype: a Cancer and Leukemia Group B study. J Clin Oncol. 2005;23(36):9234-42.

66. Heuser M, Beutel G, Krauter J, Dohner K, von Neuhoff N, Schlegelberger B, et al. High meningioma 1 (MN1) expression as a predictor for poor outcome in acute myeloid leukemia with normal cytogenetics. Blood. 2006;108(12):3898-905.

67. Langer C, Marcucci G, Holland KB, Radmacher MD, Maharry K, Paschka P, et al. Prognostic importance of MN1 transcript levels, and biologic insights from MN1-associated gene and microRNA expression signatures in cytogenetically normal acute myeloid leukemia: a cancer and leukemia group B study. J Clin Oncol. 2009;27(19):3198-204.

68. Lugthart S, van Drunen E, van Norden Y, van Hoven A, Erpelinck CA, Valk PJ, et al. High EVI1 levels predict adverse outcome in acute myeloid leukemia: prevalence of EVI1 overexpression and chromosome 3q26 abnormalities underestimated. Blood. 2008;111(8):4329-37.

69. Groschel S, Lugthart S, Schlenk RF, Valk PJ, Eiwen K, Goudswaard C, et al. High EVI1 Expression Predicts Outcome in Younger Adult Patients With Acute Myeloid Leukemia and Is Associated With Distinct Cytogenetic Abnormalities. J Clin Oncol. 2010;28(12):2101-7.

70. Barjesteh van Waalwijk van Doorn-Khosrovani, Erpelinck C, van Putten WL, Valk PJ, van der Poel-van de Luytgaarde, Hack R, et al. High EVI1 expression predicts poor survival in acute myeloid leukemia: a study of 319 de novo AML patients. Blood. 2003;101(3):837-45.

71. Kim DH, Lee NY, Sung WJ, Baek JH, Kim JG, Sohn SK, et al. Multidrug resistance as a potential prognostic indicator in acute myeloid leukemia with normal karyotypes. Acta Haematol. 2005;114(2):78-83.

72. Leith CP, Kopecky KJ, Chen IM, Eijdems L, Slovak ML, McConnell TS, et al. Frequency and clinical significance of the expression of the multidrug resistance proteins MDR1/P-glycoprotein, MRP1, and LRP in acute myeloid leukemia: a Southwest Oncology Group Study. Blood. 1999;94(3):1086-99.

73. Sievers EL, Smith FO, Woods WG, Lee JW, Bleyer WA, Willman CL, et al. Cell surface expression of the multidrug resistance P-glycoprotein (P-170) as detected by monoclonal antibody MRK-16 in pediatric acute myeloid leukemia fails to define a poor prognostic group: a report from the Childrens Cancer Group. Leukemia. 1995;9(12):2042-8.

74. Willman CL. The prognostic significance of the expression and function of multidrug resistance transporter proteins in acute myeloid leukemia: studies of the Southwest Oncology Group Leukemia Research Program. Semin Hematol. 1997;34(4):25-33.

75. Lane SW, Scadden DT, Gilliland DG. The leukemic stem cell niche: current concepts and therapeutic opportunities. Blood. 2009;114(6):1150-7.

76. Meshinchi S, Arceci RJ. Prognostic factors and risk-based therapy in pediatric acute myeloid leukemia. Oncologist. 2007;12(3):341-55.

77. Rombouts EJ, Pavic B, Lowenberg B, Ploemacher RE. Relation between CXCR-4 expression, Flt3 mutations, and unfavorable prognosis of adult acute myeloid leukemia. Blood. 2004;104(2):550-7.

78. Spoo AC, Lubbert M, Wierda WG, Burger JA. CXCR4 is a prognostic marker in acute myelogenous leukemia. Blood. 2007;109(2):786-91.

79. de Jonge HJ, Valk PJ, Veeger NJ, ter EA, den Boer ML, Cloos J, et al. High VEGFC expression is associated with unique gene expression profiles and predicts adverse prognosis in pediatric and adult acute myeloid leukemia. Blood. 2010;116(10):1747-54.

80. de Jonge HJ, de Bont ES, Valk PJ, Schuringa JJ, Kies M, Woolthuis CM, et al. AML at older age: age-related gene expression profiles reveal a paradoxical down-regulation of p16INK4A mRNA with prognostic significance. Blood. 2009;114(14):2869-77.

81. Pollack JR. A perspective on DNA microarrays in pathology research and practice. Am J Pathol. 2007;171(2):375-85.

82. Elvidge G. Microarray expression technology: from start to finish. Pharmacogenomics. 2006;7(1):123-34.

83. Hoheisel JD. Microarray technology: beyond transcript profiling and genotype analysis. Nat Rev Genet. 2006;7(3):200-10.

84. Quackenbush J. Microarray analysis and tumor classification. N Engl J Med. 2006;354(23):2463-72.

85. Debernardi S, Lillington DM, Chaplin T, Tomlinson S, Amess J, Rohatiner A, et al. Genome-wide analysis of acute myeloid leukemia with normal karyotype reveals a unique pattern of homeobox gene expression distinct from those with translocation-mediated fusion events. Genes Chromosomes Cancer. 2003;37(2):149-58.

86. D'haeseleer P. How does gene expression clustering work? Nat Biotechnol. 2005;23(12):1499-501.

87. Eisen MB, Spellman PT, Brown PO, Botstein D. Cluster analysis and display of genome-wide expression patterns. Proc Natl Acad Sci. USA 1998;95(25):14863-8.

88. Bullinger L, Dohner K, Bair E, Frohling S, Schlenk RF, Tibshirani R, et al. Use of gene-expression profiling to identify prognostic subclasses in adult acute myeloid leukemia. N Engl J Med. 2004;350(16):1605-16.

89. Haferlach T, Kohlmann A, Schnittger S, Dugas M, Hiddemann W, Kern W, et al. Global approach to the diagnosis of leukemia using gene expression profiling. Blood. 2005;106(4):1189-98.

90. Ross ME, Mahfouz R, Onciu M, Liu HC, Zhou X, Song G, et al. Gene expression profiling of pediatric acute myelogenous leukemia. Blood. 2004;104(12):3679-87.

91. Schoch C, Kohlmann A, Schnittger S, Brors B, Dugas M, Mergenthaler S, et al. Acute myeloid leukemias with reciprocal rearrangements can be distinguished by specific gene expression profiles. Proc Natl Acad Sci USA.. 2002;99(15):10008-13.

92. Valk PJ, Verhaak RG, Beijen MA, Erpelinck CA, Barjesteh van Waalwijk van Doorn-Khosrovani, Boer JM, et al. Prognostically useful gene-expression profiles in acute myeloid leukemia. N Engl J Med. 2004;350(16):1617-28.

93. Verhaak RG, Wouters BJ, Erpelinck CA, Abbas S, Beverloo HB, Lugthart S, et al. Prediction of molecular subtypes in acute myeloid leukemia based on gene expression profiling. Haematologica. 2009;94(1):131-4.

94. Vey N, Mozziconacci MJ, Groulet-Martinec A, Debono S, Finetti P, Carbuccia N, et al. Identification of new classes among acute myelogenous leukaemias with normal karyotype using gene expression profiling. Oncogene. 2004;23(58):9381-91.

95. Virtaneva K, Wright FA, Tanner SM, Yuan B, Lemon WJ, Caligiuri MA, et al. Expression profiling reveals fundamental biological differences in acute myeloid leukemia with isolated trisomy 8 and normal cytogenetics. Proc Natl Acad Sci USA. 2001;98(3):1124-9.

96. Balgobind BV, van den Heuvel-Eibrink MM, Menezes RX, Reinhardt D, Hollink IH, Peters ST, et al. Evaluation of gene expression signatures predictive for cytogenetic and molecular subtypes of pediatric acute myeloid leukemia. Haematologica. 2010 Epub ahead of print

97. Camos M, Esteve J, Jares P, Colomer D, Rozman M, Villamor N, et al. Gene expression profiling of acute myeloid leukemia with translocation t(8;16)(p11;p13) and MYST3-CREBBP rearrangement reveals a distinctive signature with a specific pattern of HOX gene expression. Cancer Res. 2006;66(14):6947-54.

98. Murati A, Gervais C, Carbuccia N, Finetti P, Cervera N, Adelaide J, et al. Genome profiling of acute myelomonocytic leukemia: alteration of the MYB locus in MYST3-linked cases. Leukemia. 2009;23(1):85-94.

99. Verhaak RG, Goudswaard CS, van Putten W, Bijl MA, Sanders MA, Hugens W, et al. Mutations in nucleophosmin (NPM1) in acute myeloid leukemia (AML): association with other gene abnormalities and previously established gene expression signatures and their favorable prognostic significance. Blood. 2005;106(12):3747-54.

100. Bullinger L, Dohner K, Kranz R, Stirner C, Frohling S, Scholl C, et al. An FLT3 gene-expression signature predicts clinical outcome in normal karyotype AML. Blood. 2008;111(9):4490-5.

101. Wilson CS, Davidson GS, Martin SB, Andries E, Potter J, Harvey R, et al. Gene expression profiling of adult acute myeloid leukemia identifies novel biologic clusters for risk classification and outcome prediction. Blood. 2006;108(2):685-96.

102. Bullinger L, Rucker FG, Kurz S, Du J, Scholl C, Sander S, et al. Gene-expression profiling identifies distinct subclasses of core binding factor acute myeloid leukemia. Blood. 2007;110(4):1291-300.

103. Bourquin JP, Subramanian A, Langebrake C, Reinhardt D, Bernard O, Ballerini P, et al. Identification of distinct molecular phenotypes in acute megakaryoblastic leukemia by gene expression profiling. Proc Natl Acad Sci. USA. 2006;103(9):3339-44.

104. Wouters BJ, Jorda MA, Keeshan K, Louwers I, Erpelinck-Verschueren CA, Tielemans D, et al. Distinct gene expression profiles of acute myeloid/T-lymphoid leukemia with silenced CEBPA and mutations in NOTCH1. Blood. 2007;110(10):3706-14.

105. Rao AV, Valk PJ, Metzeler KH, Acharya CR, Tuchman SA, Stevenson MM, et al. Age-specific differences in oncogenic pathway dysregulation and anthracycline sensitivity in patients with acute myeloid leukemia. J Clin Oncol. 2009;27(33):5580-6.

106. de Jonge HJ, Woolthuis CM, de Bont ES, Huls G. Paradoxical down-regulation of p16 mRNA with advancing age in acute myeloid leukemia. Aging (Albany NY). 2009;1(11):949-53.

107. Bovelstad HM, Nygard S, Storvold HL, Aldrin M, Borgan O, Frigessi A, et al. Predicting survival from microarray data--a comparative study. Bioinformatics. 2007;23(16):2080-7.

108. Simon R. Roadmap for developing and validating therapeutically relevant genomic classifiers. J Clin Oncol. 2005;23(29):7332-41.

109. Radmacher MD, Marcucci G, Ruppert AS, Mrozek K, Whitman SP, Vardiman JW, et al. Independent confirmation of a prognostic gene-expression signature in adult acute myeloid leukemia with a normal karyotype: a Cancer and Leukemia Group B study. Blood. 2006;108(5):1677-83.

110. Michiels S, Koscielny S, Hill C. Interpretation of microarray data in cancer. Br J Cancer. 2007;96(8):1155-8.

111. Metzeler KH, Hummel M, Bloomfield CD, Spiekermann K, Braess J, Sauerland MC, et al. An 86-probe-set gene-expression signature predicts survival in cytogenetically normal acute myeloid leukemia. Blood. 2008;112(10):4193-201.

112. Yagi T, Morimoto A, Eguchi M, Hibi S, Sako M, Ishii E, et al. Identification of a gene expression signature associated with pediatric AML prognosis. Blood. 2003;102(5):1849-56.

113. Southern E, Mir K, Shchepinov M. Molecular interactions on microarrays. Nat Genet. 1999;21(1l):5-9.

114. Lipshutz RJ, Fodor SP, Gingeras TR, Lockhart DJ. High density synthetic oligonucleotide arrays. Nat Genet. 1999;21(1):20-4.

115. Wouters BJ, Lowenberg B, Delwel R. A decade of genome-wide gene expression profiling in acute myeloid leukemia: flashback and prospects. Blood. 2009;113(2):291-8.

116. Michiels S, Koscielny S, Hill C. Interpretation of microarray data in cancer. Br J Cancer. 2007;96(8):1155-8.

117. Brazma A, Hingamp P, Quackenbush J, Sherlock G, Spellman P, Stoeckert C, et al. Minimum information about a microarray experiment (MIAME)-toward standards for microarray data. Nat Genet. 2001;29(4):365-71.

118. Edgar R, Domrachev M, Lash AE. Gene Expression Omnibus: NCBI gene expression and hybridization array data repository. Nucleic Acids Res. 2002;30(1):207-10.

119. Simon R, Radmacher MD, Dobbin K, McShane LM. Pitfalls in the use of DNA microarray data for diagnostic and prognostic classification. J Natl Cancer Inst. 2003;95(1):14-8.

120. Eisen MB, Spellman PT, Brown PO, Botstein D. Cluster analysis and display of genome-wide expression patterns. Proc Natl Acad Sci. USA 1998;95(25):14863-8.

121. Fehrmann RS, de Jonge HJ, ter Elst A, de Vries A, Crijns AG, Weidenaar AC, et al. A new perspective on transcriptional system regulation (TSR): towards TSR profiling. PLoS One. 2008;3(2):e1656.

122. Hoheisel JD. Microarray technology: beyond transcript profiling and genotype analysis. Nat Rev Genet. 2006;7(3):200-10.

123. DeRisi J, Penland L, Brown PO, Bittner ML, Meltzer PS, Ray M, et al. Use of a cDNA microarray to analyse gene expression patterns in human cancer. Nat Genet. 1996;14(4):457-60.

124. Golub TR, Slonim DK, Tamayo P, Huard C, Gaasenbeek M, Mesirov JP, et al. Molecular classification of cancer: class discovery and class prediction by gene expression monitoring. Science. 1999;286(5439):531-7.

125. Haferlach T, Kohlmann A, Schnittger S, Dugas M, Hiddemann W, Kern W, et al. Global approach to the diagnosis of leukemia using gene expression profiling. Blood. 2005;106(4):1189-98.

126. Ross ME, Mahfouz R, Onciu M, Liu HC, Zhou X, Song G, et al. Gene expression profiling of pediatric acute myelogenous leukemia. Blood. 2004;104(12):3679-87.

127. Willman CL. Has gene expression profiling improved diagnosis, classification, and outcome prediction in AML? Best Pract Res Clin Haematol. 2008;21(1):21-8.

128. Wouters BJ, Lowenberg B, Delwel R. A decade of genome-wide gene expression profiling in acute myeloid leukemia: flashback and prospects. Blood. 2009;113(2):291-8.

129. Willman CL. Has gene expression profiling improved diagnosis, classification, and outcome prediction in AML? Best Pract Res Clin Haematol. 2008;21(1):21-8.

130. Bonnet D, Dick JE. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. Nat Med. 1997;3(7):730-7.

131. Lapidot T, Sirard C, Vormoor J, Murdoch B, Hoang T, Caceres-Cortes J, et al. A cell initiating human acute myeloid leukaemia after transplantation into SCID mice. Nature. 1994;367(6464):645-8.

132. Taussig DC, Miraki-Moud F, Anjos-Afonso F, Pearce DJ, Allen K, Ridler C, et al. Anti-CD38 antibody-mediated clearance of human repopulating cells masks the heterogeneity of leukemia-initiating cells. Blood. 2008;112(3):568-75.

133. Wang JC, Dick JE. Cancer stem cells: lessons from leukemia. Trends Cell Biol. 2005;15(9):494-501.

134. Warner JK, Wang JC, Hope KJ, Jin L, Dick JE. Concepts of human leukemic development. Oncogene. 2004;23(43):7164-77.

135. Cozzio A, Passegue E, Ayton PM, Karsunky H, Cleary ML, Weissman IL. Similar MLL-associated leukemias arising from self-renewing stem cells and short-lived myeloid progenitors. Genes Dev. 2003;17(24):3029-35.

136. Krivtsov AV, Twomey D, Feng Z, Stubbs MC, Wang Y, Faber J, et al. Transformation from committed progenitor to leukaemia stem cell initiated by MLL-AF9. Nature. 2006;442(7104):818-22.

137. Taussig DC, Vargaftig J, Miraki-Moud F, Griessinger E, Sharrock K, Luke T, et al. Leukemia-initiating cells from some acute myeloid leukemia patients with mutated nucleophosmin reside in the CD34(-) fraction. Blood. 2010;115(10):1976-84.

138. Gentles AJ, Plevritis SK, Majeti R, Alizadeh AA. Association of a leukemic stem cell gene expression signature with clinical outcomes in acute myeloid leukemia. JAMA. 2010;304(24):2706-15.

139. Becker H, Marcucci G, Maharry K, Radmacher MD, Mrozek K, Margeson D, et al. Favorable prognostic impact of NPM1 mutations in older patients with cytogenetically normal de novo acute myeloid leukemia and associated gene- and microRNA-expression signatures: a Cancer and Leukemia Group B study. J Clin Oncol. 2010;28(4):596-604.

140. Jongen-Lavrencic M, Sun SM, Dijkstra MK, Valk PJ, Lowenberg B. MicroRNA expression profiling in relation to the genetic heterogeneity of acute myeloid leukemia. Blood. 2008;111(10):5078-85.

141. Li Z, Lu J, Sun M, Mi S, Zhang H, Luo RT, et al. Distinct microRNA expression profiles in acute myeloid leukemia with common translocations. Proc Natl Acad Sci USA.. 2008;105(40):15535-40.

142. Marcucci G, Radmacher MD, Maharry K, Mrozek K, Ruppert AS, Paschka P, et al. MicroRNA expression in cytogenetically normal acute myeloid leukemia. N Engl J Med. 2008;358(18):1919-28.

143. Figueroa ME, Lugthart S, Li Y, Erpelinck-Verschueren C, Deng X, Christos PJ, et al. DNA methylation signatures identify biologically distinct subtypes in acute myeloid leukemia. Cancer Cell. 2010;17(1):13-27.

144. Hoheisel JD. Microarray technology: beyond transcript profiling and genotype analysis. Nat Rev Genet. 2006;7(3):200-10.

145. Metzker ML. Sequencing technologies – the next generation. Nat Rev Genet. 2010;11(1):31-46.

146. Shendure J, Ji H. Next-generation DNA sequencing. Nat Biotechnol. 2008;26(10):1135-45.

147. Mardis ER, Wilson RK. Cancer genome sequencing: a review. Hum Mol Genet. 2009;18(R2):R163-R168.

148. Wold B, Myers RM. Sequence census methods for functional genomics. Nat Methods. 2008;5(1):19-21.

149. Metzker ML. Sequencing technologies – the next generation. Nat Rev Genet. 2010;11(1):31-46.

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REVIEW

Autoimmune haemolytic anaemia – a practical guide to cope with a diagnostic and therapeutic challenge

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ABSTRACT

Autoimmune haemolytic anaemia (AIHA) is a rare disease. In clinical practice, diagnosis and treatment of AIHA turns out to be troublesome. Correct diagnosis is dependent on proper comprehension of the pathophysiology and the laboratory tests performed by the transfusion laboratory. The present review provides a short overview on the pathogenesis of autoimmune haemolytic anaemia. The diagnostic pitfalls will be discussed and a diagnostic algorithm for proper diagnosis of AIHA will be given. Moreover, a brief overview on the treatment of different forms of AIHA is given.

KEYWORDS

Autoimmune hemolytic anemia, hemolysis, cold-autoantibodies, warm-autoantibodies, complement, autoantibodies

INTRODUCTION

The diagnosis of autoimmune haemolytic anaemia (AIHA) is a challenge for both the immunohaematology laboratory and the clinician as the laboratory investigation can be troublesome and often requires extensive time-consuming serological testing, especially when a blood transfusion is needed. Frequently, there is a need to start therapy rapidly. Therefore, close collaboration and a good communication between laboratory and clinician is a 'sine qua non'. The aim of the present review is to give an overview of the laboratory techniques used for the diagnosis of AIHA. Moreover, a short overview on therapeutic options in AIHA will be provided.

OVERVIEW

AIHA is characterised by an increased breakdown of red blood cells (RBC) due to autoantibodies (auto-Ab's) with or without complement activation. The diagnostic features of AIHA include the combination of clinical and laboratory signs of RBC haemolysis together with the detection of auto-Ab's and/or complement deposition on RBC as mostly evidenced by a positive direct antiglobulin test (DAT) also known as direct Coombs test. A negative direct Coombs test using standard techniques does not exclude the diagnosis of AIHA.[1]

In more than 50% of the patients the development of AHIA is associated with an underlying disease *(secondary AIHA)*, but can occur without any evidence of an underlying disorder *(idiopathic or primary AIHA, table 1)*.[2] Based on the optimal temperature for autoantibody binding to RBC, AIHA is divided into a warm antibody AIHA (WA-AIHA), cold antibody AIHA (CA-AIHA) or AIHA due to biphasic auto-Ab (paroxysmal cold haemoglobinuria, PCH). With an incidence of 1:100,000 WA-AIHA is a rare disease, the incidence of CA-AIHA is even lower (1:1,000,000).[1] In contrast, 10% of patients suffering from lupus erythematosus develop an AIHA.[3,4] Occasionally, lymphoma is complicated by AIHA, but it can also be a herald of a lymphoma that has not yet been diagnosed. This is evidenced by the fact that 18% of patients with primary AIHA develop overt lymphoma at a later date.[5]

PATHOGENESIS

Autoantibodies directed to epitopes on RBC consisting in sugar and/or protein structures are crucial in the pathogenesis of AIHA. The *isotype* is important for the clinical significance of an autoantibody. Immunoglobulins

Table 1. *Aetiologies of autoimmune haemolytic anaemia*

Autoantibody (incidence)
Warm antibody AIHA (1:100000)
Primary (idiopathic)
Secondary
Lymphoproliferative disease (lymphoma)
Autoimmune diseases (SLE, colitis ulcerosa)
Acute leukaemia
Solid malignancy (ovarian carcinoma)
Cold antibody AIHA (1:1000000)
Primary (idiopathic): frequently herald of occult lymphoma
Secondary
Lymphoproliferative disease (M. Waldenstrom, lymphoma)
Infection (mycoplasma, EBV)
Biphasic haemolysins (rare)
Idiopathic
Secondary
Postviral,siphilis
Mixed forms with warm and cold antibodies
Idiopathic
Secondary
Autoimmune diseases (SLE)

EBV: Epstein-Barr virus, SLE: systemic lupus erythematosus

of IgM isotype form a pentameric structure and are therefore very efficient in complement activation. IgG1 and IgG3 are efficient complement activators as well, whereas IgG2 and IgA have only a weak capacity to activate complement. IgG4 does not activate complement. Generally, the complement system is not completely activated and complement degradation products (C3c, C3d) can be detected as traces on RBC's ('Complement footprints'). However, complement activation may proceed until the formation and introduction of the membrane attack complex C6-9 (MAC) leading to RBC lysis. The optimal temperature of auto-Ab's to bind to RBC is of clinical relevance as well. Cold autoantibodies (CA-Ab) show optimal binding to RBC below 30 °C and are mostly of IgM isotype. CA-Ab having an optimal binding around 30 °C are clinically relevant since they may induce complement activation *in-vivo*.[6] Warm autoantibodies (WA-Ab) show optimal binding at 37 °C and are mostly IgG, less commonly IgM and rarely IgA.[1] Biphasic auto-Ab's are IgG which show optimal binding below 30 °C and induce complement activation at 37 °C.[6] RBC coated with IgG with/without C3c/C3d are preferentially removed by via Fc-gamma receptor mediated phagocytosis in the spleen, whereas RBC coated with C3c/C3d in the absence of IgG are destroyed via complement-receptor mediated phagocytosis in the liver (*extravascular haemolysis)*. In the presence of IgM which is reactive above 30 °C, complement activation may proceed till the insertion of MAC leading to intravascular RBC destruction *(intravascular haemolysis)*.

DIAGNOSIS

Clinical considerations

The clinical presentation of AIHA is not different from other forms of acute haemolytic anaemia or acute crisis of a chronic haemolytic anaemia. Frequently, patients are icteric and suffer from clinical signs of anaemia, such as pallor, fatigue, shortness of breath and palpitations. In contrast, haemoglobinuria as a sign of intravascular haemolysis is rare, but the patient must explicitly be asked for that symptom. In case of cold agglutinins, cold exposure may lead to agglutination of RBC in the circulation as reflected by cyanotic discolouring of the acra, such as toes, fingers, ears and nose. After warming up, the cyanotic discolouring disappears quickly and in contrast to a Raynaud phenomenon, no reactive hyperaemia occurs. The presence of a disease frequently reported to be associated with AIHA supports the suspected diagnosis. Since many of these diseases are accompanied by anaemia, the diagnosis of a mild AIHA can easily be missed. An overview on the different forms and aetiologies of AIHA is shown in *table 1*.

General laboratory findings

Besides a careful evaluation of the clinical history, laboratory diagnostics play a central role in the diagnosis of AIHA in order to detect both haemolysis and auto-Ab's to RBC. Increased levels of lactate dehydrogenase (LDH), indirect hyperbilirubinaemia, decreased haptoglobulin and reticulocytosis reflect increased RBC breackdown either due to intra- or extravascular haemolysis. Normal levels of LDH do not exclude the presence of haemolysis! Reticulocytosis might be absent in the beginning of AIHA and/or in case of decreased functional capacity of the bone marrow, as seen after chemotherapy. Frequently, microspherocytes can be detected in the peripheral blood smear. Microspherocytes are autoantibody-coated RBC, which have lost their biconcave shape due to loss of part of their membrane upon passage through the spleen.[7] In case of intravascular haemolysis, haemoglobin is released by destructed RBC and cleared by the kidney leading to a brownish discolouring of the urine (haemoglobinuria). Even days after the haemolytic episodes haemosiderin can be detected in the urine.

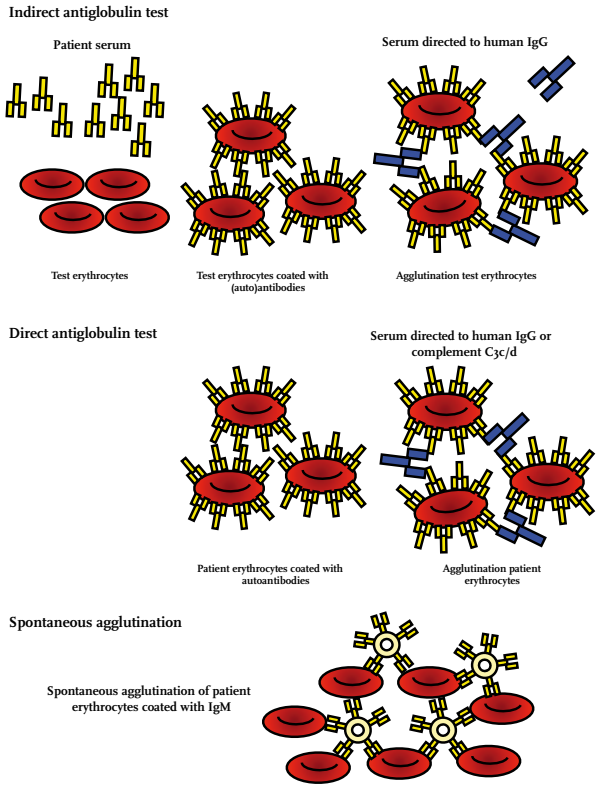
Immunohaematological diagnostics

The immunohaematological diagnosis in AIHA aims to detect auto-Ab's to RBC. In a first approach the indirect antiglobulin test (IAT) and the DAT are performed. In the IAT, auto-Ab's to RBCs present in patient's serum are detected. In a first step, standardised test RBC (test panel) are incubated with the patient's serum. In a second step, after removing the unbound immunoglobulins by washing polyspecific antihuman globulin reagent directed to both,

human IgG and complement (complement component C3) are added. If RBCs have been coated by auto-Ab's present in the patient serum, the RBC will agglutinate indicating a positive result (positive IAT, *figure 1*, above). In contrast, by means of the direct Coombs test, auto-Ab's bound *in-vivo* to patients RBC are directly detected by adding polyspecific antihuman globulin reagent (*figure 1*, middle). In rare situations the clinical picture is highly suggestive for an AIHA, but the direct Coombs is negative. As a polyspecific anti-human globulin reagent does not contain anti-IgA it is important to repeat the DAT with anti-IgG, anti-IgA, anti-IgM, anti-C3c and anti-C3d to confirm the DAT to be negative. In the situation the DAT remains negative the presence of microspherocytes in the peripheral blood smear may help to support the suspected diagnosis AIHA without detectable antibodies.

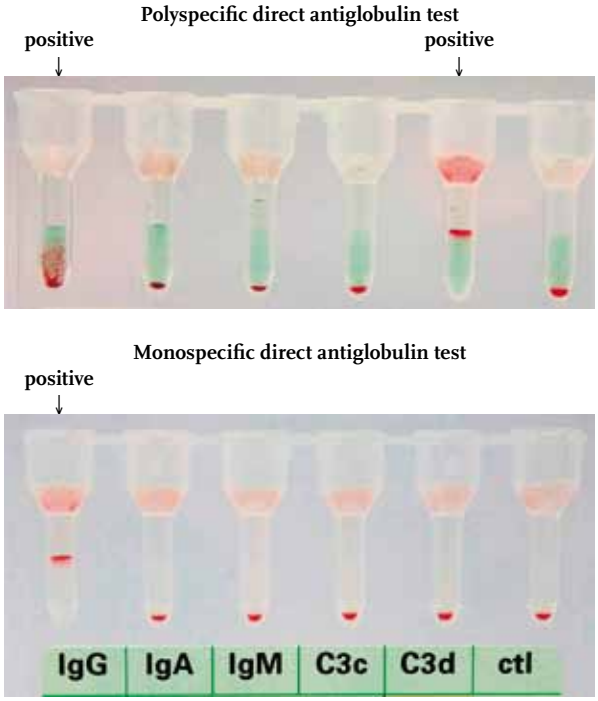
In daily practice fully automated laboratory analysing systems are used to perform DAT and IAT. All these systems are based on the detection of agglutination of RBC. Frequently, column tests with gel-containing microtubes are used. RBC and antiserum are incubated in a reaction chamber followed by a controlled centrifugation of the microtube containing anti human globulin. If agglutination occurred in the reaction chamber, the RBC-antiserum complexes will be trapped in the column upon centrifugation and the test is positive. If no agglutination occurred, the RBC pass the column upon centrifugation resulting in a pellet on the bottom of the microtube, the test is negative (*figure 2*). In some laboratories flow cytometry is used to detect RBC coated with either auto-Ab's or complement, respectively. However, in special situations RBC agglutination is still performed visually in glass tubes by an analyst.

Figure 1. *Direct and indirect antiglobulin test*



By means of the indirect antiglobulin test (IAT, indirect Coombs test) circulating allo- and autoantibodies present in patient serum are detected. In a first step treated or untreated test erythrocytes are incubated with patient serum. Allo- and autoantibodies present in the patient serum will bind to the test erythrocytes. In case of IgM present in patient serum, test erythrocytes may agglutinate directly, the test is positive. Antibodies type IgG are incomplete antibodies which do not lead to direct agglutination of test erythrocytes. In a second step test erythrocytes coated with IgG are incubated with antiserum against human IgG. In case of agglutination, the test is positive.
By means of the direct antiglobulin test (DAT, direct Coombs test) patient erythrocytes coated with either auto- or alloantibodies and/or complement are detected. Patient erythrocytes are incubated with a polyspecific serum directed to human IgG and complement (C3d). If there is an agglutination, the test is considered to be positive indicating patient erythrocytes to be coated with IgG and/or C3d.

Figure 2. *Direct antiglobulin test*



The gel system consists in a microtube containing a reaction chamber (R) and a gelmatrix (G). The reaction chamber either contains a polyspecific (top) or monospecific (bottom) human antiserum directed to immunoglobulins or complement (C3c/C3d). Patient erythrocytes are added to the reaction chamber and after a short incubation the microtube is centrifuged. If agglutination in the reaction chamber occurred, patient erythrocytes will be trapped in the gel matrix upon centrifugation, the test is positive (arrow). If no agglutination occurred, the erythrocytes pass the gel matrix forming a pellet at the bottom of the microtube, the test is negative. (Figure kindly provided by E. Schaeffer and G.J. van den Akker, AMC.)

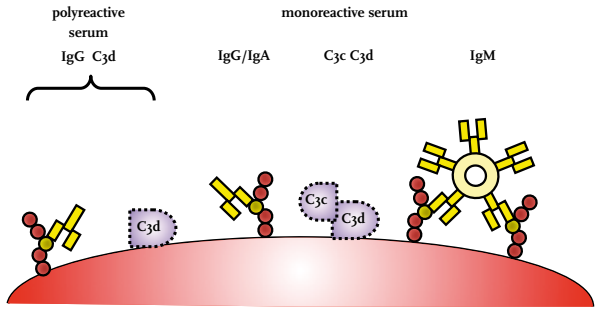
Positive direct Coombs: what to do next?

If the DAT proves to be positive when using a polyspecific antihuman globulin reagent, further specification with a monospecific reagent is needed in order to detect whether RBC are coated with IgG, IgA, IgM and C3C or/and C3d, respectively (*figure 3*). If complement deposition (C3c/C3d) can be detected in the absence of an autoantibody, the presence of CA-Ab (IgM), WA-Ab (IgM, IgA) or biphasic antibodies must be considered. In that situation further laboratory diagnostics are also mandatory, to investigate the presence of either IgM or IgA. IgA auto-Ab's without IgG auto-Ab's are very rare. However they show an optimal binding at 37 °C and can lead to fulminant and fatal haemolysis.[8,9] Due to their size (pentamer) IgM auto-Ab's are difficult to detect because they are removed by the washing procedures while performing the DAT. In addition, the optimal temperature for IgM binding and the temperature at which the DAT is performed are crucial.

In a next step, the properties of IgM to directly agglutinate RBC due to its size (pentamer) can be utilised (complete antibody). If there is spontaneous agglutination after incubation of patient serum with test RBC at 16 °C, a CA-Ab IgM must be suspected. A potentially clinically relevant cold antibody must be considered if agglutination occurs at 30 °C. Another useful test to detect complement binding antibodies in serum is a haemolysis test using RBC pretreated with enzymes (being much more sensitive for complement-mediated lysis as compared with normal RBC) incubated with patient serum at both 16 °C and 37 °C. Thereafter, standard serum with a lower pH after adding acid is added as complement source and incubation is performed (*figure 4*). If lysis occurs a clinically relevant antibody which can potentially cause haemolysis or shortening of the life span of the RBC must be considered. In case of fulminant intravascular haemolysis auto-Ab's frequently have the potential to induce lysis even in non-pretreated RBC *in-vitro*. If a CA-Ab is suspected, the pre-analytical handling of the patient samples is crucial. After venipuncture the blood sample must immediately be put on 37 °C, since the auto-Ab's will bind to RBC at room temperature thereby decreasing the auto-Ab's concentration in the serum, bearing the risk of a false-negative result.

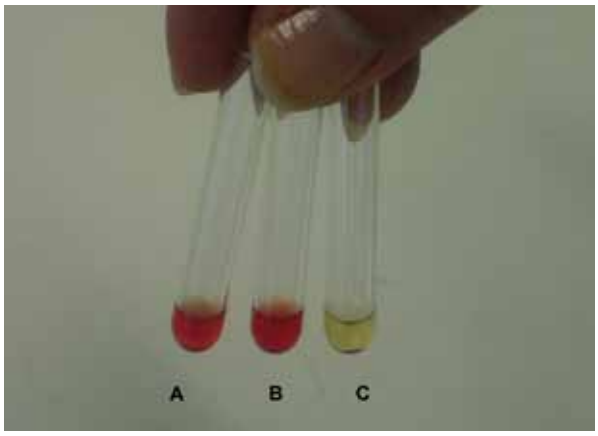
In order to identify the specificity, the warm auto-Ab's can be separated from the RBC by means of laborious elution techniques. In analogy to the IAT the eluate (containing the auto-Ab's which were bound to RBC) is tested in a standard panel of RBC. If a specificity of the eluted antibody can be identified, this will be indicated in the diagnostic rapport (e.g. specific autoantibody, anti-C). However, in many cases no specificity can be identified (non-specific antibody). Specific WA-Ab's are frequently directed to parts or to the entire Rhesus system, rarely to the Kell system.[1] CA-Ab are frequently directed to I-antigen or H antigen, whereas biphasic auto-Ab's have anti-P specificity.[6]

Figure 3. *Monospecific direct antiglobulin test (Coombs test)*



In case of a positive polyspecific antiglobulin test the components on the patient erythrocytes need further specification. Patient erythrocytes are incubated with monospecific serum directed to human IgG, IgA, IGM or complement components (C3c, C3d). If there is agglutination with one of the antisera, the test is positive indicating the presence of the respective immunoglobulin or complement component on the patient erythrocytes.

Figure 4. *Detection of autoantibodies potentially able to induce haemolysis*



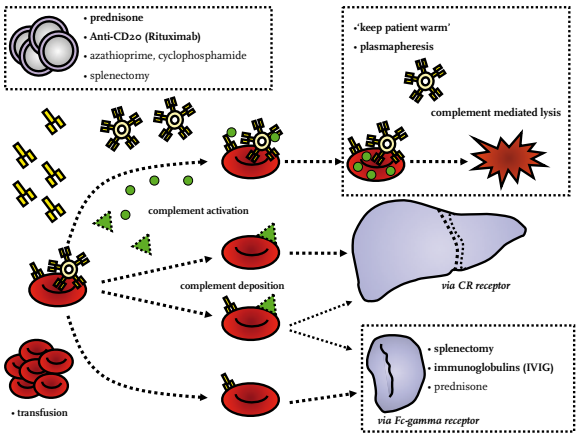
Pretreated test erythrocytes, which are more sensitive for haemolysis than normal test erythrocytes are incubated with patient serum first at 16 °C (A) and 37 °C (B) (control: C). After addition of standard serum as source of fresh complement, the sensibilised test erythrocytes are incubated at 37 °C. If haemolysis occurs, the autoantibody may potentially induce haemolysis in vivo. Rarely, an autoantibody may induce haemolysis in non-pretreated test erythrocytes (figure kindly provided by P. Ligthart, Sanquin).

Type and screen: remains challenge in AIHA

In case of a planned transfusion, type and screen has to be performed. Besides the characterisation of the auto-Ab's, detection of alloantibodies is of outstanding importance. Literature suggests that alloantibodies can be detected in 15 to 43% of patients suffering from AIHA, mostly after receiving transfusions.[10] Moreover,

patients with one alloantibody have a significantly increased risk to develop additional alloantibodies.[10,11] The presence of auto-Ab's in serum complicates the type and screen procedure. The determination of the patient's blood group *(Type)* by serological methods remains difficult, especially in patients with CA-Ab, and requires time-consuming washing steps (extensive washing to get rid of the RBC-bound auto-Ab's). Sometimes, serological blood group determination is not possible. Genotyping for the most important blood groups (Rhesus, Kell, Duffy, Kidd, SS's) may offer a solution. In case of WA-Ab bound to the RBC typing with monoclonal reagents is a possibility to avoid genotyping. The detection of alloantibodies remains difficult, since patient auto-Ab's react with test RBCs. This is also illustrated by the fact that in some cases crossmatching is positive for all selected RBC concentrates. With different absorption techniques (auto- and alloabsorption) auto-Ab's can be removed from patient serum in order to perform a proper screening for alloantibodies. However, these techniques are time-consuming, require abundant patient material and can only be performed by specialised reference laboratories.

Figure 5. *Mechanisms of red blood cell removal in autoimmune haemolytic anaemia*



Erythrocytes coated with IgG autoantibodies are mainly removed via Fc-gamma receptors on macrophages in the spleen. Complement deposition on erythrocytes in the absence of IgG leads to red blood cell removal in the liver via complement receptors on Kupfer cells. In case of fulminant haemolysis, red blood cells are destructed in the circulation.

THERAPY

If possible, transfusion should be avoided! There is a significant risk for alloantibody formation upon transfusion in that situation. Moreover, ongoing haemolysis can be exacerbated by transfusion, since auto-Abs also react with transfused red blood cells. Anaemia should only be corrected in case of clinical symptoms. Transfusion must be performed under control of vital parameters, such as cardiac function (ECG), renal function and diuresis. If there is no vital indication for a transfusion it is prudent to wait for the results of the immunohaematological tests and the ensuing transfusion advice based on this. In a second approach the process of haemolysis must be stopped or at least be attenuated via an inhibition of autoantibody production and/or inhibition of premature RBC destruction. Successful treatment of secondary AIHA is only possible when the underlying disease is treated. *Figure 5* provides an overview on the different therapeutic approaches in AIHA. Due to the availability of a therapy efficiently targeting autoantibody-producing B-cells (anti-CD20 antibody therapy), the significance of splenectomy is a matter of debate. Prospective randomised trials evaluating the efficacy of different treatment modalities are not widely available since AIHA is a rare disease and affects a heterogeneous patient population. Moreover, the interpretation of the efficacy of the treatment effects in these studies is difficult since there are no uniform definitions for response to therapy, complete and partial remission, respectively. In the following section, therapeutic approaches for WA-AIHA and CA-AIHA will be discussed. The definitions partial and complete response are adopted from the publication cited in the text.

Treatment of WA-AIHA

Transfusion

The blood product must be compatible with respect to complement-activating alloantibodies present in patient's serum. If possible the selected product must be negative for the antigens, to which alloantibodies have been identified in the antibody screening. In addition, the development of new or additional alloantibodies must be prevented. Therefore, a blood product as compatible as possible with the recipient antigens will be selected. The minimal requirement is that the selected product must be compatible to Rhesus and Kell antigens. In case of severe haemolysis blood product selection may also consider the specificity of auto-Ab's. When there is a conflict making the right choice to select RBC it is important to keep in mind that in case of transfusion alloantibodies are more important than auto-Ab's. If there is no time to wait for the result of the serological investigations, it must be considered to prevent alloantibody formation by matching patient and donor for the most important RBC antigens: Rhesus, Kell, Kidd, Duffy, Ss.

Steroids

Steroids are effective in the treatment of AIHA and therefore are the treatment of choice. Steroids decrease the production of auto-Ab's by B-cells.[12] Moreover, steroids reduce the density of Fc-gamma receptors on phagocytes

in the spleen.[13,14] Steroids induce a partial remission in 60 to 70% of the patients, in 10 to 15% a complete remission is achieved.[1,15,16] Commonly, prednisolone, 1 mg/kg/day is started, and depending on the clinical response is tapered slowly. After stabilisation of the haemoglobin a scheme frequently used at our department is to taper prednisolone to a dosage to 20 mg/day in two weeks. If the haemoglobin level remains stable, dosage can further be reduced to 10 mg/day after a month. Thereafter, the steroid dosage can further be tapered and be stopped after two weeks. In order to diagnose steroid-induced diabetes mellitus early, blood glucose levels must be monitored regularly. Moreover, osteoporosis prophylaxis must be started since the patients suffering from AIHA receive steroids over a long period of time. The psychological side effects of steroid treatment are frequently underestimated (e.g. agitation, lack of self-control, psychosis) and might become an incriminatory problem for the patient and social environment. Therefore steroid doses have to be reduced often or the therapy has even to be stopped.

Cytotoxic drugs

Azathioprine and cyclophosphamide are both immune suppressors leading to a decrease of autoantibody production. The addition of these drugs can be considered if steroid therapy does not lead to a sufficient result, when a steroid maintenance dose of more than 20 mg/day is needed or steroid doses must be tapered due to side effects.[17,20] Cyclophosphamide (100 mg/d) or azathioprine (100-150 mg/d) can be administered as monotherapy or in combination with steroids. Due to their myelosupressive effects peripheral blood cell counts must be controlled regularly and if needed dosage must be adapted. In refractory AIHA pulse therapy with cyclophosphamide (50 mg/kg over 4 days) in combination with mesna and G-CSF might be successful.[21] In desperate cases vincristine might be a valuable alternative bearing the advantage of being less myelotoxic than cyclophosphamide.[22] Immunosuppressive drugs, such as cyclosporine or mycophenolate-mofetil seem to be effective in some case series.[23,24]

Splenectomy

By means of splenectomy RBC destruction is abated and the production of auto-Ab's is decreased. Two weeks after splenectomy anaemia has stabilised in more than 50% of the patients.[25-27] Approximately 20% of the patients reach long-time remissions or are even cured from the disease. In half of the patients steroids can further be tapered. However, one-third of the patients do not reach a substantial remission. The mortality of splenectomy by laparatomy is around 1%, in laparoscopic splenectomy it is about 0.5%.[28,29] Patients after splenectomy have an increased risk for infections as compared with the normal population.[30,31] Vaccination against *N. menigitidis, Str. pneumoniae, H. influenzae,* if possible prior to splenectomy, significantly decreases the risk for infection in these patients.[32]

Anti-C20 antibody

Rituximab is a chimeric, monoclonal antibody targeting CD20 expressed on all B-cells except plasma cells.[33] Administration of rituximab decreases autoantibody production by targeted destruction of B cells. The efficacy of rituximab in WA-AIHA is difficult to assess due to the presence of a considerable publication bias and the lack of controlled prospective studies. Retrospective studies report a complete remission in 20 to 70% of the patients. In prospective studies, >60% of the patients achieve a complete remission, but most patients will relapse sooner or later (>24 months).[34-38] Rituximab is well tolerated, occasionally allergic reactions with hives, chills and hypotension occur. As a very rare but fatal complication, progressive multifocal leucoencephalopathy after rituximab therapy in patients suffering from systemic lupus erythematosus has been reported.[34,39] Despite the lack of controlled prospective studies rituximab has to be considered to replace splenectomy as therapy of choice in steroid-resistant WA-AIHA. If splenectomy is reconsidered after failure of rituximab therapy, it must be kept in mind that vaccination to encapsulated bacteria might be ineffective after Rituximab therapy.

Immunoglobulins

In approxinately 40% of cases, administration of immunoglobulins improves anaemia temporarily. This is mainly attributed to a reduction of RBC destruction in the spleen.[40] In addition, immunomodulatory effects of gammaglobulins might contribute to the beneficial effect as well. Therapy with immunoglobulins might be considered in acute life-threatening situations in order to reduce breakdown of patients or donor erythrocytes.

Treatment of CA-AIHA

Fortunately, anaemia in CA-AIHA is usually mild and there is no need for correction. The basic treatment in that situation is quite simple: 'keep it warm'. Patients must protect themselves properly against the cold by wearing gloves, a hat and warm shoes. If necessary, transfusion must be performed under controlled conditions at 37 °C by means of a controlled heating system.[6,34] During surgery, body temperature must be kept at 37 °C. The criteria to choose a blood product are similar to those in WA-AIHA. However, the treatment of CA-AIHA remains a frustrating issue. Moreover, only a modicum of controlled studies are available. Steroids are clearly less effective than in WA-AIHA.[6,41-43] The same holds for cyclophosphamide and azathioprine.[6] In CA-AIHA there is no role for

splenectomy.[6] A couple of studies report some beneficial effects of gammaglobulins. In two controlled trials, rituximab was demonstrated to induce a response in 40 to 50%, but again achievement of complete remission is rare and relapses are common.[44,45] Since IgM are mainly located intravascularly, plasmapheresis induces a quick reduction of IgM levels and may therefore contribute to a short-term stabilisation of an AIHA.[46] Since plasmapheresis has to be performed at 37 °C, the technical procedure remains a challenge.

Treatment options in case of intravascular haemolysis

The treatment options in case of fulminant intravascular haemolysis are restricted. There are no controlled studies. Therapy focuses on supportive care with a close monitoring of vital functions, renal function and haemolysis parameters. In the literature, gammaglobulins and plasmapheresis have been reported as therapeutic options. In selected cases an inhibitor of the activation of complement component C5 (eculizumab) has been administered thereby attenuating the formation of the membrane attack complex.[47]

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REFERENCES

1. Packman CH. Hemolytic anemia due to warm autoantibodies. Blood Rev. 2008 January;22(1):17-31.
2. Engelfriet CP, van t'Veer MB, Maas N, Ouwehand WH, Beckers DO, von den Borne AEG. Autoimmune haemolytic anemias. In: Kay AB, Denman AM, Wright R, editors. Clinical Immunology and Allergy. London: Baillieres Tindall;1987:251-67.
3. Jeffries M, Hamadeh F, Aberle T, Glenn S, Kamen DL, Kelly JA, et al. Haemolytic anaemia in a multi-ethnic cohort of lupus patients: a clinical and serological perspective. Lupus 2008 August;17(8):739-43.
4. Nossent JC, Swaak AJ. Prevalence and significance of haematological abnormalities in patients with systemic lupus erythematosus. Q J Med. 1991 July;80(291):605-12.
5. Arndt PA, Leger RM, Garratty G. Serologic findings in autoimmune hemolytic anemia associated with immunoglobulin M warm autoantibodies. Transfusion. 2009 February;49(2):235-42.
6. Petz LD. Cold antibody autoimmune hemolytic anemias. Blood Rev. 2008 January;22(1):1-15.
7. LoBuglio AF, Cotran RS, Jandl JH. Red cells coated with immunoglobulin G: binding and sphering by mononuclear cells in man. Science. 1967 December 22;158(808):1582-5.
8. Petz LD. Diagnostic complexities in autoimmune hemolytic anemias. Transfusion. 2009 February;49(2):202-3.
9. Bardill B, Mengis C, Tschopp M, Wuillemin WA. Severe IgA-mediated auto-immune haemolytic anaemia in a 48-yr-old woman. Eur J Haematol. 2003 January;70(1):60-3.
10. Engelfriet CP, Reesink HW, Garratty G, Knight R, de SM, Contreras M, et al. The detection of alloantibodies against red cells in patients with warm-type autoimmune haemolytic anaemia. Vox Sang. 2000;78(3):200-7.
11. Schonewille H, de Vries RR, Brand A. Alloimmune response after additional red blood cell antigen challenge in immunized hematooncology patients. Transfusion. 2009 March;49(3):453-7.
12. Evans RS, Bingham M, Boehni P. Autoimmune hemolytic disease. Antibody dissociation and activity. Arch Intern Med. 1961 September;108:338-52.
13. Fries LF, Brickman CM, Frank MM. Monocyte receptors for the Fc portion of IgG increase in number in autoimmune hemolytic anemia and other hemolytic states and are decreased by glucocorticoid therapy. J Immunol. 1983 September;131(3):1240-5.
14. Schreiber AD, Parsons J, McDermott P, Cooper RA. Effect of corticosteroids on the human monocyte IgG and complement receptors. J Clin Invest. 1975 November;56(5):1189-97.
15. Allgood JW, Chaplin H, Jr. Idiopathic acquired autoimmune hemolytic anemia. A review of forty-seven cases treated from 1955 through 1965. Am J Med. 1967 August;43(2):254-73.
16. Meyer O, Stahl D, Beckhove P, Huhn D, Salama A. Pulsed high-dose dexamethasone in chronic autoimmune haemolytic anaemia of warm type. Br J Haematol. 1997 September;98(4):860-2.
17. Serrano J. [Autoimmune hemolytic anemia. Review of 200 cases studied in a period of 20 years (1970-1989)]. Sangre. 1992 August;37(4):265-74.
18. Zupanska B, Sylwestrowicz T, Pawelski S. The results of prolonged treatment of autoimmune haemolytic anaemia. Haematologia. 1981 December;14(4):425-33.
19. Sakalova A, Hrubisko M. [Cyclophosphamide in the treatment of immune hemocytopenias]. Folia Haematol Int Mag Klin Morphol. 1975;102(5):559-64.
20. Worlledge SM, Brain MC, Cooper AC, Hobbs JR, Dacie JV. Immmunosuppressive drugs in the treatment of autoimmune haemolytic anaemia. Proc R Soc Med. 1968 December 12;61(12):1312-5.
21. Moyo VM, Smith D, Brodsky I, Crilley P, Jones RJ, Brodsky RA. High-dose cyclophosphamide for refractory autoimmune hemolytic anemia. Blood. 2002 July 15;100(2):704-6.
22. Shvidel L, Sigler E, Shtalrid M, Berrebi A. Vincristine-loaded platelet infusion for treatment of refractory autoimmune hemolytic anemia and chronic immune thrombocytopenia: rethinking old cures. Am J Hematol. 2006 June;81(6):423-5.
23. Emilia G, Messora C, Longo G, Bertesi M. Long-term salvage treatment by cyclosporin in refractory autoimmune haematological disorders. Br J Haematol. 1996;93(2):341-4.
24. Howard J, Hoffbrand AV, Prentice HG, Mehta A. Mycophenolate mofetil for the treatment of refractory auto-immune haemolytic anaemia and auto-immune thrombocytopenia purpura. Br J Haematol. 2002 June;117(3):712-5.
25. Allgood JW, Chaplin H, Jr. Idiopathic acquired autoimmune hemolytic anemia. A review of forty-seven cases treated from 1955 through 1965. Am J Med. 1967 August;43(2):254-73.
26. Cherthow G, Dacie JV. Results of splenectomy in auto-immune haemolytic anaemia. Br J Haematol. 1956 July;2(3):237-49.
27. Coon WW. Splenectomy in the treatment of hemolytic anemia. Arch Surg. 1985 May;120(5):625-8.
28. Casaccia M, Torelli P, Squarcia S, Sormani MP, Savelli A, Troilo B, et al. Laparoscopic splenectomy for hematologic diseases: a preliminary analysis performed on the Italian Registry of Laparoscopic Surgery of the Spleen (IRLSS). Surg Endosc. 2006 August;20(8):1214-20.
29. Kojouri K, Vesely SK, Terrell DR, George JN. Splenectomy for adult patients with idiopathic thrombocytopenic purpura: a systematic review to assess long-term platelet count responses, prediction of response, and surgical complications. Blood. 2004 November 1;104(9):2623-34.

30. Yong M, Thomsen RW, Schoonen WM, Farkas DK, Riis A, Fryzek JP, et al. Mortality risk in splenectomised patients: a Danish population-based cohort study. Eur J Intern Med. 2010 February;21(1):12-6.

31. Thomsen RW, Schoonen WM, Farkas DK, Riis A, Jacobsen J, Fryzek JP, et al. Risk for hospital contact with infection in patients with splenectomy: a population-based cohort study. Ann Intern Med. 2009 October 20;151(8):546-55.

32. Ejstrud P, Kristensen B, Hansen JB, Madsen KM, Schonheyder HC, Sorensen HT. Risk and patterns of bacteraemia after splenectomy: a population-based study. Scand J Infect Dis. 2000;32(5):521-5.

33. Reff ME, Carner K, Chambers KS, Chinn PC, Leonard JE, Raab R, et al. Depletion of B cells in vivo by a chimeric mouse human monoclonal antibody to CD20. Blood. 1994 January 15;83(2):435-45.

34. Garvey B. Rituximab in the treatment of autoimmune haematological disorders. Br J Haematol. 2008 April;141(2):149-69.

35. Bussone G, Ribeiro E, Dechartres A, Viallard JF, Bonnotte B, Fain O, et al. Efficacy and safety of rituximab in adults' warm antibody autoimmune haemolytic anemia: retrospective analysis of 27 cases. Am J Hematol. 2009 March;84(3):153-7.

36. Dierickx D, Verhoef G, Van HA, Mineur P, Roest A, Triffet A, et al. Rituximab in auto-immune haemolytic anaemia and immune thrombocytopenic purpura: a Belgian retrospective multicentric study. J Intern Med. 2009 November;266(5):484-91.

37. Narat S, Gandla J, Hoffbrand AV, Hughes RG, Mehta AB. Rituximab in the treatment of refractory autoimmune cytopenias in adults. Haematologica. 2005 September;90(9):1273-4.

38. Shanafelt TD, Madueme HL, Wolf RC, Tefferi A. Rituximab for immune cytopenia in adults: idiopathic thrombocytopenic purpura, autoimmune hemolytic anemia, and Evans syndrome. Mayo Clin Proc. 2003 November;78(11):1340-6.

39. Carson KR, Evens AM, Richey EA, Habermann TM, Focosi D, Seymour JF, et al. Progressive multifocal leukoencephalopathy after rituximab therapy in HIV-negative patients: a report of 57 cases from the Research on Adverse Drug Events and Reports project. Blood. 2009 May 14;113(20):4834-40.

40. Majer RV, Hyde RD. High-dose intravenous immunoglobulin in the treatment of autoimmune haemolytic anaemia. Clin Lab Haematol. 1988;10(4):391-5.

41. Dausset J, Colombani J. The serology and the prognosis of 128 cases of autoimmune hemolytic anemia. Blood. 1959 December;14:1280-301.

42. Firkin BG, Blackwell JB, Johnston GA. Essential eryoglobulinaemia and acquired haemolytic anaemia due to cold agglutinins. Australas Ann Med. 1959 May;8(2):151-7.

43. Pisciotta AV. Cold hemagglutination in acute and chronic hemolytic syndromes. Blood. 1955 April;10(4):295-311.

44. Berentsen S, Ulvestad E, Gjertsen BT, Hjorth-Hansen H, Langholm R, Knutsen H, et al. Rituximab for primary chronic cold agglutinin disease: a prospective study of 37 courses of therapy in 27 patients. Blood. 2004 April 15;103(8):2925-8.

45. Schollkopf C, Kjeldsen L, Bjerrum OW, Mourits-Andersen HT, Nielsen JL, Christensen BE, et al. Rituximab in chronic cold agglutinin disease: a prospective study of 20 patients. Leuk Lymphoma. 2006 February;47(2):253-60.

46. Siami FS, Siami GA. A last resort modality using cryofiltration apheresis for the treatment of cold hemagglutinin disease in a Veterans Administration hospital. Ther Apher Dial. 2004 October;8(5):398-403.

47. Roth A, Huttmann A, Rother RP, Duhrsen U, Philipp T. Long-term efficacy of the complement inhibitor eculizumab in cold agglutinin disease. Blood. 2009 April 16;113(16):3885-6.

PHOTO QUIZ

A postoperative puzzle

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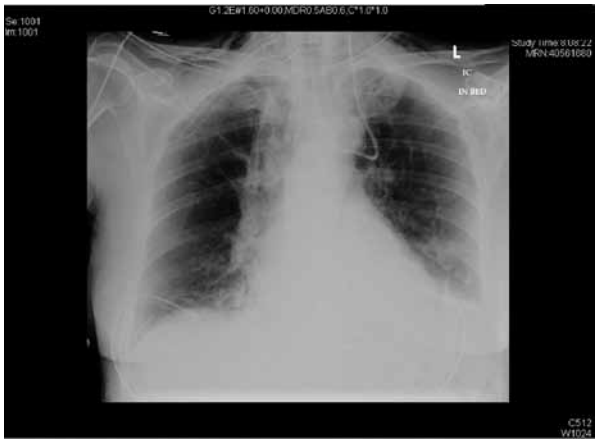
CASE REPORT

A 73-year-old woman was admitted to the intensive care unit (ICU) after surgery for an adenocarcinoma of the oesophagus. A routine postoperative chest radiograph revealed an unusual finding (*figure 1*).

Figure 1. *Postoperative chest X-ray*



WHAT IS YOUR DIAGNOSIS?

See page 195 for the answer to this photo quiz.

Netherlands
The Journal of Medicine

A nonproductive cough that would give most people a headache, but not this patient!

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CASE REPORT

A 54-year-old woman presented with a nonproductive cough that started ten weeks ago, accompanied by a slight shortness of breath, fatigue and fever. On suspicion of a respiratory tract infection the general practitioner had prescribed amoxicillin, without any effect. Next, she was seen by a pulmonologist, who ordered a chest X-ray and lung function test. These tests showed no abnormalities. The patient's symptoms were attributed to (post)infectious bronchial inflammation possibly combined with gastric asthma, for which she was treated unsuccessfully with doxycycline and pantoprazol. Thereupon, the patient was referred to the department of internal medicine.

Repeated history taking was noncontributory. Physical examination was unremarkable, except for a temperature of 38.0 °C. Laboratory analysis showed an erythrocyte sedimentation rate of 120 mm/hour, a C-reactive protein level of 178 mg/l and a normocytic anaemia (haemoglobin level 6.1 mmol/l) without thrombocytosis or leucocytosis.

Our differential diagnosis consisted of autoimmune diseases such as systemic lupus erythematodes or a vasculitis, malignancies such as a lymphoma or pulmonary metastasised solid tumour, chronic pulmonary embolism and atypical infections such as tuberculosis.

Immunological investigation showed only borderline presence of antinuclear antibodies and no anti-double-stranded DNA or antineutrophil cytoplasmatic antibodies. Computed tomography (CT) pulmonary angiography and an abdominal ultrasound were normal. Blood cultures and a Mantoux test were negative. Finally, positron emission tomography (PET)/CT was performed (*figures 1A and 1B*)

WHAT IS YOUR DIAGNOSIS?

See page 199 for the answer to this photo quiz.

Figure 1A. *Coronal image of whole body PET with increased uptake in the aorta, the carotid arteries, subclavian arteries and iliac arteries*

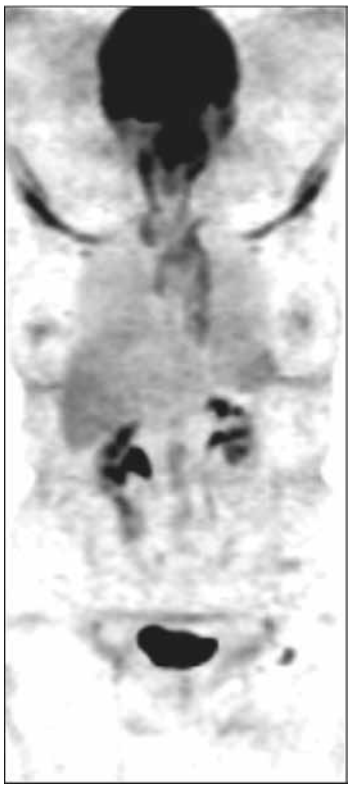
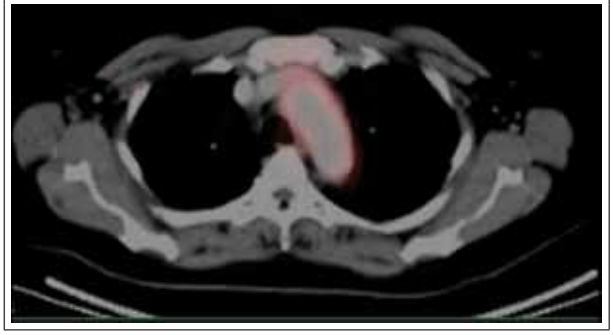


Figure 1B. *Transversal fused PET/CT image with increased uptake in the aortic arch*



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ORIGINAL ARTICLE

Cardiopulmonary events during primary colonoscopy screening in an average risk population

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ABSTRACT

Background: Large colorectal cancer screening studies using primary colonoscopy have reported a low risk of major complications. Studies on diagnostic and therapeutic colonoscopy have pointed to a frequent occurrence of (minor) cardiopulmonary events, and with the steady increase of colonoscopy screening, it is important to investigate their occurrence in colonoscopy screening.

Methods: This study describes the frequency of bradycardia (pulse rate <60 min^{-1}), hypotension (systolic blood pressure (SBP) <90 mmHg), hypoxaemia (blood oxygenation, SaO_2 <90%) and ECG changes during colonoscopy screening in an average-risk population (hospital personnel, n=214, mean age 54.0±3.8, 39.3% male), without significant comorbidity) and aims at identifying subject-related and/or endoscopic factors associated with their occurrence. All data were collected prospectively. During 214 consecutive primary screening colonoscopies under conscious sedation (midazolam and pethidine), on top of pulse rate and SaO_2, blood pressure and a three-channel ECG were recorded every five minutes.

Results: No major complications or relevant ECG changes occurred. Hypoxaemia occurred in 119 (55.6%), hypotension in 19 (8.9%) and bradycardia in 12 subjects (5.6%). In multivariate analysis, the sedation level 3 increased the risk of hypoxaemia (OR 4.8, CI 1.7-13.7), and incomplete colonoscopy (OR 5.3, CI 1.6-18.1) was associated with hypotension. Subjects with bradycardia had a longer mean procedure time (38±12 vs. 29±12 min, p<0.05), which did not turn out as a risk factor in a multivariate analysis.

Conclusions: Mainly procedure-related and not subject-related factors were found to be associated with the occurrence of cardiopulmonary events in primary colonoscopy screening in this relatively healthy screening population.

KEYWORDS

Colonoscopy, monitoring, cardiopulmonary events, complications, sedation

INTRODUCTION

Colorectal cancer (CRC) is posing a major health issue, with each year over 1.2 million cases and an estimated 608,000 deaths worldwide.[1] CRC mortality can be lowered by CRC screening, as early detection of tumours improves disease outcome[2] and the removal of adenomatous polyps is able to reduce CRC mortality.[3] Several CRC screening methods are currently accepted, such as the Faecal Occult Blood Test (FOBT), sigmoidoscopy and colonoscopy, each characterised by advantages and disadvantages.[2] Colonoscopy is currently considered the standard for the detection of colorectal neoplasia and in case of positive findings detected by FOBT or sigmoidoscopy, a colonoscopy has to be performed for verification and possible removal of the lesion.

Colonoscopy as a primary screening method has been implemented in an increasing number of countries, and the US Centre of Disease Control has reported a rising adherence to endoscopy screening in the country.

Colonoscopy is considered a rather safe procedure. Large screening studies have reported a low risk of major complications, such as perforation, bleeding and serious cardiopulmonary complications (e.g. myocardial infarction, arrhythmia).[4,5] However, several studies on clinical diagnostic and therapeutic colonoscopies have reported the occurrence of one or several cardiopulmonary events (e.g. dysrhythmias, ST elevations/depressions, hypoxaemia, bradycardia and hypotension)[2,6] and pointed especially to the high occurrence of hypoxaemia in 64% of the examinations.[7] Such cardiopulmonary events may increase the risk of serious cardiopulmonary complications.[8] In a recent survey of over 12,000 diagnostic and/or therapeutic colonoscopies, the occurrence of cardiopulmonary complications necessitated termination of the examination in 0.25%.[9] With the steady increase of primary and follow-up screening colonoscopies, performed in relatively healthy populations, it is important that data are obtained on the frequency and severity of cardiopulmonary events and to identify factors associated with their occurrence. The primary aim of the present observational study was to assess the frequency of cardiopulmonary events during screening with primary colonoscopy and to identify subject- and procedure-related factors associated with these events.

POPULATION AND METHODS

Study population and colonoscopic procedure

Employees (50 to 65 years) of the Academic Hospital Maastricht, the Netherlands, were invited for colorectal cancer screening by primary colonoscopy. During an appointment prior to the colonoscopy, a standardised short medical history, medication used, and weight were registered by an experienced nurse. Subjects were excluded if they had undergone a colonoscopy within the previous five years, reported severe comorbidity increasing the risk of colonoscopy, were under surveillance for colorectal neoplasia and/or had onset of acute gastrointestinal symptoms in the previous three months.

The study protocol was approved by the Dutch Health Council (Ministry of Health) and the local medical ethics committee. All participants gave written informed consent.

Colonoscopic procedure

A total of 214 unselected, consecutive screening colonoscopies were performed by four experienced endoscopists and information on the endoscopic procedure, including objective colonoscopy quality indicators[10] and reasons for incomplete procedures, were registered using a standardised form.

A polyethylene glycol-based electrolyte solution (Klean Prep, Norgine b.v., Higher Denham, UK) had been given as bowel cleansing, starting 24 hours before colonoscopy. All participants were offered conscious sedation, consisting of midazolam and pethidine. A starting dose was administered intravenously (i.v.) prior to colonoscopy and in case of discomfort during the procedure an additional dose could be given. After caecal intubation, the antispasmodic/anticholinergic agent scopolamine butyl bromide 20 mg was given i.v. in order to relax the colonic wall before instrument retraction for thorough control of the mucosal surface. In case of contraindications medication was not administered. Sedation levels were registered using level 1 for 'awake', level 2 for 'sleepy', level 3 for 'eyes closed, reacts to verbal stimuli' (level 2+3 are targets of conscious sedation), level 4 for 'eyes closed, reacts to physical stimuli' (deep sedation), and level 5 for 'eyes closed, no reaction to verbal or physical stimuli' (general anaesthesia).[11,12]

Cardio respiratory monitoring

Prior to, during and 10 minutes after colonoscopy, pulse rate, blood oxygenation, and a three-channel electrocardiogram (ECG) were monitored continuously in all procedures. Furthermore, systolic (SBP) and diastolic (DBP) blood pressure were measured every five minutes. Hypoxaemia was defined as oxygen saturation (SaO_2) below 90%, lasting several seconds, oxygen being supplemented by nasal catheter if the SaO_2 did not immediately normalise spontaneously. A pulse rate below 60 min^{-1} was defined as bradycardia. Hypotension was defined as an SBP below 90 mmHg. Mean arterial pressure (MAP) was calculated as: MAP= DPB + 1/3 x (SBP-DBP).

Questionnaires

Before colonoscopy, a standardised questionnaire was completed by the participants on medical history (e.g. smoking, hypertension, pulmonary and/or cardiac disease) and after colonoscopy another questionnaire on symptoms during as well as complications and/or symptoms in the first month after the procedure.

Statistical analysis

Dichotomous variables were compared using a χ^2 test, with Fisher's exact test when necessary. Parametric continuous variables were compared using a Student's t-test. Significant variables identified were subsequently included in multivariate logistic regression models, adjusted for age and gender for the following outcome measures: hypoxaemia, bradycardia, and hypotension. All tests were conducted using SPSS version 15.0 (SPSS inc, 2006) and a p-value below 0.05 was considered to be statistically significant (using two-sided tests).

As hypoxaemia was expected to be the most frequent cardiopulmonary event, its frequency of occurrence was defined as the primary outcome measure. Bradycardia and hypertension were secondary outcome measures.

With an α of 0.05 and power of 80%, we were able to detect a minimal difference of 20% in characteristics between groups for hypoxaemia (group sizes 119 and 95), of 25% for hypotension (group sizes 19 and 195), and of 32% for bradycardia (group sizes 12 and 202).

RESULTS

Study population and colonoscopy procedure

The study population had a mean age of 54.0±3.8 years, and consisted of 84 men (39.3%) and 130 women (60.7%). A medical history of hypertension, pulmonary or cardiac disease was present in 41 (19.3%), 11 (5.1%), and 8 (3.8%) subjects, respectively. Furthermore, 36 (16.8%) participants were current smokers. In total, the American Society of Anesthesiologists (ASA) physical status was classified as I/II in 85.5% and III in 14.5% of participants. Medical history was the reason for exclusion in only one subject.

In total, 214 subsequent and unselected screening colonoscopies were monitored. Caecal intubation rate was 92.0%. Adenomas were detected and removed in 51 participants (23.8%). In total 211 (98.6%) participants had chosen to undergo colonoscopy under conscious sedation. No major complications such as bleeding or perforation occurred during or were reported up to one month after colonoscopy.

Cardiopulmonary events

Hypoxaemia, bradycardia, and hypotension, as previously defined, occurred in 119 (55.6%), in 12 (5.6%), and in 19 (8.9%) subjects, respectively, during the colonoscopy procedure. Apart from bradycardia, no relevant ECG changes occurred during or up to ten minutes after colonoscopy. Major cardiopulmonary complications (e.g. symptomatic myocardial ischaemia or dysrhythmias) did not occur during colonoscopy, nor were they reported by participants in the one month follow-up period.

Mean baseline values just before the start of the colonoscopy were 97.4±1.8% for oxygen saturation, 77.3±14.9 min-1 for pulse rate, 147.8±20.8 mmHg for SBP, and 108.2±13.9 mmHg for MAP.

In the group with hypoxaemia, the mean of the lowest SaO_2 value reached was 86.7±2.9% with a mean time of occurrence of 13.2±8.3 min after start of the procedure. Oxygen was supplemented in 82 of these 119 cases (68.6%). In colonoscopies in which hypoxaemia occurred compared with those without hypoxaemia, the mean procedure time was longer (31±12 *vs* 28±12 min, p=0.046), mean dosages of midazolam (0.06±0.02 *vs* 0.05±0.02 mg/kg, p=0.000) and pethidine (0.71±0.18 *vs* 0.58±0.22 mg/kg, p=0.000) were higher, sedation level 3 was more frequent (63.6 *vs* 25.0%, p=0.000), level 1 (10.2 *vs* 32.6%,p=0.00) and level 2 (22.9 *vs* 42.4%, p=0.003) were less frequent, and severe abdominal pain during colonoscopy was more frequent (15.0 *vs* 3.7%, p=0.012) (*table 1*). In a multivariate regression analysis only sedation level 3 (conscious sedation) was associated with hypoxaemia (OR 4.8, CI 1.7 to 13.7).

When using a lower cut-off level for hypoxaemia, as recently proposed by Cotton *et al.*,[13] 19 participants (8.9%) had an SaO_2 below 85%. In this group no statistically significant subject- or procedure-related differences were found compared with the group with an SaO_2 ≥85%.

Table 1. *Differences between participants with or without hypoxaemia (SaO_2<90) during colonoscopy*

	Oxygen saturation		
	Hypoxaemia n=119	Normal n=95	p value*
Participants			
Age	54.4 ±3.9	53.6 ±3.6	0.128
Gender: % women	63.0	57.9	0.483
BMI (kg/m²)	24.7	25.0	0.585
Current smoking %	14.3	20	0.276
History of pulmonary disease %	5.0	5.3	1.000
History of cardiac disease %	2.6	5.3	0.471
History of hypertension %	20.4	18.5	0.730
ASA classification			
- I/II	87.4	84.0	0.560
- III	12.6	16.0	
Procedures			
Procedure time (min)	31 ±12	28 ±12	0.046
Caecal intubation rate %	89.8	94.7	0.214
Sedation medication			
- Midazolam (mg/kg)	0.06 ±0.02	0.05± 0.02	0.000
- Pethidine (mg/kg)	0.71 ±0.18	0.58± 0.22	0.000
Sedation level %			
1. Awake	10.2	32.6	0.000
2. Sleepy (anxiolysis)	22.9	42.4	0.003
3. Eyes closed, reacts to verbal stimuli (conscious sedation)	63.6	25.0	0.000
4. Eyes closed, reacts to physical stimuli (deep sedation)	2.5	0	0.258
5. Eyes closed, unarousable (general anaesthesia)	0.8	0	1.000
Polypectomy and/or biopsies %	52.9	50.5	0.784
Severe abdominal pain during colonoscopy^ %	15.0	3.7	0.012

Variables presented as mean ± SD, or %; ^no significant findings for all other symptoms during colonoscopy; *based on χ^2 or Student's t-test

In the group with bradycardia, the mean lowest value was 43.6 min^{-1} ± 4.0 and the mean time of occurrence was 7.0±3.3 min after procedure start. The mean procedure time was longer compared with those without bradycardia (38±12 *vs* 29±12 min, p=0.014) *(table 2)*. This factor was not significant in the multivariate regression analysis. Two participants had a pre-colonoscopy bradycardia, but had normal pulse rates during colonoscopy.

In the entire study group, blood pressure values were higher before (148±20.8 mmHg for SBP) than at the end of colonoscopy (125±16.4 mmHg for SBP). With respect to hypotension (n=19), the mean nadir SBP was 81.7±7.8 mmHg. The mean procedure time after which hypotension occurred was 19.0±16.6 min. In all participants with hypotensive events, blood pressure normalised spontaneously without i.v. fluid administration. In 13 out of the 19 subjects (68.4%) the pulse rate remained within the normal range, the remainder showed a bradycardia during the hypotensive event. In one case, the hypotensive event was registered as reason for not completing the colonoscopy. The mean decrease in MAP during colonoscopy compared with the baseline MAP was 13±2.3%. A relative decrease of more than 40% occurred in 20 patients (9.3%).
Colonoscopies in which hypotension occurred were less often complete (68.4 *vs* 94.3%, p=0.001), and biopsies and/or polypectomies were less frequently performed (26.3 *vs* 54.4%, p=0.029) **(***table 3***)**. In multivariate regression analysis, only incomplete colonoscopy (OR 5.3, CI 1.6 to 18.1) was associated with hypotension.

Table 2. *Differences between participants with or without bradycardia (pulse rate <60min^{-1}) during colonoscopy*

	Pulse rate		
	Brady-cardia n=12	**Normal n=202**	**p value***
Participants			
Age	54.6 ±4.3	54.0 ±3.7	0.600
Gender: % women	66.7	60.4	0.768
BMI (kg/m^2)	24.9	24.2	0.517
Current smoking %	0	17.8	0.225
History of pulmonary disease %	0	5.4	1.000
History of cardiac disease %	0	4.0	1.000
History of hypertension %	33.3	18.5	0.253
ASA classification			
- I/II	85.6	83.3	0.687
- III	14.4	16.7	
Procedures			
Procedure time (min)	38 ±12	29 ±12	0.014
Caecal intubation rate %	83.3	92.5	0.246
Sedation medication			
- Midazolam (mg/kg)	0.06± 0.03	0.06± 0.02	0.801
- Pethidine (mg/kg)	0.67± 0.22	0.65± 0.21	0.715
Sedation level %			
1. Awake	16.7	20.2	1.000
2. Sleepy (anxiolysis)	16.7	32.3	0.347
3. Eyes closed, reacts to verbal stimuli (conscious sedation)	58.3	46.0	0.553
4. Eyes closed, reacts to physical stimuli (deep sedation)	8.3	1.0	0.163
5. Eyes closed, unarousable (general anaesthesia)	0	0.5	1.000
Polypectomy and/or biopsies %	50.0	52.0	1.000
Severe abdominal pain during colonoscopy^ %	22.2	9.3	0.221

Variables presented as mean ± SD, or %; ^no significant findings for all other symptoms during colonoscopy; *based on χ^2 or Student's t-test

DISCUSSION

With the steady increase of primary and follow-up screening colonoscopies, performed in average risk subjects, data on the frequency and severity of cardiopulmonary events and on factors associated with their occurrence in a screening setting, are of clinical importance. In 214 consecutive screening colonoscopies, no major complications occurred; however, monitoring revealed a frequent occurrence of minor cardiopulmonary events. Mainly procedure-related and not subject-related factors were found to be associated with their occurrence.
Of all cardiopulmonary events, hypoxaemia (<90%) occurred most frequently in more than half of the colonoscopies. It should be noted that the clinical relevance of these hypoxaemic events and the clinical relevance of the cut-off level to be used are still under debate. With a cut-off level of SaO_2 <85%, as recently proposed during an American Society for Gastrointestinal Endoscopy (ASGE) workshop,[13] only 8.9% of the participants would have had such an event. However, it should be taken into account that oxygen administration was immediately started upon an SaO_2 <90 % and this might have prevented a further decrease of the SaO_2 to <85%.
A conscious sedation level, which is usually reached when using moderate doses of midazolam and pethidine, increased the risk for the occurrence of hypoxaemia (defined as SaO_2 <90%).[11] No differences were found in person- and procedure-characteristics between participants with SaO_2 below or above 85%. This may be due to small sample size (i.e. 19 subjects with SaO_2 <85%).
A high frequency of oxygen desaturation occurring in colonoscopies under conscious sedation has been reported by others, although with a substantial

Table 3. *Differences between participants with or without hypotension (SBP <90 mmHg) during colonoscopy*

	Blood pressure		
	Hypo-tension N=19	Normal N=195	p value*
Participants			
Age	55.0 ±4.2	53.9 ±3.7	0.293
Gender: % women	63.2	60.5	1.000
BMI (kg/m²)	24.9	24.2	0.349
Current smoking %	5.3	17.9	0.210
History of pulmonary disease %	0	5.6	0.604
History of cardiac disease %	10.5	3.1	0.154
History of hypertension %	15.8	19.7	1.000
ASA classification			
- I/II	85.1	89.5	1.000
- III	14.9	10.5	
Procedures			
Procedure time (min)	34 ±15	29 ±12	0.120
Caecal intubation rate %	68.4	94.3	0.001
Sedation medication			
- Midazolam (mg/kg)	0.06± 0.02	0.06± 0.02	0.495
- Pethidine (mg/kg)	0.71± 0.13	0.65± 0.22	0.220
Sedation level %			
1. Awake	5.3	21.5	0.132
2. Sleepy (anxiolysis)	42.1	30.4	0.308
3. Eyes closed, reacts to verbal stimuli (conscious sedation)	52.6	46.1	0.635
4. Eyes closed, reacts to physical stimuli (deep sedation)	0	1.6	1.000
5. Eyes closed, unarousable (general anaesthesia)	0	0.5	1.000
Polypectomy and/or biopsies %	26.3	54.4	0.029
Severe abdominal pain during colonoscopy^ %	23.1	8.9	0.125

Variables presented as mean ± SD, or %; ^no significant findings for all other symptoms during colonoscopy; *based on χ^2 or Student's t-test

variation (33 to 64%).[7,14,15] This variation may result from differences in medication (e.g. propofol), differences in population characteristics or use of various cut-off levels. Furthermore, in some studies O_2 was administered preventively or hypoxaemia was defined as such only if it lasted for a certain predefined period of time. Since many studies have reported high frequencies of hypoxaemia, it has been suggested that preventive O_2 administration should be considered, but results from studies are conflicting.[11,16] Some studies have been shown a reduction of the frequency and/or the magnitude of desaturation,[17] whereas others have reported a higher frequency of cardiopulmonary 'unforeseen' events when preventive O_2 was administered.[18]

Bradycardia and hypotension occurred in 6% and 9% of colonoscopies, respectively. This is in line with literature data showing rates of 12% for bradycardia and 6 to 19% for hypotension during diagnostic and therapeutic colonoscopies using various sedatives.[6,14,19-21] It should be noted that for the detection of differences in subject- or procedure-related factors, the sizes of the groups with hypotension and bradycardia were small. Therefore, some potential risk factors, with a weaker association, might have been missed. However, differences in procedure-related factors were found for these group. In the subsequent multivariate analysis no association of subject- and/or procedure-related factors with bradycardia were identified but an incomplete colonoscopy was found to be associated with the occurrence of hypotension. Hypotension was the reason to interrupt the procedure in only one subject. Therefore, occurrence of hypotension is not an explanation for incomplete colonoscopy procedures. A more plausible explanation might be that in incomplete colonoscopies abdominal pain was more frequently present, the dosages of sedatives used were higher, and the sedation level reached was deeper (*data not shown*). Therefore we hypothesise that hypotension may have occurred as a vaso-vagal reaction due to pain and/or as a consequence of higher dosages of sedatives used.

In general, no association was found between pre-existing morbidity and the occurrence of hypoxaemia, hypotension or bradycardia. It has, however, to be considered that this workplace-based population consisted of relatively healthy and health-conscious subjects, in whom the severity of morbidity was probably lower than in many other screening and in most diagnostic and therapeutic colonoscopy populations, in whom a higher ASA classification has been shown to increase the risk for cardiopulmonary events.[22] Furthermore, in the present study, having the advantage of the application of a pre-screening medical interview, one subject was excluded based on severe comorbidity. Exclusion of such subjects with severe comorbidity might further reduce the incidence cardiopulmonary events.

We conclude that, even though the population was relatively healthy, hypoxaemia, arterial hypotension and bradycardia frequently occur during CRC screening with primary colonoscopy under conscious sedation. Procedure-related and not subject-related factors were associated with their occurrence.

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REFERENCES

1. Ferlay J, Shin HR, Bray F, et al. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. International Journal of Cancer. 2010;epub ahead:NA.
2. Levin B, Lieberman DA, McFarland B, et al. Screening and Surveillance for the Early Detection of Colorectal Cancer and Adenomatous Polyps, 2008: A Joint Guideline From the American Cancer Society, the US Multi-Society Task Force on Colorectal Cancer, and the American College of Radiology. Gastroenterology. 2008;134(5):1570-95.
3. Winawer SJ, Zauber AG, Ho MN, et al. Prevention of Colorectal Cancer by Colonoscopic Polypectomy. N Engl J Med. 1993 December 30, 1993;329(27):1977-81.
4. Sieg A, Theilmeier A. [Results of coloscopy screening in 2005--an Internet-based documentation]. Dtsch Med Wochenschr. 2006 Feb 24;131(8):379-83.
5. Wilkins T, LeClair B, Smolkin M, et al. Screening Colonoscopies by Primary Care Physicians: A Meta-Analysis. Ann Fam Med. 2009 January 1, 2009;7(1):56-62.
6. McQuaid KR, Laine L. A systematic review and meta-analysis of randomized, controlled trials of moderate sedation for routine endoscopic procedures. Gastrointestinal Endoscopy. 2008;67(6):910-23.
7. Jaffe P, Fennerty M, Sampliner R, Hixson L. Preventing hypoxemia during colonoscopy. A randomized controlled trial of supplemental oxygen. J Clin Gastroenterol. 1992 Mar;14(2):114-6.
8. Lazzaroni M, Bianchi Porro G. Preparation, Premedication and Surveillance. Endoscopy. 2003;35(02):103-11.
9. Radaelli F, Meucci G, Minoli G. Colonoscopy practice in Italy: A prospective survey on behalf of the Italian Association of Hospital Gastroenterologists. Digestive and Liver Disease. 2008;40(11):897-904.
10. Rex DK, Petrini JL, Baron TH, et al. Quality indicators for colonoscopy. Am J Gastroenterol. 2006 Apr;101(4):873-85.
11. Cohen LB, DeLegge MH, Aisenberg J, et al. AGA Institute Review of Endoscopic Sedation. Gastroenterology. 2007;133(2):675-701.
12. Commissie kwaliteit en richtlijnen Nederlands Genootschap van artsen voor Maag- Darm- en Leverziekten. Richtlijn sedatie en /of analgesie door Maag-, Darm- en Leverartsen bij endoscopische ingrepen. http://wwwmdlnl/uploads/240/121/Richtlijn_sedatie_en_of_analgesie_door_mdl-artsen_bij_endoscopische_ingrepenpdf.
13. Cotton PB, Eisen GM, Aabakken L, et al. A lexicon for endoscopic adverse events: report of an ASGE workshop. Gastrointestinal Endoscopy. 2010;71(3):446-54.
14. Froehlich F, Thorens J, Schwizer W, et al. Sedation and analgesia for colonoscopy: patient tolerance, pain, and cardiorespiratory parameters. Gastrointest Endosc. 1997 Jan;45(1):1-9.
15. O'Connor K, Jones S. Oxygen desaturation is common and clinically underappreciated during elective endoscopic procedures. Gastrointest Endosc. 1990 May-Jun;36(3 Suppl):S2-4.
16. Lichtenstein DR, Jagannath S, Baron TH, et al. Sedation and anesthesia in GI endoscopy. Gastrointestinal Endoscopy. 2008;68(5):815-26.
17. Rozario L, Sloper D, Sheridan MJ. Supplemental Oxygen During Moderate Sedation and the Occurrence of Clinically Significant Desaturation During Endoscopic Procedures. Gastroenterology Nursing. 2008;31(4):281-5.
18. Sharma VK, Nguyen CC, Crowell MD, et al. A national study of cardiopulmonary unplanned events after GI endoscopy. Gastrointestinal Endoscopy. 2007;66(1):27-34.
19. Ristikankare M, Julkunen R, Mattila M, et al. Conscious sedation and cardiorespiratory safety during colonoscopy. Gastrointestinal Endoscopy. 2000;52(1):48-54.
20. Poon C, Leung T, Wong C, et al. Safety of nurse-administered propofol sedation using PCA pump for outpatient colonoscopy in Chinese patients: a pilot study. Asian J Surg. 2007 Oct;30(4):239-43.
21. Lee D, Chan A, Wong S, et al. The safety, feasibility, and acceptability of patient-controlled sedation for colonoscopy: prospective study. Hong Kong Med J. 2004 Apr;10(2):84-8.
22. Vargo JJ, Holub JL, Faigel DO, Lieberman DA, Eisen GM. Risk factors for cardiopulmonary events during propofol-mediated upper endoscopy and colonoscopy. Alimentary Pharmacology & Therapeutics. 2006;24(6):955-63.

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CASE REPORT

Cyclophosphamide-induced symptomatic hyponatraemia

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ABSTRACT

Cyclophosphamide is an alkylating agent used in antineoplastic and immunosuppressive therapies. Symptomatic hyponatraemia is a rare but life-threatening complication in patients treated with cyclophosphamide. We report the case of a 64-year-old woman with breast cancer who developed severe symptomatic hyponatraemia with a generalised seizure and convulsions after a second cycle of adjuvant chemotherapy with 5-fluouracil, epirubicin and cyclophosphamide. She completely recovered after correction of the serum sodium concentration without neurological deficits. Physicians prescribing cyclophosphamide, irrespective of the treatment indication and dosage, should be aware of this potentially life-threatening complication.

KEYWORDS

Cyclophosphamide, adverse effects, hyponatraemia

What was known on this topic?
Severe hyponatraemia after administration of low-dose cyclophosphamide therapy (<15 mg/kg) is extremely rare. The exact mechanism of action is unclear. A direct toxic effect of cyclophosphamide or its metabolites on renal collecting tubules or an antidiuretic hormone-like activity of cyclophosphamide metabolites has been suggested.

What does this case add?
In this case, severe hyponatraemia with neurological symptoms occurred shortly after administration of low-dose cyclophosphamide. No definite mechanism of action could be elucidated. A potential role for citalopram as a contributing causal factor can not be excluded. Physicians should be aware of contributing factors, such as renal failure, drug interactions and extreme water intake.

INTRODUCTION

Severe hyponatraemia (serum sodium <120 mmol/l) is a serious electrolyte disorder with potential life-threatening neurological complications. It has been reported in association with a variety of anticancer drug regimens including cytotoxic agents as vinca alkaloids, platinum compounds and alkylating agents.[1]
Cyclophosphamide, an alkylating agent, is widely used to treat malignant neoplasms and can be effective in the treatment of several rheumatic diseases. We report a patient with severe, symptomatic hyponatraemia which occurred during the second chemotherapy cycle containing cyclophosphamide.

CASE REPORT

A 64-year-old woman, suffering from a pT1cN1aG1M0 carcinoma of the left breast, was planned to receive three cycles of adjuvant chemotherapy containing 5-fluouracil 500 mg/m^2, epirubicin 100 mg/m^2 and cyclophosphamide 500 mg/m^2 (FEC) with a three-week time interval. Her medical history included depression and anxiety disorder for which she was treated with flurazepam and alprazolam. Three months before chemotherapy, citalopram was prescribed with a stepwise increase in dosage. Seven years before she also had been treated with citalopram in a dose of 40 mg for her depression, without any side effects.

The first cycle of chemotherapy was uneventful. Ten days before the second cycle the dosage of citalopram was increased from 30 mg to 40 mg daily. On day 1 of the second cycle, normal renal function and serum potassium were observed. The serum sodium concentration was 134 mmol/l (normal 135 to 145 mmol/l). Concomitant with chemotherapy, the patient was hydrated with 0.5 litre of isotonic saline. Antiemetic therapy consisted of dexamethasone and ondansetron. Furthermore, the patient ingested approximately 1.5 to 2 litre of tea and water after administration of chemotherapy. She reported dizziness in the evening of day 1 and was advised to take extra dexamethasone. On the second day, 28 hours after chemotherapy, she developed a generalised seizure with convulsions, after a period of impaired consciousness and incoherent speech. At the emergency ward a Glasgow Coma Score of 3 was observed. Her blood pressure was 128/54 mmHg with a pulse of 68 beats/min. She was euvolaemic and had an urine output of 80 ml in the first hour after admission. Laboratory tests showed a serum sodium of 107 mmol/l, urinary sodium of 29 mmol/l and serum potassium at 4.6 mmol/l (normal 3.5 to 5.0 mmol/l). The CT scan of the brain revealed no abnormalities.
Her sodium deficit was calculated at 480 mmol, with a desired serum sodium of at least 120 mmol/l. Because of the severity of the symptoms, urgent intervention with hypertonic saline infusion (800 ml NaCl 3% at 100 ml/h) was initiated on the intensive care unit and the citalopram was discontinued. Within 12 hours, the serum sodium concentration rose gradually from 104 mmol/l to 120 mmol/l and the patient slowly recovered from her neurological symptoms. During the next five days, the serum sodium concentration was slowly corrected up to 135 mmol/l by infusion of isotonic saline (*table 1*). The patient was discharged asymptomatically after seven days.
Reintroduction of citalopram in a dose of 20 mg did not induce a fall of the serum sodium concentration.
On day 22 the patient received the third chemotherapy cycle without administration of cyclophosphamide. This cycle was well tolerated without neurological symptoms or electrolyte imbalances.

DISCUSSION AND REVIEW OF THE LITERATURE

A deep hyponatraemia with severe neurological symptoms was observed in our patient within 28 hours after administration of the second cycle of FEC chemotherapy. In the absence of structural brain lesions, no evidence for renal, heart or liver failure, no hypothyroidism, and adrenal insufficiency highly improbable with dexamethasone gifts before and after chemotherapy, the hyponatraemia is considered to be chemotherapy related and very likely cyclophosphamide related.

Cyclophosphamide can induce severe hyponatraemia. This life-threatening side effect was first described in patients treated with high-dose i.v. cyclophosphamide (30 to 40 mg/kg), and later in patients treated with moderate doses (20 to 30 mg/kg).[2,3] There are a small number of cases of severe hyponatraemia after administration of low-dose cyclophosphamide therapy (<15 mg/kg).[4-11] These data are summarised in *table 2*.

The exact mechanism of action is unclear. The syndrome of inappropriate antidiuretic hormone secretion (SIADH) has been proposed in a fatal case of severe hyponatraemia in a patient who had received high-dose i.v. cyclophosphamide.[3] Post-mortem examination revealed

Table 1. *Serum electrolytes, clinical chemistry values and neurological state pre and post chemotherapy*

	Normal range	1 hour before chemotherapy	28 hours post chemotherapy	42 hours post chemotherapy	5 days post chemotherapy
Haemoglobin	7.2-9.8 mmol/l	7.4	6.7	ND	ND
Haematocrit	0.35-0.47 l/l	0.34	0.29	0.30	ND
Glucose	3.5-7.8 mmol/l	8.0	7.5	6.3	ND
Sodium	135-145 mmol/l	134	107	120	135
Potassium	3.5-5.0 mmol/	4.8	4.6	4.3	ND
Creatinine	50-90 µmol/l	60	54	ND	ND
Urea	2.0-4.0 mmol/	ND	3.8	ND	ND
Urinary sodium	(variable) mmol/	ND	29	ND	ND
TSH	mU/	ND	ND	2.6	ND
Neurological symptoms		Generalised seizure and convulsions	Sedated and intubated	Sedated and intubated	No neurological deficits

ND = not determined, TSH = thyroid-stimulating hormone.

Table 2. *Published reports of hyponatraemia after low-dose i.v. cyclophosphamide*

Indication of treatment	Age (years) and sex	Cyclophosphamide dosage	Serum sodium (mmol/l)	Possible influencing factors	(Estimated) fluid intake (l/h)	References
Multiple myeloma	68, male	500 mg, iv	108	Concomitant use of indomethacin	3l/24h	4
SLE	59, female	10 mg/kg, iv	116	-	2.4l/24h	5
Sjögren's disease	57, female	780 mg, iv	117	-	>0.95l/6h	6
SLE	48, female	750 mg, iv	119	-	3l/24h	7
SLE	53, female	500 mg, iv	119	-	3l/2h	7
ANCA-related glomerulonephritis	70, female	50 mg, iv	108	Renal failure and hypoalbuminaemia	>2l/12h	8
Neuro-Behcet	43, male	15 mg/kg	107	High fluid intake	6l/6h	9
Polyarteritis nodosa	46, female	15 mg/kg	112	High fluid intake	10l/12h	9
SLE	30, female	15 mg/kg	106	Renal involvement in SLE, high fluid intake	5l/8h	9
Diffuse cutaneous systemic sclerosis	49, female	500 mg	106	-	Unknown	10
Metastatic adenocarcinoma of the small salivary glands	69, female	500 mg/m^2	116	Concomittant administration of cisplatin	Unknown	11
Breast cancer	64, female	500 mg/m^2	107	-	2l/24h	This case

ANCA = antineutrophil cytoplasmic autoantibodies, iv = intravenous, SLE = systemic lupus erythematosus.

loss of Herring's bodies and degranulation of various hypothalamic neurosecretory organelles, which supported this hypothesis. In other cases, no rise of antidiuretic hormone (ADH) concentrations could be demonstrated.[2,8] Interesting is the case of a girl with established diabetes insipidus who developed hyponatraemia after cyclophosphamide infusion despite an inability to secrete ADH.[12] A direct toxic effect of cyclophosphamide or its metabolites on renal collecting tubules or an antidiuretic hormone-like activity of cyclophosphamide metabolites, has been suggested.[4] Solely based on the euvolaemic state of our patient and the urinary sodium of >20 mmol/l neither mechanism can be confirmed or ruled out in this case.

Patients in a recent series of three cases of severe hyponatraemia were reported to have ingested extreme amounts of fluids in a short time after cyclophosphamide infusion (*table 2*). Since our patient drank only two litres of fluids after the cyclophosphamide, this is insufficient to explain her deep hyponatraemia. In general, physicians should be aware of extreme water intake in patients treated with cyclophosphamide. Not seldom patients are advised to drink substantial amounts of water to reduce the risk of the side effect of haemorrhagic cystitis.

Other factors that may contribute to the severity of the hyponatraemia as described in previous cases are the presence of renal failure and hypoalbuminaemia and drug interactions with non-steroidal anti-inflammatory drugs or concomitant administration of platinum compounds, such as cisplatin (*table 2*). In our case, a potential role for citalopram in the induction of the severe hyponatraemia can not be excluded, although treatment with citalopram in the past was uneventful and rechallenge with citalopram did not induce a rebound hyponatraemia. Based on the Naranjo causality scale, a ten-question-based method for estimating the causality of adverse reactions and drug use, the causal relationship between cyclophosphamide and citalopram and the hyponatraemia is estimated as probable and possible, respectively.[13] Causality of an interaction phenomenon between cyclophosphamide and citalopram using the drug interaction probability scale of Horn *et al.* was estimated as doubtful.[14]

In conclusion, physicians prescribing cyclophosphamide, irrespective of the treatment indication and dosage, should be aware of the acute, potentially life-threatening complication of severe hyponatraemia.

REFERENCES

1. Berghmans T. Hyponatremia related to medical anticancer treatment. Support Care Cancer. 1996 Sep;4(5):341-50.
2. Bressler RB, Huston DP. Water intoxication following moderate-dose intravenous cyclophosphamide. Arch Intern Med. 1985 Mar;145(3):548-9.
3. Harlow PJ, DeClerck YA, Shore NA, Ortega JA, Carranza A, Heuser E. A fatal case of inappropriate ADH secretion induced by cyclophosphamide therapy. Cancer. 1979 Sep;44(3):896-8.
4. Webberley MJ, Murray JA. Life-threatening acute hyponatraemia induced by low dose cyclophosphamide and indomethacin. Postgrad Med J. 1989 Dec;65(770):950-2.
5. McCarron M, Wright GD, Roberts SD. Water intoxication after low dose cyclophosphamide. BMJ. 1995 Jul;29;311(7000):292.
6. Spital A, Ristow S. Cyclophosphamide induced water intoxication in a woman with Sjogren's syndrome. J Rheumatol. 1997 Dec;24(12):2473-5.

7. Salido M, Macarron P, Hernandez-Garcia C, D'Cruz DP, Khamashta MA, Hughes GR. Water intoxication induced by low-dose cyclophosphamide in two patients with systemic lupus erythematosus. Lupus. 2003;12(8):636-9.

8. Kato A, Sugiura T, Yamamoto T, Misaki T, Tsuji T, Sakao Y, et al. Water intoxication induced by low-dose oral cyclophosphamide in a patient with anti-neutrophil cytoplasmic antibody-related glomerulonephritis. NDT Plus. 2008 Oct;1(5):286-8.

9. Alilou M, Awab A, Zarouf M, Moussaoui RE, Hijri AE, Azzouzi A, et al. [Severe hyponatraemia secondary to cure of cyclophosphamide (about three cases)]. Ann Fr Anesth Reanim. 2009 Jan;28(1):103-4.

10. Jayachandran NV, Chandrasekhara PK, Thomas J, Agrawal S, Narsimulu G. Cyclophosphamide-associated complications: we need to be aware of SIADH and central pontine myelinolysis. Rheumatology. (Oxford) 2009 Jan;48(1):89-90.

11. Berger AK, Bellos F, Siegmund A, Eisenbach C, Lordick F. Symptomatic hyponatraemia caused by cylophosphamide. Onkologie. 2009 May;32(5):280-2.

12. Campbell DM, Atkinson A, Gillis D, Sochett EB. Cyclophosphamide and water retention: mechanism revisited. J Pediatr Endocrinol Metab. 2000 Jun;13(6):673-5.

13. Naranjo CA, Busto U, Sellers EM, Sandotr P, Ruiz I, Roberts EA, et al. A method for estimating the probability of adverse drug reactions. Clin Pharmacol Ther. 1981;30:239-45.

14. Horn JR, Hansten PD, Chan LN. Proposal for a new tool to evaluate drug interaction cases. Ann Pharmacother. 2007 Apr;41(4):674-80. Epub 2007 Mar 27.

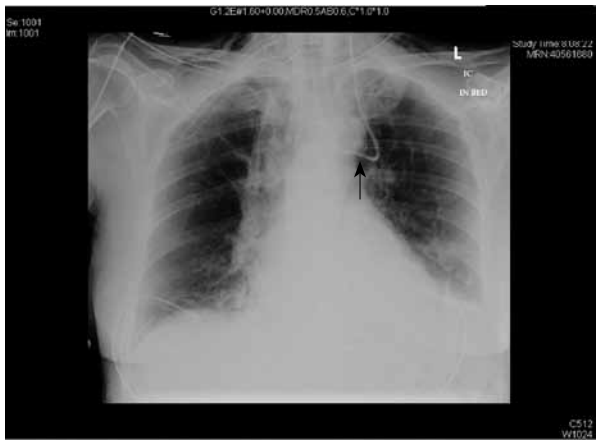
ANSWER TO PHOTO QUIZ (PAGE 184)

A POSTOPERATIVE PUZZLE

DIAGNOSIS

An aberrant location of a central venous catheter is observed in approximately 5 to 10% of all procedures.[1] The majority of malpositions concern the descending aorta, a persistent left superior vena cava or one of the local smaller veins (e.g., the left internal thoracic vein, the cardiophrenic vein or the left superior intercostal vein).[2] Among the more serious complications of malpositioning are hydromediastinum after perforation of a small vein and pericardial tamponade due to a lesion of the pericardiophrenic vein. Extravascular (e.g. mediastinal, pericardial or pleural) positioning of the venous catheter has also been described.[3] Extravascular malpositions are excluded in the presence of smooth aspiration of blood through all lumina. Additionally, diagnostic procedures such as a chest radiography, administration of intravenous contrast, blood gas analysis, and assessment of the venous pressure, can clarify the situation. In the present case the malposition, in a superior intercostal vein (*figure 2*), did not have consequences.

Figure 2. *Chest X-ray showing central venous catheter in left superior intercostal vein (arrow)*



ACKNOWLEDGEMENT

A. Sikkenk, radiologist, evaluated the chest radiograph.

REFERENCES

1. Ruesch S, Walder B, Tramèr MR. Complications of central venous catheters: Internal jugular versus subclavian access – A systematic review. Crit Care Med. 2002;30(2):454-60.

2. Dunbar RD, Mitchell R, Lavine M. Aberrant locations of central venous catheters. Lancet. 1981:317(8222):711-5.

3. Langston C. The aberrant central venous catheter and its complications. Radiology. 1971;100:55-9.

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CASE REPORT

Recovery from near drowning and postanoxic status epilepticus with controlled hypothermia

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ABSTRACT

A diver was resuscitated after cardiac arrest due to near drowning and was hypothermic on hospital arrival. During rewarming, status epilepticus occurred, previously identified as a predictor of poor outcome. The seizures responded well to treatment with antiepileptic drugs and controlled hypothermia. After six weeks, the patient had completely recovered. This case supports the hypothesis that hypothermia offers neuroprotection, even in the presence of status epilepticus. We recommend that near-drowning victims who are comatose after resuscitation for cardiac arrest be treated with controlled mild hypothermia for 12 to 24 hours.

KEYWORDS

Brain hypoxia, induced hypothermia, near drowning, resuscitation, status epilepticus

INTRODUCTION

Controlled hypothermia is recommended in the resuscitation guidelines created by the International Liaison Committee on Resuscitation (ILCOR) to limit neurological damage in patients resuscitated for out-of-hospital cardiac arrest.[1] Although evidence about temperature management in resuscitated near-drowning victims is lacking, it has been recommended to treat these patients in a similar way.[2] Nevertheless, reports about the results of controlled hypothermia in near-drowning victims are scarce.[3-6] In this case report, we present a near-drowning victim who recovered completely after treatment with controlled hypothermia, despite postanoxic status epilepticus, which has previously been identified as a predictor of poor outcome.

CASE REPORT

A 44-year-old male diver lost his mouthpiece and was found pulseless with asystolic heart activity 18 minutes later. Ten minutes after resuscitation was started, spontaneous circulation returned. On hospital arrival, the Glasgow Coma Score was 3 and the body temperature 30.1 °C. The patient was mechanically ventilated and haemodynamically stable under sedation with propofol. The pupils were dilated and not reactive to light, while corneal and oculocephalic reflexes were absent. Laboratory results showed lactic acidosis (pH 7.01, lactate 20.3 mmol/l). Since the clinical situation was stable and the core temperature was below 32 °C, the patient was allowed to rewarm according to international guidelines.[1] However, the temperature accidentally rose to 38 °C and recurrent tonic-clonic seizures occurred, compatible with status epilepticus.[7] Controlled hypothermia with a target of 33 °C was applied for 24 hours and the seizures were treated with valproic acid and levetiracetam. Three days later, somatosensory evoked potentials revealed a bilateral intact N20 response and the electroencephalogram showed a slow background pattern without ictal activity. A week later, the patient regained consciousness and six weeks after the accident, he had completely recovered. Neuropsychological assessment six months after the accident showed no deficits.

DISCUSSION

Controlled hypothermia limits neurological damage in resuscitated patients and might therefore also be beneficial in resuscitated near-drowning victims.[2] Successful use of controlled hypothermia in these patients has been reported previously.[3-6] It is hypothesised that controlled hypothermia not only decreases cerebral metabolism, but also limits

the effects of ischaemia and reperfusion on the brain. Animal studies have shown that controlled hypothermia postpones ischaemic depolarisation and inhibits the increase in excitatory neurotransmitters such as glutamate and dopamine.[8,9] In addition, controlled hypothermia inhibits the metabolism of arachidonic acid, limiting the production of cell membrane damaging metabolites such as prostaglandins and eicosanoids.[10] Finally, controlled hypothermia has an anti-inflammatory effect: it inhibits the production of cytokines and adhesion molecules, thereby limiting polymorphonuclear cell infiltration and oxygen radical production.[11]

Controlled hypothermia might have added to the favourable outcome in our patient. To our knowledge, this is the first report of a near-drowning victim who recovered completely despite postanoxic status epilepticus, which previously has been identified as an independent predictor of poor neurological outcome when occurring within 24 hours after cardiopulmonary arrest.[12] In addition, a recent paper described two patients with postanoxic status epilepticus after resuscitation for primary cardiac arrest, who experienced a favourable outcome after treatment with controlled hypothermia.[13]

Whether the fever occurring during rewarming precipitated the occurrence of status epilepticus in our patient remains speculative. Although it is well known that fever can induce seizures in animals and children, this has never been demonstrated in adults. Therefore, it seems unlikely that fever caused epilepsy in our patient. However, fever has been described as a phenomenon accompanying the presentation of status epilepticus.[14]

A limitation of the current case report is the fact that status epilepticus was not confirmed electrographically before treatment with anticonvulsive agents. The value of continuous amplitude-integrated electroencephalography in patients with postanoxic status epilepticus has been described previously.[15-17] In a recent study, 26 of 95 resuscitated patients treated with hypothermia experienced postanoxic status epilepticus. The outcome of these patients was related to the way status epilepticus developed: two of ten patients with status epilepticus developing from a continuous background regained consciousness, whereas none of 16 patients with status epilepticus developing from suppression burst background did.[15] In the two patients who regained consciousness, status epilepticus occurred after rewarming, just as in our patient.

In summary, the current case supports the hypothesis that controlled hypothermia may offer neuroprotection in patients with postanoxic encephalopathy, even in the presence of status epilepticus. We recommend that near-drowning victims who are comatose after resuscitation for cardiac arrest be treated with controlled mild hypothermia for 12 to 24 hours.

REFERENCES

1. Morrison LJ, Deakin CD, Morley PT, et al. on behalf of the Advanced Life Support Chapter Collaborators. Part 8: Advanced Life Support: 2010 International Consensus on Cardiopulmonary Resuscitation and Emergency Cardiovascular Care Science with treatment recommendations. Circulation. 2010;122(16 Suppl 2):S345-421.
2. Layon AJ, Modell JH. Drowning: Update 2009. Anesthesiology. 2009;110:1390-401.
3. Mizobuchi M, Nakamura S, Muranishi H, et al. Hypothermia with extracorporeal membrane oxygenation for sudden cardiac death and submersion. Am J Emerg Med. 2010;28:115.
4. Friberg H, Rundgren M. Submersion, accidental hypothermia and cardiac arrest, mechanical chest compressions as a bridge to final treatment: a case report. Scand J Trauma Resusc Emerg Med. 2009;17:7-10.
5. Varon J, Marik PE. Complete neurologic recovery following delayed initiation of hypothermia in a victim of warm water near-drowning. Resuscitation. 2006;68:421-3.
6. Williamson JP, Illing R, Gertler P, Braude S. Near-drowning treated with therapeutic hypothermia. Med J Aust. 2004;181:500-1.
7. Feen ES, Bershad EM, Suarez JI. Status epilepticus. S Med J. 2008;101:400-6.
8. Takeda Y, Namba K, Higuchi T, et al. Quantative evaluation of the neuroprotective effects of hypothermia ranging from 34 degrees C to 31 degrees C on brain ischemia in gerbils and determination of the mechanism of neuroprotection. Crit Care Med. 2003;31:255-60.
9. Hachimi-Idrissi S, van Hemelrijck A, Michotte A, et al. Postischemic mild hypothermia reduces neurotransmitter release and astroglial cell proliferation during reperfusion after asphyxial cardiac arrest in rats. Brain Res. 2004;1019:217-25.
10. Kubota M, Nakane M, Narita K, et al. Mild hypothermia reduces the rate of metabolism of arachidonic acid following postischemic reperfusion. Brain Res. 1998;779:297-300.
11. Wang GJ, Deng HY, Maier CM, Sun GH, Yenari MA. Mild hypothermia reduces ICAM-1 expression, neutrophil infiltration and microglial monocyte accumulation following experimental stroke. Neuroscience. 2002;114:1081-90.
12. Wijdicks EF, Hijdra A, Young GB, Bassetti CL, Wiebe S. Practice parameter: prediction of outcome in comatose survivors after cardiopulmonary resuscitation (an evidence based review): report of the Quality Standards Subcommittee of the American Academy of Neurology. Neurology. 2006;67:203-10.
13. Rossetti AO, Oddo M, Liauder L, et al. Predictors of awakening from postanoxic status epilepticus after therapeutic hypothermia. Neurology. 2009;72:744-9.
14. El-Ad B, Neufeld Y. Periodic febrile confusion as a presentation of complex partial status epilepticus. Acta Neurol Scand. 1990;82:350-2.
15. Rundgren M, Westhall E, Cronberg T, Rosén I, Friberg H. Continuous amplitude-integrated electroencephalogram predicts outcome in hypothermia-treated cardiac arrest patients. Crit Care Med. 2010;38:1838-44.
16. Legriel S, Bruneel F, Sediri H, et al. Early EEG monitoring for detecting postanoxic status epilepticus during therapeutic hypothermia: a pilot study. Neurocrit Care. 2009;11:338-44.
17. Wennervirta JE, Ermes MJ, Tiainen M, et al. Hypothermia treated cardiac arrest patients with good neurological outcome differ early in quantitative variables of EEG suppression and epileptiform activity. Crit Care Med. 2009;37:2427-35.

PHOTO QUIZ

Tropical fever

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CASE REPORT

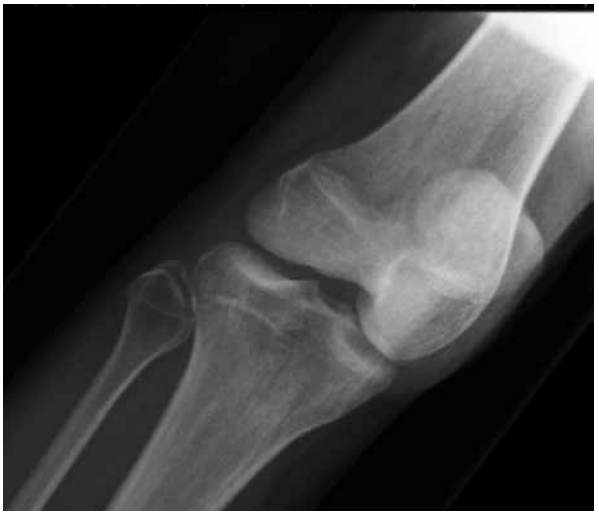
A 58-year-old Caucasian woman with no medical history presented with diarrhoea and fever after a short visit to the North-Western region of Thailand. She complained of pain in her lower legs and joints and altered fingers, toes and nails. A positive history for smoking (40 pack-years) was present. Physical examination showed clubbed fingers and toes with eye-glass shape of the nails, painful joints with no signs of arthritis *(figure 1A)*. Body temperature was 38.5 °C; no haemodynamic and respiratory instability was found. Additional laboratory investigation showed an erythrocyte sedimentation rate of 42 mm/U, leucocytes of 13.6 x 10^9/l, platelet count of 419 x 10^9/l and a C-reactive protein of 48 mg/l. Because of her recent visit to the tropics, infectious disease was suspected, but blood and stool cultures were negative. No parasites were found in the stools. Serological and endoscopic examination for Whipple's disease, Yersinia and HIV were negative. An X-ray of her lower legs was performed which revealed a periostitis *(figure 1B)*.

WHAT IS YOUR DIAGNOSIS?

See page 200 for the answer to this photo quiz.

Figure 1A. *Clubbed fingers with eye-glass shape of the nails*

Figure 1B. *Periosteal reaction in the proximal tibia and fibula and the distal femur*



ANSWER TO PHOTO QUIZ (PAGE 185)

A NONPRODUCTIVE COUGH THAT WOULD GIVE MOST PEOPLE A HEADACHE, BUT NOT THIS PATIENT!

DIAGNOSIS

The PET/CT revealed increased uptake of 18-fluorodeoxyglucose in the aorta and its large branches. This aortitis in a female patient beyond the age of 50 years made us assume the diagnosis of giant cell arteritis (GCA). A temporal biopsy was taken at random, since the temporal arteries were pulsatile and nontender. It confirmed the diagnosis GCA by showing mononuclear cell infiltration of the arterial wall and intima proliferation *(figures 2A and 2B)*. Treatment with prednisolone 60 mg/day made the symptoms disappear within one week, including the cough.

GCA is a relatively common vasculitis of the medium and large arteries. The most frequent symptoms include a new-onset headache, jaw claudication and stiffness and/or pain in the shoulder and pelvic girdles. This patient had none of these symptoms. Instead, she presented with a persistent nonproductive cough. Respiratory tract symptoms are unusual manifestations of GCA. Nevertheless, it has been estimated that respiratory tract symptoms affect 9% of the patients with GCA, while being the initial manifestation in 4%.[1] Besides a (non) productive cough and dyspnoea, the reported respiratory tract symptoms include pleuritic pain, a sore throat and hoarseness.[1] Radiological changes of the lungs are rare, but can occur as nodules of variable size, reticular infiltrates, pleural effusions and pleural thickening.[2] Involvement of the aorta can currently be visualised with a PET/CT, which shows inflammation of the aorta and its large branches in up to 76% of the patients with GCA.[3]

The respiratory symptoms in this patient can be explained by inflammation of the aorta and peribronchial vasculature causing stimulation of the bronchial cough receptors.[4] Awareness that GCA can present with atypical symptoms such as a nonproductive cough, can facilitate rapid diagnosis and treatment.

Figure 2A. *Overview of the temporal artery biopsy showing GCA (haematoxylin-eosin stain), the single arrow marks intima proliferation and the double arrow marks mononuclear cell invasion of the arterial wall*

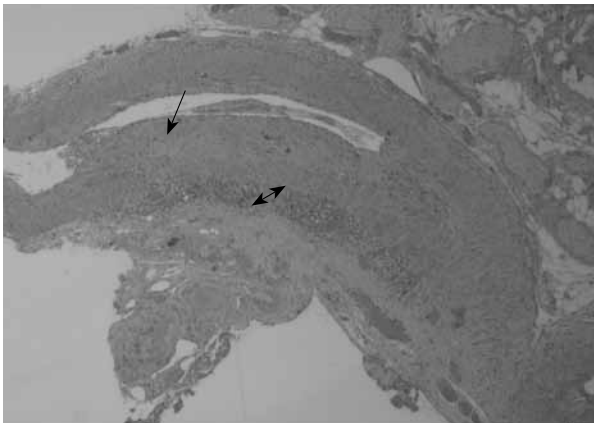
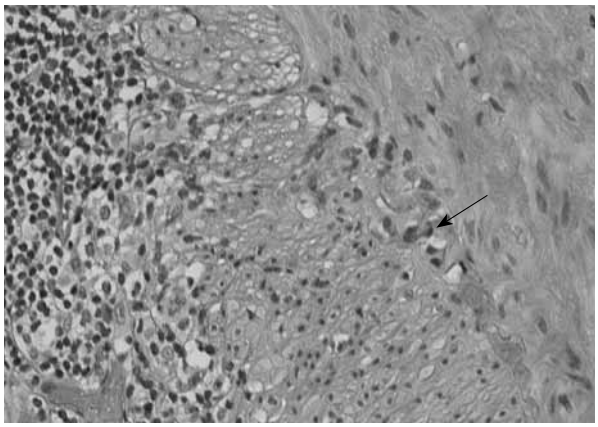


Figure 2B. *A detail of the temporal artery biopsy, the single arrow and the double arrow mark infiltration of the cell wall by a group of macrophages and lymphocytes, respectively*



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REFERENCES

1. Larson TS, Hall S, Hepper NG, Hunder GG. Respiratory tract symptoms as a clue to giant cell arteritis. Ann Intern Med. 1984;101:594-7.
2. Carassou P, Aletti M, Cinquetti G, et al. Respiratory manifestations of giant cell arteritis: 8 cases and review of the literature. Presse Med. 2010 Apr 16 [Epub ahead of print].
3. Blockmans D, Stroobants S, Maes A, Mortelmans L. Positron emission tomography in giant cell arteritis and polymyalgia rheumatica: evidence for inflammation of the aortic arch. Am J Med. 2000;108:246-9.
4. Irwin RS, Rosen MJ, Braman SS. Cough: a comprehensive review. Arch Intern Med. 1977;1371186-91.

ANSWER TO PHOTO QUIZ (PAGE 198)

TROPICAL FEVER

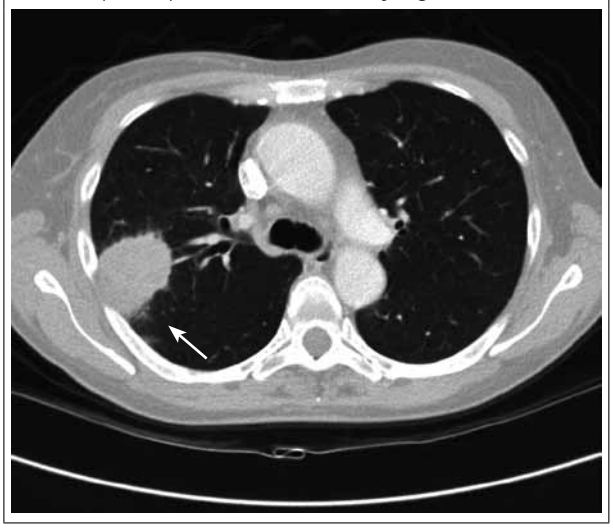
DIAGNOSIS

The symptoms and clinical signs of the patient were caused by a paraneoplastic sign called hypertrophic pulmonary osteoarthropathy (HPOA), also known as the Pierre-Marie-Bamberger syndrome. Additional investigation by chest X-ray and subsequent CT thorax revealed a mass in the right upper lung *(figure 2)*. A lymph node biopsy confirmed adenocarcinoma of the lung. Typical signs of HPOA are symmetric periostoses on the diaphyses of the long tubular bones, clubbed fingers and toes with eye-glass shape of the nails, neuro-vegetative disturbances and dysproteinaemia. HPAO is strongly associated with lung carcinoma, but may also occur in a primary form, which is often familial and more common in males. Other secondary forms include carcinomas of the liver and gut, inflammatory bowel disease, liver cirrhosis, congenital cyanotic heart disease, pulmonary fibrosis, Graves' disease, thalassaemia and many other rarer conditions.[1] The incidence of HPAO associated with lung carcinoma is reported between 0.8 and 10%.[2,3] The prevalence is higher in non-small cell lung carcinoma (NSCLC) than in small cell lung carcinoma (SCLC).[4] HPAO is associated with arteriovenous shunting, but the exact cause of HPAO is still unclear. Besides arteriovenous shunting humoral factors may play a role in HPOA. Production of growth factors such as platelet-derived growth factor and vascular-endothelial growth factor (VEGF), leading to angiogenesis, endothelial hyperplasia and clubbing may contribute to the onset of HPOA. Production of growth factor by malignant cells is the main source of endothelial stimulation and development of distal changes, although shunting due to local tissue destruction may contribute. Treatment is based on expert opinion as no clinical trials have been performed.[5] Treatment is primarily focused on eliminating the aetiology of the HPOA (e.g. resection of the malignancy) and secondarily on treatment of symptoms of HPOA with NSAIDs, bisphosphonates, octreotide, vagotomy, and even chemotherapy with VEGF antagonists.[5]

This patient was treated for her adenocarcinoma with concurrent chemotherapy and radiotherapy and subsequently underwent surgery for lobectomy of the right upper lobe.

In conclusion, patients with a history of smoking and signs of HPOA should be screened for primary or secondary lung cancer.

Figure 2. *CT scan of chest, showing right upper lobe tumour (arrow) with involvement of right hilar nodes*



REFERFENCES

1. Armstrong DJ, McCausland EM, Wright GD. Hypertrophic pulmonary osteoarthropathy (HPOA) (Pierre Marie-Bamberger syndrome): two cases presenting as acute inflammatory arthritis. Description and review of the literature. Rheumatol Int. 2007;27:399-402.
2. Izumi M, Takayama K, Yabuuchi H, Abe K, Nakanishi Y. Incidence of hypertrophic pulmonary osteoarthropathy associated with primary lung cancer. Respirology. 2010;15:809-12.
3. Segal AM, Mackenzie AH. Hypertrophic osteoarthropathy: a 10-year retrospective analysis. Semin Arthritis Rheum. 1982;12:220-32.
4. Sridhar KS, Lobo CF, Altman RD. Digital clubbing and lung cancer. Chest. 1998;114:1535-7.
5. Nguyen S, Hojjati M. Review of current therapies for secondary hypertrophic pulmonary osteoarthropathy. Clin Rheumatol. 2011;30:7-13.

Netherlands
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SPECIAL ARTICLE

Leptospirosis in a Dutch catfish farm

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ABSTRACT

A 51-year-old farm worker presented with jaundice and fever. There had been a rat infestation around the farm ponds and in the shed. He was admitted to our hospital with acute renal and liver failure, thrombocytopenia and rhabdomyolysis. Because of the clinical clues, leptospirosis was suspected and diagnosed in blood by polymerase chain reaction and serology. Also his son, a co-worker on the farm, showed a positive serology. Clinicians should be aware of these occupational outbreaks and should recognise the clinical picture.

KEYWORDS

Jarisch-Herxheimer, leptospirosis, outbreak, rhabdomyolysis, Weil's disease

What was known on this topic?
Leptospirosis is a zoonotic bacterial disease caused by pathogenic *Leptospira,* frequently carried by rodents. After infection, only a subset of patients develop the icteric form of the disease with severe late manifestations (Weil's disease). In the Netherlands, leptospirosis is most frequently associated with recreational exposure or travelling to (sub)tropical countries.

What does this case add?
We describe a small outbreak of leptospirosis on a catfish farm and show that the severity of infection may range from subclinical to life-threatening. The icteric leptospirosis in our patient was complicated by rhabdomyolysis and a Jarisch-Herxheimer reaction, both of which are uncommon findings. Clinicians should be aware of this clinical picture. Additionally, the value of polymerase chain reaction for early diagnosis of leptospirosis was established. This case report stipulates the importance of exposure history, even in low-endemic countries such as the Netherlands.

INTRODUCTION

Leptospirosis has been classified as an emerging infectious disease, particularly in (sub)tropical areas.[1] In the Netherlands, leptospirosis historically has been associated with agricultural or recreational exposure risks.[2] Exposures related to travelling to endemic countries have recently emerged as an important new cause of infection.[3] However, occupational exposures continue to exist, which requires an on-going alertness in low endemic locations.
Fish workers are at considerable risk of leptospirosis as a result of rats attracted to the ponds and sheds where the fish food is stored. We describe a small outbreak among workers on a catfish farm and present a case of a patient with an acute life-threatening form of leptospirosis.

CASE REPORT

A 51-year-old man who had been ill for five days with fever, sweating, headache, myalgia and limb weakness was admitted to our hospital. The patient's medical history was, besides a history of fibromyalgia, unremarkable. He worked on a family-owned catfish farm. Physical examination showed a blood pressure of 134/87 mmHg, pulse of 110 beats/min, oxygen saturation of 96% and temperature of 36.4 °C. There was an obvious jaundice and extreme tenderness of the legs. Lung and heart sounds were normal. Abdominal examination showed right upper quadrant pain without rebound tenderness or guarding. The laboratory findings were: creatine kinase

(CK) 3547 U/l (<400 U/l), serum bilirubin 219 µmol/l (total), 193 µmol/l (conjugated), aspartate aminotransferase 169 U/l (<40), alanine aminotransferase 37 U/l (<45), alkaline phosphatase (AF) 82 U/l (<150), gamma-glutamyltransferase 71 U/l (<65), serum urea 24.7 mmol/l (2.5 to 7.5), creatinine 262 µmol/l (60 to 110), C-reactive protein 348 mg/l (<5), white cell count 16.2 x 10^9/l, and platelets 30 x 10^9/l. Chest radiography and abdominal ultrasound showed no abnormalities. He was admitted to the intensive care unit with acute renal and liver failure, thrombocytopenia and signs of rhabdomyolysis. Heteroanamnestic information revealed that there had been a recent rat plague on the catfish farm. The rats were eradicated by poisoning and subsequently eliminated from the shed with a high-pressure sprayer by our patient. Of the other employees on the family-owned fish farm, only the patient's son had a history of an influenza-like illness during the previous month. The patient's occupation and clinical presentation suggested the possibility of leptospirosis and intravenous cefotaxim was started. Rehydration, dialysis and platelet transfusion were necessary. One hour after the infusion of cefotaxim, he suddenly experienced rigors and a rapid decline in blood pressure which was attributed to a Jarisch-Herxheimer reaction. After ten days of treatment, the serum bilirubin, CK and platelets nearly normalised but the creatinine increased to 499 µmol/l. In total, the patient received dialysis for approximately six weeks. His urine output gradually increased and his kidney function slowly recovered.

The DNA of pathogenic leptospires was detected by polymerase chain reaction (PCR) in the blood (i.e. five days after the onset of illness). At that time, serological tests on the same blood sample were negative. A second serum sample was tested on hospital day 9, which showed a positive ELISA IgM with a titre of 1:1280. The microscopic agglutination test (MAT) was moderately positive, demonstrating weak reactions with eight of the ten leptospiral serogroup antigens but gave the strongest reaction with serotype *icterohaemorrhagiae* of the Icterohaemorrhagiae serogroup. Two months after the onset of illness, the MAT showed reactions with the serotype *icterohaemorrhagiae* of the Icterohaemorrhagiae serogroup and the serotype *poi* of the serogroup Javanica (*table 1*). Additionally, sera from the other family members were tested. The MAT of the son who had experienced an influenza-like illness showed an antibody titre of 1:2560 against serotype *icterohaemorrhagiae* of the Icterohaemorrhagiae serogroup. The IgM was positive as well, indicating a very recent infection. The sera of the remaining family members were negative (*table 1*).

DISCUSSION

The diagnosis of an uncommon disease usually depends on recognising an unusual combination of clinical findings. In the present case, jaundice, isolated hyperbilirubinaemia, high creatine kinase levels and renal failure following a febrile illness could be recognised as a pattern characteristic of leptospirosis. Crucial to these clinical findings is to realise the importance of the patient's exposure history. Detailed information regarding the rat plague on the catfish farm was a major clue to the correct diagnosis.

Leptospirosis is a bacterial infectious disease caused by pathogenic leptospires of the genus *Leptospira*.[4] The disease is maintained in nature by chronic renal infection of carrier animals, such as rodents, which transmit them through urine and consequently contaminate lakes or standing water. The portal of entry is generally through cuts in the skin or via conjunctiva, but inhalation of aerosols also may result in infection.[1]

After a seven to ten day incubation period, leptospirosis starts with a bacteraemic phase marked by non-specific influenza-like illness of approximately one week followed by a second phase with production of antibodies, disappearance of leptospires from the blood and the appearance of spirochetes in urine (*figure 1*). In humans, the majority of infections caused by leptospires are subclinical. However, a subset (5 to 15%) of patients develop the icteric form of the disease with severe late

Table 1. *Diagnostic tests on the family members of the catfish farm*

Farm workers	Age (years)	Symptoms	IgM ELISA (titre)	MAT (titre)	Serogroup (serotype)	PCR (blood)	Culture (blood)
Father (patient)	51	Jaundice, renal failure	640-1280	160-320 320-1280	Icterohaemorrhagiae (*icterohaemorrhagiae*) Javanica *(poi)*	Positive	Negative
Mother	49	Healthy	Negative	Negative	-	n.p.	n.p.
Child 1	21	Healthy	Negative	Negative	-	n.p.	n.p.
Child 2	19	Flu-like illness	320	2560	Icterohaemorrhagiae *(icterohaemorrhagiae)*	n.p.	n.p.
Child 3	16	Healthy	Negative	Negative	-	n.p.	n.p.

n.p. = not performed, MAT = microscopic agglutination test.

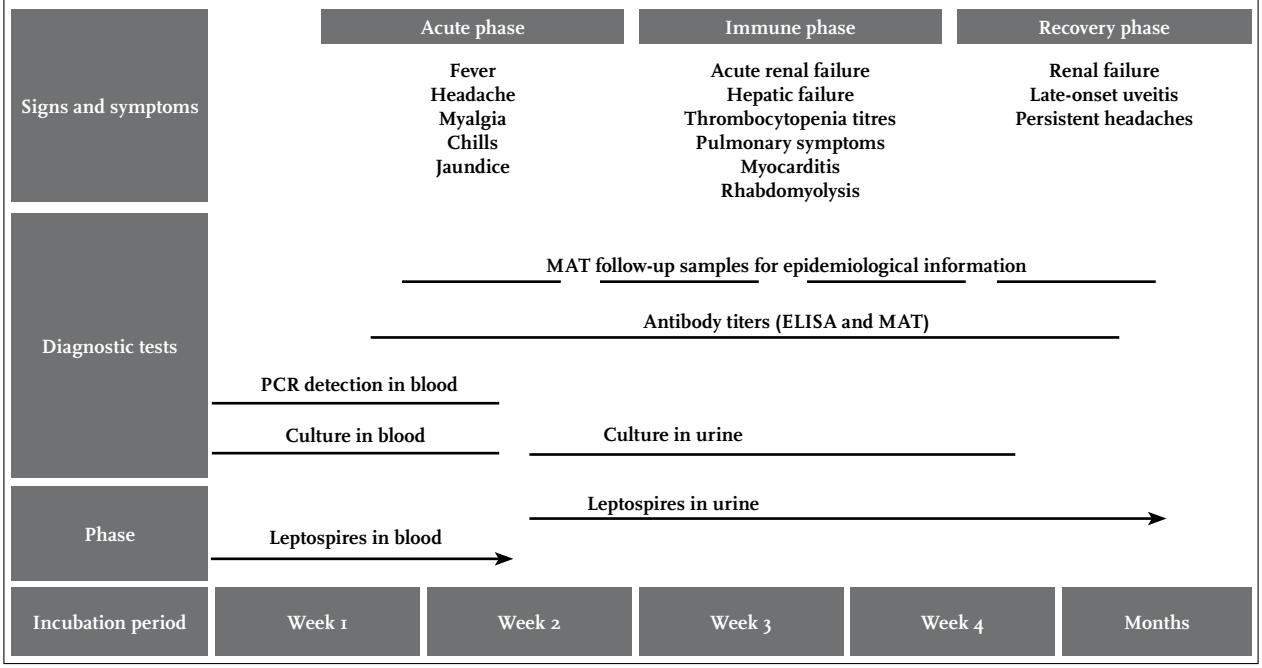
manifestations four to six days after the onset of illness. The complications of icteric leptospirosis (Weil's disease) emphasise the multisystemic nature of the disease, which is characterised by reversible generalised vasculitis and endothelial damage. The liver, kidneys and lungs are most frequently involved. In our patient, serum bilirubin levels were markedly elevated compared with the moderate rises in transaminase levels and AF, which is typical for leptospirosis.[5] Acute renal failure is a result of interstitial nephritis caused by the invasion of leptospires in the interstitial tissue and tubules. In our patient, the renal failure was probably also the result of rhabdomyolysis. Rhabdomyolysis is characterised by extremely high serum levels of muscle components, due to focal muscle necrosis, which might precipitate in the glomerular filtrate, resulting in renal tubular obstruction and direct nephrotoxicity.[6]

Because *Leptospira* take weeks to grow on specialised media, the diagnosis of leptospirosis is usually made by serological testing (*figure 1*). The current reference method is the microscopic agglutination test (MAT), in which patient sera are incubated with live antigen suspensions of multiple leptospiral serovars. Interpretation of the MAT is complicated by the high degree of cross-reaction that occurs between different serovars, especially in the acute phase samples.[7] Two months after the onset of illness, the MAT of our patient's serum showed reactions with two different serotypes. This might be explained by either cross-reaction or exposure to more than one serotype. ELISA has repeatedly been shown to be more sensitive than MAT in the acute phase of the disease. However, ELISA only detects antibodies reacting with a broadly reactive genus-specific antigen and thus gives no indication of the causative serovar or serogroup.[7] In the Netherlands PCR has not been used routinely but a recent study showed that RT-PCR on blood samples was highly sensitive during the first four days of illness.[8] This was confirmed in our study as leptospirosis was diagnosed by PCR on the fifth day of illness, whereas serology still remained negative at that time. PCR thus facilitates early diagnosis and enables starting treatment at the most effective time point, which is essential for optimal antibiotic therapy.[9]

The management of severe leptospirosis requires antibiotic treatment and supportive care. Antibiotic therapy may consist of third-generation cephalosporines, doxycycline or penicillin G, which have all been shown to be equally effective.[10,11] A rare complication of antimicrobial treatment in leptospirosis, as is the case with other diseases caused by spirochetes such as secondary syphilis or relapsing fever, may be a Jarisch-Herxheimer reaction. This is a systemic reaction resembling a severe inflammatory response that usually begins one to two hours after initial treatment with effective antibiotics, especially penicillins. It consists of the abrupt onset of fever, rigors, tachycardia and hypotension, as was found in our patient.[12]

This report demonstrates that leptospirosis may range from a subclinical to a life-threatening infection. The two cases of leptospirosis on a family-owned fish farm emphasise the danger associated with rat infestation and elimination, even in low-endemic countries. Although

Figure 1. *Course of leptospirosis and relevant diagnostic tests at different stages of disease*



rodent control has reduced the incidence of leptospirosis in the Netherlands,[3] there still is a significant risk associated with occupation and recreational exposures occurring in water sports. Therefore, a patient's exposure history and recognition of the clinical picture is of major importance for the diagnosis of leptospirosis.

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The authors wish to thank Dr. R. Hartskeerl of the Tropical Institute of Biomedical Research (KIT), Amsterdam, the Netherlands, for performing the diagnostic part of this study.

REFERENCES

1. Levett PN. Leptospirosis. Clin Microbiol Rev. 2001;14:296-326.
2. Olszyna DP, Jaspars R, Speelman P, et al. Leptospirose in Nederland 1991-1995. Ned Tijdschr Geneeskd. 1998;142:1270-3.
3. Hartskeerl R, Goris M. Leptospirose in Nederland in 2008 en 2009. Infectieziekten Bulletin. 2010;6:185-7.
4. Morey RE, Galloway RL, Bragg SL, et al. Species-specific identification of Leptospiraceae by 16S rRNA gene sequencing. J Clin Microbiol. 2006;44:3510-6.
5. Heath CW, Alexander AD, Galton MM. Leptospirosis in the United States: analysis of 483 cases in man, 1949-1961. N Engl J Med. 1965;273:857-64, 915-22.
6. Kahn FY. Rhabdomyolysis: a review of the literature. Neth J Med. 2009;67:272-82.
7. World Health Organisation, International Leptospirosis Society. Human leptospirosis: guidance for diagnosis, surveillance and control. Geneva; 2003: pp 1-109.
8. Ahmed A, Engelberts MF, Boer KR, et al. Development and validation of a real-time PCR for detection of pathogenic Leptospira species in clinical materials. PLoS ONE. 2009;4:e7093.
9. Katz AR, Ansdell VE, Effler OV, et al. Assessment of the clinical presentations and treatment of 353 cases of laboratory-confirmed leptospirosis in Hawaii, 1974-1988. Clin Infect Dis. 2001;33:1834-41.
10. Panaphut T, Domrongkitchaiporn S, Vibhagool A, et al. Ceftriaxone compared with sodium penicillin G for treatment of severe leptospirosis. Clin Infect Dis. 2003;36:1507-13.
11. Suputtamongkol Y, Niwattayakul K, Suttinont C, et al. An open, randomized, controlled trial of penicillin, doxycycline, and cefotaxime for patients with severe leptospirosis. Clin Infect Dis. 2004;39:1417-24.
12. Pound MW, May DB. Proposed mechanisms and preventative options of Jarisch-Herxheimer reactions. J Clin Pharm Therap. 2005;30:291-5.

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SPECIAL ARTICLE

The success of a weekly medical quiz. Test-based medical education

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ABSTRACT

Background: **Clinical images and tests are considered useful tools to enhance the memorisation of facts and information in medical education. Therefore, we initiated a weekly medical quiz for our department of Internal Medicine.**
Methods: **Every week, a new case on a single slide with relevant information and a representative image, is sent by e-mail to staff, residents and others. All are requested on a voluntary basis to e-mail the presumed diagnosis within one week.**
Results: **After two years, 100 cases were presented to 452 registered participants. On average, only 33 of 452 (range 14 to 59) participants (7.3%; 95% CI 4.9 to 9.7) responded per case. Most presumed diagnoses were submitted on the same day the case was sent (OR 0.81; 95% CI 0.69 to 0.94; p<0.01). Cases with a high response rate were associated with relatively more correct answers than cases with a low response rate. In addition, it was striking that participants in some subspecialities, particularly specialists in infectious diseases, were much more likely to respond to cases in their own subspecialty.**
Conclusion: **Our experience with a weekly medical quiz demonstrates rather low response rates. This could be due to time restraints, but could also be due to the fact that doctors do not like to be wrong, and are afraid to fail among their peers. Hence, although images and tests may be helpful learning tools, the success and contribution of such clinical-based quizzes to medical education are difficult to determine.**

KEYWORDS

Clinical education, continuing medical education, problem-based learning, testing/assessment

INTRODUCTION

Clinical images and case-based material are considered to be efficient tools in medical education.[1,2] Images aid in memorising facts and enhance the process of clinical reasoning. It has also been published that the use of tests promotes better retention of information.[3] Therefore, some journals have created features using clinical images, such as 'Images in Clinical Medicine' in the *New England Journal of Medicine*, with an occasional query to test participants on their knowledge and learning. Images and tests also play an increasingly important role in Continuing Medical Education (CME), where credits can be earned by successfully completing accompanying quizzes. To exploit these theories, we initiated a weekly medical quiz for our department of Internal Medicine, which is part of a tertiary teaching hospital.

METHODS

We created a weekly medical quiz, presented on a single slide that contains all relevant information and a representative image. Every Monday during the department's morning report, a new case is presented and the diagnosis, including a short explanation of the previous case, is given. In addition, the case is sent by e-mail to staff, residents and others who expressed interest (e.g. researchers, interns, students). All are requested on a voluntary basis to submit the presumed diagnosis within one week by e-mail to one of us (GEL). Cases encompass the broad spectrum of general internal medicine and its subspecialities, and are obtained from our own institution and local training hospitals.

Table 1. *Relative response ratios of subspecialists on cases in their own speciality*

Subspecialty	Number of cases on subspecialty	Number of participants registered with subspeciality	Relative response ratio on cases in own subspeciality*
General internal medicine	21	66	2.4
Endocrinology	12	13	2.7
Haematology	6	15	0.8
Infectious diseases	21	25	4.4
Gastroenterology	7	7	0.6
Nephrology	4	13	0.4
Rheumatology	8	9	1.2
Vascular medicine	2	26	0.3
Cardiology	6	9	0.9
Intensive care medicine	2	8	0.4
Oncology	6	11	0.8

***Calculated as ratio of response on cases in own subspeciality to total response of participants within the specific subspeciality (with exclusion of general internal medicine cases in all other subspecialities).**

RESULTS

After two years, we have presented 100 cases to 452 registered participants. On average, only 33 of 452 (range 14 to 59) registered participants (7.3%; 95% CI 4.9 to 9.7) responded per case. Response levels per participant varied from one to almost all cases (range 1 to 81), while residents proved to be more loyal participants than members of staff. Of all response, 46% was submitted by residents, 35% by staff members and the remainder by others. Most presumed diagnoses were submitted on the same day the case was sent (OR 0.8; 95% CI 0.7 to 0.9; p<0.01). Staff members submitted a correct diagnosis in 61.2% of cases, as did 56.3% of residents. Cases with a high (≥40 respondents) and low (<25 respondents) response rate were compared. This demonstrated that cases with a high response rate were generally associated with a higher percentage of correct answers (mean 63.8%; range 20.0 to 100%) than cases with a low response rate (mean 45.0%; range 0.0 to 87.5%).

For 202 of 452 participants (44.7%), a subspecialty was registered. It was striking that in some subspecialities, participants, both residents and staff, were much more likely to submit answers for cases in their own subspecialty (*table 1*). Specialists in infectious diseases serve as an example: they were almost five times more likely to respond to infectious diseases cases than to others.

DISCUSSION

Case-based images and tests may be useful tools in medical education and the training of Internal Medicine, by direct recognition of clinical diagnoses. However, our experience with a weekly medical quiz also demonstrates that interaction is limited due to rather low response rates. This could be due to time restraints, but could also be explained by the fact that our participants do not like the possibility of being wrong. This hypothesis is supported by the observation that participants were more likely to submit answers on the same day they received the case, and to cases concerning their own subspecialty. In addition, cases with a high response rate were associated with a relatively higher number of correct answers than cases with a low response rate, possibly reflecting the difficulty of the case. Apparently, participants are more likely to submit a diagnosis if they are (more) convinced of having the correct answer. It is therefore tempting to conclude that doctors are afraid to fail among their peers. Yet, these observations also hinder in determining the success and contribution of such clinical case-based quizzes to medical education.

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No conflict of interest relevant to this manuscript for any of the authors.

REFERENCES

1. Kassirer JP. Teaching clinical reasoning: case-based and coached. Acad Med. 2010 Jul;85(7):1118-24.
2. Usatine R. Learning from images in clinical medicine. J Fam Pract. 2003 Jan;52(1):52.
3. Larsen DP, Butler AC, Roediger HL, III. Test-enhanced learning in medical education. Med Educ. 2008. Oct;42(10):959-66.

LETTER TO THE EDITOR

False elevation of chromogranin A due to proton pump inhibitors

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Dear Editor,

In their review on the diagnostic approach of neuroendocrine tumours (NET) Kuiper *et al* state that chromogranin A (CgA) is the most specific (86%) and sensitive (68%) diagnostic serum marker.[1] However, CgA may be elevated in a number of other endocrine, gastrointestinal, malignant and even cardiovascular disorders. We want to draw attention to one of the most frequent causes of false elevation of CgA, namely the use of H2 blockers or proton pump inhibitors (PPI).[2]

Patient A, a 49-year-old woman, was evaluated for the presence of NET because of vegetative symptoms and profuse watery diarrhoea. The urinary excretion of 5-HIAA was normal, while serum CgA (4960 µg/l (normal 20 to 100)) and gastrin (0.67 µg/l (normal <0.15)) were strongly elevated. The subsequent somatostatin receptor scintigraphy was normal. After discontinuation of the long-term esomeprazol (40 mg twice daily), both serum CgA (84 µg/l) and gastrin (0.10 µg/l) levels normalised. Re-treatment with esomeprazol led to a serum CgA level of 3090 µg/l.

Patient B is a 58-year-old woman on long-term esomeprazol (20 mg) treatment because of gastro-oesophageal reflux. Because of profound flushes, palpitations and abdominal complaints, serum CgA was determined to exclude NET. The elevated (543 µg/l) serum CgA level prompted a somatostatin receptor scintigraphy without abnormalities. After discontinuation of the esomeprazol, the serum CgA level normalised (43 µg/l) with a marked increase to 1360 µg/l several weeks after reinstitution.

Patient C, a 35-year-old woman, was evaluated for NET because of episodes of sweating, palpitations and abdominal cramps. While taking 40 mg pantoprazol, the serum CgA level was 271 µg/l. No imaging studies were done as the serum CgA dropped to 44 µg/l after discontinuation of pantoprazol.

These three cases illustrate that CgA may strongly rise during long-term treatment with PPI. Treatment with gastric pH increasing drugs such as PPI and to a lesser extent H2 blockers leads to gastrin production by the antral G-cells with subsequent stimulation of the gastric enterochromaffin-like cells and release of CgA. In most patients treated with PPI a two- to fourfold increase in CgA is found.[3,4] The increase in CgA seems related to the dosage and duration of PPI treatment. A more than tenfold increase in CgA levels, as in two of our patients, has occasionally been reported.[2] One to two weeks after discontinuation of PPI the CgA levels return to normal. It is therefore advocated to stop PPI treatment for at least two weeks before determination of CgA to avoid unnecessary imaging studies.

REFERENCES

1. Kuiper P, Verspaget HW, Overbeek LIH, Biemond I, Lamers CB. An overview of the current diagnosis and recent developments in neuroendocrine tumours of the gastroenteropancreatic tract: the diagnostic approach. Neth J Med. 2011;69:4-20.
2. Modlin IM, Gustafsson BI, Moss SF, Pavel M, Tsolakis AV, Kidd M. Chromogranin A – biological function and clinical utility in neuro endocrine tumor disease. Ann Surg Oncol. 2010;17:2427-43.
3. Giusti M, Sidoti M, Augeri C, Rabitti C, Minuto F. Effect of short-term treatment with low dosages of the proton-pump inhibitor omeprazole on serum chromogranin A levels in man. Eur J Endocrinol. 2004;60:299-303.
4. Sanduleanu S, Stridsberg M, Jonkers D, et al. Serum gastrin and chromogranin A during medium- and long-term acid suppressive therapy. A case-control study. Aliment Pharmacol Ther. 1999;13:145-53.

INFORMATION FOR AUTHORS

Aims and scope

The *Netherlands Journal of Medicine* publishes papers in all relevant fields of internal medicine. In addition to reports of original clinical and experimental studies, reviews on topics of interest or importance, case reports, book reviews and letters to the editor are welcomed.

Manuscripts

Manuscripts submitted to the Journal should report original research not previously published or being considered for publication elsewhere. Submission of a manuscript to this Journal gives the publisher the right to publish the paper if it is accepted. Manuscripts may be edited to improve clarity and expression.

Language

The language of the Journal is English. English idiom and spelling is used in accordance with the Oxford dictionary. Thus: Centre and not Center, Tumour and not Tumor, Haematology and not Hematology.

Submission

All submissions to the *Netherlands Journal of Medicine* should be submitted online through Manuscript Central at http://mc.manuscriptcentral.com/nethjmed. Authors should create an account and follow the instructions. If you are unable to submit through Manuscript Central contact the editorial office at m.m.levi@amc.uva.nl, tel.: +31 (0)20-566 21 71, fax: +31 (0)20-691 96 58.

Preparation of manuscripts

Type all pages with double spacing and wide margins on one side of the paper. To facilitate the reviewing process, number the lines in the margin and the pages.

Subheadings should not exceed 55 characters, including spaces.

Abbreviations: Measurements should be abbreviated according to SI units. All other abbreviations or acronyms should be defined on the first appearance in the text. Use a capital letter for generic names of substances and materials.

A *Covering letter* should accompany the manuscript, identifying the corresponding person (with the address, telephone number, fax number and e-mail address). Conflicts of interest, commercial affiliations, consultations, stock or equity interests should be specified. In the letter one to three sentences should be dedicated to what this study adds. The letter should make it clear that the final manuscript has been seen and approved by all authors. All authors should sign the letter. The letter should either be submitted through http://mc.manuscriptcentral.com/nethjmed or faxed to the editorial office (+31 (0)20-691 96 58).

Divide the manuscript into the following sections: Title page, Abstract, Keywords, Introduction, Materials and Methods, Results, Discussion, Acknowledgements, References, Tables and Figures with Legends.

The *Title page* should include authors' names, degrees, academic addresses, correspondence address, including telephone number, fax number, e-mail address and grant support. Also the contribution of each author should be specified.

The title should be informative and not exceed 90 characters, including spaces. Avoid use of extraneous words such as 'study', 'investigation' as well as priority claims (new, novel, first). Give a running title of less than 50 characters. If data from the manuscript have been presented at a meeting, list the name, date and location of the meeting and reference and previously published abstracts in the bibliography. Give a word count (including references, excluding tables and legends) at the bottom of this page.

The *Abstract*, not exceeding 250 words, should be written in a structured manner and with particular care. In original articles, the Abstract should consist of the following paragraphs: Background, Methods, Results and Conclusion. They should briefly describe the problem being addressed in the study, how the study was performed and which measurements were carried out, the most relevant results, and what the authors conclude from the results.

Keywords: Include three to five keywords in alphabetical order.

The *Introduction* should be brief and set out the purposes for which the study has been performed.

The *Materials and methods* should be sufficiently detailed so that readers and reviewers can understand precisely what has been done without studying the references directly. The description may be abbreviated when well-accepted techniques are used.

The *Results* should be presented precisely, without discussion.

The *Discussion* should directly relate to the study being reported. Do not include a general review of the topic, but discuss the pertinent literature.

Acknowledgement: All funding sources should be credited here. Also a statement of conflicts of interest should be mentioned.

References should be numbered consecutively as they appear in the text (after the punctuation and in square brackets). Type the reference list with double spacing on a separate page. References should be in the language they are published in, conform the 'Vancouver' style for biomedical journals (N Engl J Med. 1991;324:424-8).

Journal abbreviations should conform to the style used in the Cumulated Index Medicus. Examples:

1. Smilde TJ, van Wissen S, Wollersheim H, Kastelein JJP, Stalenhoef AFH. Genetic and metabolic factors predicting risk of cardiovascular disease in familial hypercholesterolemia. Neth J Med. 2001;59:184-95.
2. Kaplan NM. Clinical Hypertension. 7th ed. Baltimore: Williams & Wilkins; 1998.
3. Powell LW, Isselbacher KJ. Hemochromatosis. In: Braunwald E, Fauci AS, Kasper DL, et al., editors. Harrison's Principles of Internal Medicine. 15th edition. New York: McGraw-Hill; 2001. p. 2257-61.

Please note that all authors should be listed when six or less; when seven or more, list only the first three and add et al. Do not include references to personal communications, unpublished data or manuscripts either 'in preparation' or 'submitted for publication'. If essential, such material may be incorporated into the appropriate place in the text. Recheck references in the text against the reference list after your manuscript has been revised.
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Tables should be typed with double spacing each on a separate page, numbered consecutively with Arabic numerals, and should contain only horizontal lines. Provide a short descriptive heading above each table with footnotes and/or explanation underneath.

Figures must be suitable for high-quality reproduction (>300 DPI). Submit line drawings made in Word or other computer programmes but not in a PowerPoint file. Colour figures are occasionally possible and will be charged to the authors. *Legends for figures* should be typed, with double spacing, on a separate page.

Case reports
Case reports containing concise reports on original work will be considered for publication. Case reports which are relevant for understanding the pathophysiology or clinical presentation of disease may also be accepted under this heading. Selection of case reports will be based on criteria as outlined in a special report by the editors (Drenth et al. The case for case reports in *the Netherlands Journal of Medicine*. Neth J Med. 2006;64(7):262-4). We advise potential authors to take notice of the instructions in this report. Articles published in this section should be no longer than 1000 words, and supplied with a summary of about 60 words, preferably no more than two figures and/or tables, and no more than 15 references. In addition, we require that authors of case reports answer the following two questions (Neth J Med. 2008;66(7):289-90): 1) What was known on this topic? and 2) What does this add? The answers will appear in a separate box in the text.

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Mini reviews are concise notes that bring the reader up to date with the recent developments in the field under discussion. The review article should mention any previous important reviews in the field and contain a comprehensive discussion starting with the general background of the field. It should then go on to discuss the salient features of recent developments. The authors should avoid presenting material which has already been published in a previous review. The manuscript should be divided as follows: title page, abstract and main text. The text may be subdivided further according to the areas to be discussed. The text should not exceed 2500 words.

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