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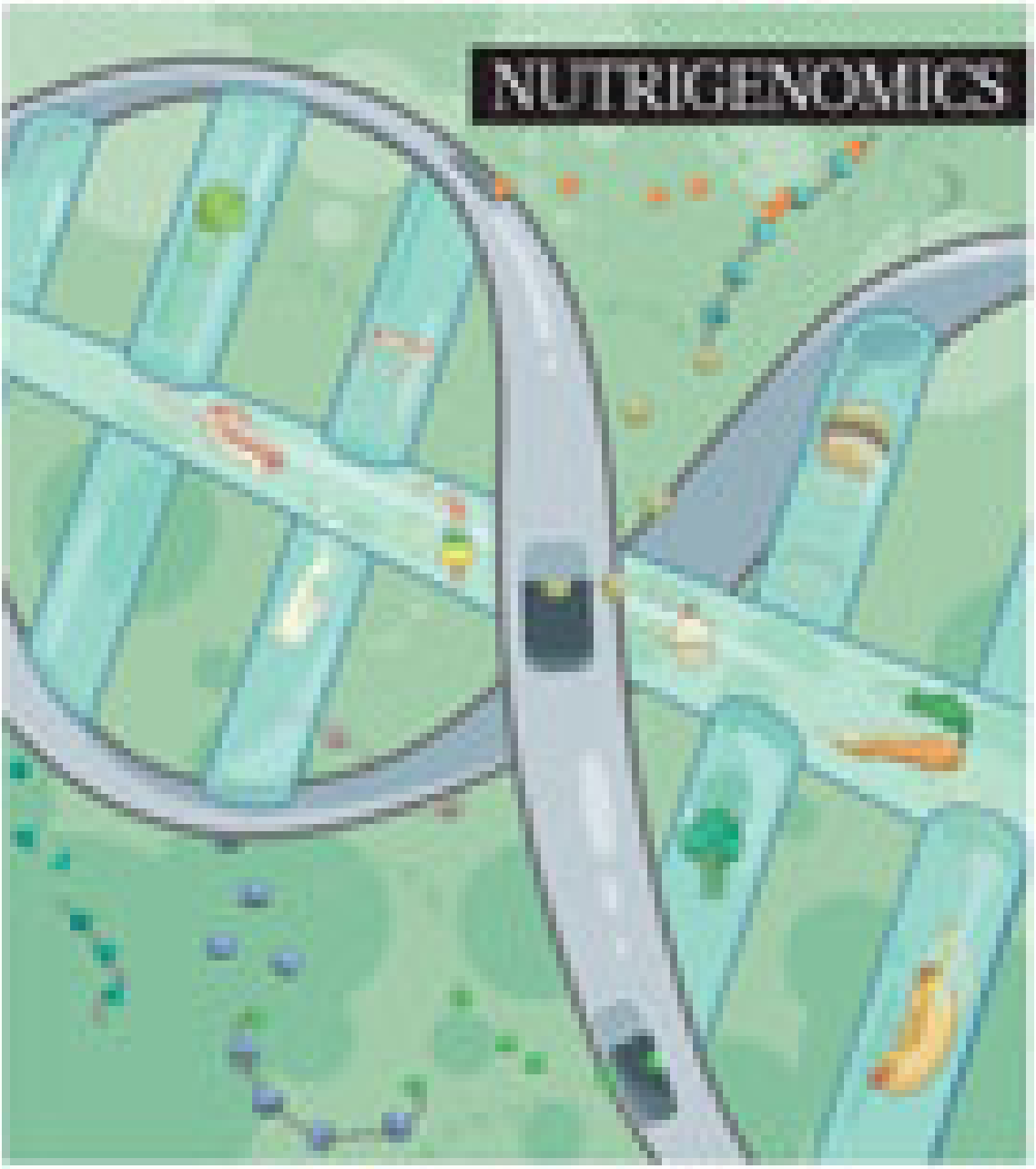

How food shapes health and evolution

nature OUTLOOK

NUTRIGENOMICS

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Food. Our need for it is primal, but our relationship with it is complex and ever-changing.

For many in the developed world, eating has become a leisure pursuit, and cooking a hobby. But our bodies are still hard-wired for a tougher world where food means survival. Our sense of taste, for example, evolved to be a front-line defence against toxins and a sensor to help detect the most energy-rich fare. However, our innate craving for sweets and fats now seems to be leading us down a path of bodily destruction.

Food affects people differently. Current nutritional research involves looking beyond ingredients in an attempt to understand the effects of food at genetic and epigenetic levels. From the first milk meal we take, through feast and famine; our genes influence our diet, and nutrients — or lack of them — affect gene expression.

Regional differences in food and culture have left their mark on our genome. Around the world, populations have adapted to their diet to make the most of local resources. In some instances, a foodstuff can protect against deadly infection, giving selective advantage to those who can readily digest it.

Nutrition has also directed the evolution of our species. Only *Homo sapiens* and our extinct hominin cousins have used fire to manipulate raw food, thereby creating safer, easily digestible and tastier recipes. Combined with the use of tools and an omnivorous, wide-ranging appetite, the advent of cooking increased the energy yield for metabolism and fed our enlarging brains.

Because food is packed full of complex, biologically active molecules, the fact it has an impact on our health is no surprise. Yet teasing apart the effects of each component on the body is a tall task, and one that will continue for many years to come. Some people predict an age of diets customized to individual energy needs and disease susceptibility. But no matter how good the science is, or how well we are able to exploit food as an agent of healthfulness, we will still be eating for pleasure for some time yet.

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Michelle Grayson
Associate Editor, Nature Outlook.

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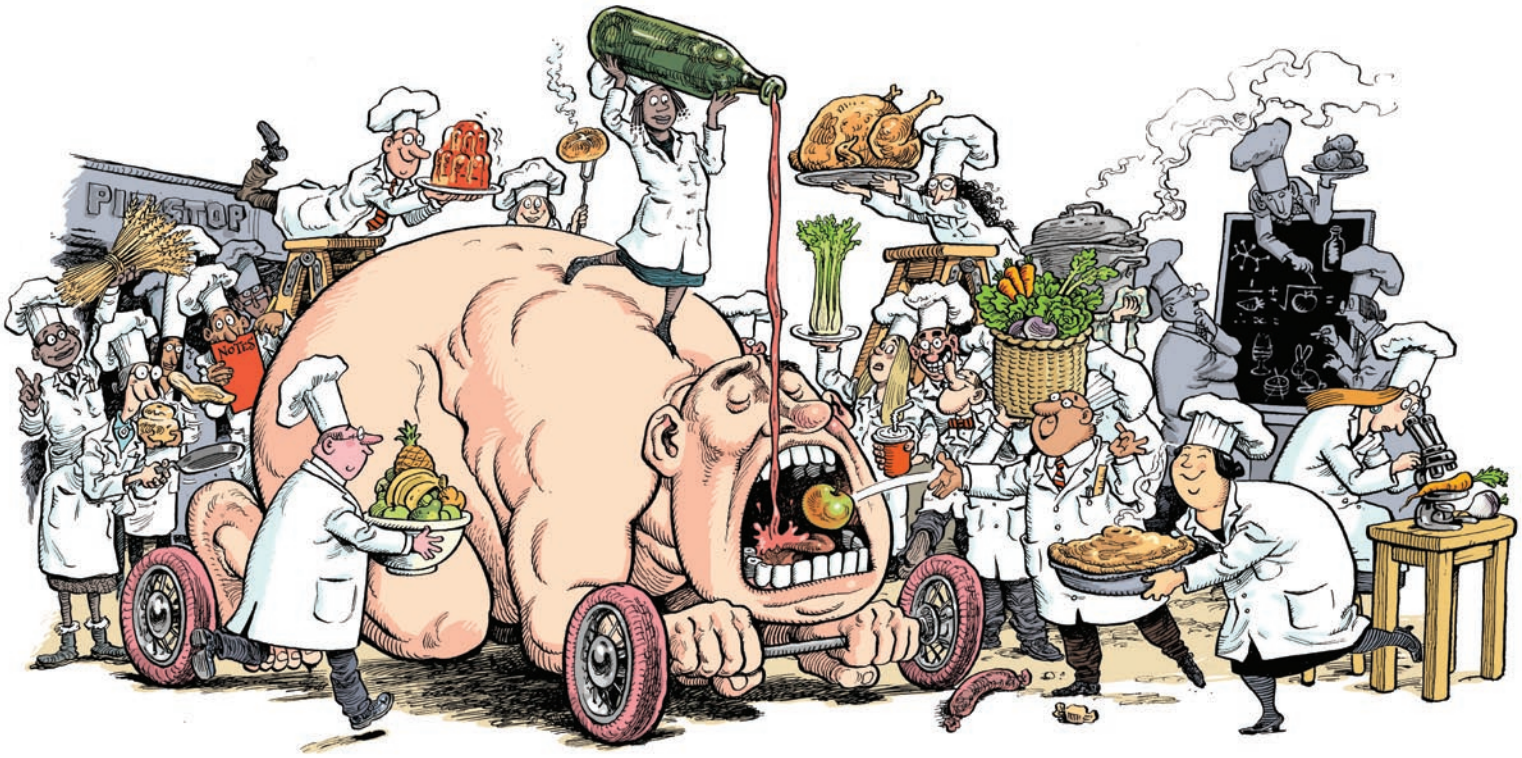

INTERDISCIPLINARY RESEARCH

Big science at the table

Researchers are adopting the tools of bioinformatics and pharmaceuticals to study and interpret the ever-growing body of data on the interplay between diet and genes.

BY LUCAS LAURSEN

José Ordovas sips a mint tea in a languid café in Madrid, Spain. His eyes scan two mobile phones as he confirms his next appointments. In conversation, he switches effortlessly between Spanish and English to find the right expressions. If the geneticist seems to be moving on a different wavelength from the other patrons, he could blame it on the jet lag: he has just flown from Boston where it's now 5am. This is his third oversees trip this month, but Ordovas contends his frequent visits from Tufts University, where he's based, to Europe have no adverse effects. "For me the time difference doesn't matter, I'm up at 4am to make calls to Europe when I'm home anyway, and then I'm up late on calls to California," he says.

Ordovas embodies the hustle and bustle of the 'big science' approach that has changed nutrition research in the past decade. This field, once confined to small groups of researchers studying the effects of single nutrients — such as particular vitamins or proteins — on a few dozen volunteers, is now adopting the heavy-lifting tools developed for genetics and pharmaceutical research. It also has a catchy name: nutrigenomics. And the more that researchers learn how our genes interact with our diet, the more they appreciate the deeper insight gained by an interdisciplinary approach. Such knowledge could lead to breakthroughs in our understanding of risk factors for diabetes and cardiovascular disease (see Edible advice, page S10) or, for example, improve the design of weight-loss diets.

Nutrigenomics is starting to reveal that a person's diet is more than the number of calories they eat or the ratio of proteins to carbohydrates or fats. Those are important, but the analogy of human metabolism as a car engine that requires a certain type and amount of fuel does not hold up in the age of whole-genome analysis. Nutrition researchers are realizing that our diet does more than just fire our pistons. It is as if the fuel we consume can reach out from the combustion chambers in the engine — through the genetic pathways that govern our metabolism — and tune the engine mid-race.

Multiply those fine adjustments by every possible mutation in each gene of the human genome, perhaps 10 million tweaks in total, and you have an idea of the scale of Ordovas' task. "The only way to realize this concept is via big science," he says.

Ordovas studies how food influences cholesterol and other cardiovascular health indicators in large groups of people. "You take large numbers of individuals with a well-characterized

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ILLUSTRATIONS BY DAVID PARKINS

diet," says his collaborator John Mathers, a nutrition scientist at the University of Newcastle, UK, "and you do a genomic study to ask the question: how does diet interact with the genome to produce a particular phenotype?" Cardiovascular health might be nutrigenomics' strongest application to date, Mathers says, but researchers are also beginning to study the long-term effects of nutrition on the brain and on ageing.

ADOPTING AND ADAPTING

Answering these questions requires examining how small genetic mutations, called single nucleotide polymorphisms (SNPs), affect the production of enzymes and hormones that control metabolism. There are thousands of these mutations in each individual and countless feedback loops, meaning that researchers in the emerging area of metabolomics must employ sophisticated bioinformatics models. "A lot of those tools were developed for pharmaceutical studies and now have become almost commonplace in all areas of biology, including nutrition," says Mathers.

Progress in pharmaceutical research has stimulated improvements in microarrays, high-throughput sequencing, polymorphism identification and DNA methylation technologies, used to scan for novel receptors that might respond to potential drug molecules, says bioinformaticist Chris Evelo of Maastricht University in the Netherlands. In large clinical trials, researchers often collect information about multiple levels of an individual's health before and after the trial in case a drug targeting the heart has an unanticipated effect on the liver, for instance. Likewise, nutrigenomics specialists are concerned with the broader effects of any experimental dietary intervention. "This system-wide approach has been the rule in nutrigenomics research all along. There often are no clear target genes for diet changes," says Evelos. This problem forces researchers to seek out subtle interactions among many elements of the metabolic system and related genes.

In addition to epidemiological studies, which examine global populations without interfering with anybody's diet, many researchers in nutrigenomics are employing intervention studies, which are more like the clinical trials used by drug and medical device makers. "In this other type of study you deliberately modify the nutritional exposure of cells, animals or people," explains Mathers, "and then measure the expression of genes using whole genome expression arrays to try to understand how altered nutritional exposure regulates gene expression and, ultimately, phenotype."

Nutrition research has experienced the move towards more pharma-like genome-wide intervention studies.

Nutrition researcher Lynnette Ferguson at the University of Auckland in New Zealand has experienced the move towards more pharma-like genome-wide intervention studies. She notes that, as recently as 2003, she and her colleagues were "talking about single genes, single nutrients." Yet many promising treatments based on single molecules had clear effects in the lab but never passed animal trials. This is because, as Evelo says, "if you push the system in one place it will compensate through another mechanism and in the end the wished for effect does not occur." Since then, rapid improvements in microarrays and 'deep sequencing' technologies have enabled researchers to consider the impact of food down to the level of individual SNPs. It has also given them a more objective tool to measure what volunteers are actually eating, rather than relying on self-reporting.

Adopting technology from outside traditional nutrition science means adopting new research methods. "My own advantage was that I had been part of a cancer research programme," says Ferguson. "I've watched the development of pharmaceuticals, seen my colleagues work with them and seen the sorts of models they use." Ferguson's team used high-throughput sequencing to screen human cells for modifications to the interleukin-12/23 receptor pathway — important for bowel health — that they suspected were caused by certain foods. This work helped them develop a cellular assay for measuring the effect of particular food components on gene expression in human cells. The next step is to validate whether such nutrient-genome interactions exist in animal models, before planning human trials, just as if they were testing a new drug.

GENETIC PROFILING

These tests will not be straightforward as not all people respond to dietary changes in the same way that not all people react to a particular medicine. Identifying different populations based on their genetic responsiveness is starting to show promise, according to Ordovas. In the best case scenario, researchers would screen individuals against panels of genetic risk factors. In the case of cholesterol, Ordovas and colleagues have found specific genetic differences between people whose cholesterol levels are affected by changing their diet and those who only respond to medication. Right now, doctors try patients on multiple diets before prescribing cholesterol-reducing drugs to avoid side effects. But with a reliable genetic screening test, doctors could prescribe drugs to patients unlikely to respond to dietary changes, saving time and helping reduce the harm caused by living with elevated cholesterol levels.

The majority of dietary effects are subtle, however, and certain genetic profiles might be relatively rare and more difficult to screen. This requires large cohorts to detect and identify signals. "In any gene, there are a few key polymorphisms that we scan, but others will be less common, may not be on the chip we use, or in the specific ethnic group that we are studying, but could still cause disease," says Ordovas. That means he may need to scan ever-larger numbers of volunteers — perhaps into the hundreds of thousands. Unlocking the massive datasets that will emerge will, of course, require dozens of researchers — outnumbering the volunteers that participated in Ordovas' studies in the 1980s (a fact he mentions when he gives presentations about this burgeoning field).

MEETING OF MINDS

New conferences catering for nutrigenomics

4th Asia Pacific Nutrigenomics Conference
21–25 February 2010, Auckland, New Zealand
Exploring the theme of gut health as influenced by both genetics and the microbiota. Around 200 people attended from 19 countries.

7th NuGO Week
31 August – 3 September 2010, Glasgow, UK
An overarching theme of metabolic health, with sessions on biomarkers, modelling tools and personalized nutrition. Around 130 people attended.

1st International Conference on Nutrigenomics
26–29 September 2010, Sao Paulo, Brazil
Discussions centred on the interaction between diet and genes, and how this enables personalized health and disease prevention, particularly in Latin America.

1st Global HealthShare Initiative Workshop
18–20 October 2010, Davis, California
An invitation-only event that jointly addressed issues of nutrition and immunity in the developing world.

4th Congress of the International Society of Nutrigenetics/Nutrigenomics
17–20 November 2010, Pamplona, Spain
Reviewing developments in the related fields of nutrigenomics, nutrigenetics and nutriepigenomics, in disease prevention.

On top of the new mentality and tools, any new scientific discipline needs a way to share data. Through a collaboration called the European Nutrigenomics Organisation (NuGO), Ben van Ommen at the Netherlands Organisation for Applied Scientific Research (TNO) recruits contributors to the Nutritional Phenotype Database (dbNP). Its goal is to combine data from many different areas of biology, including genetics, transcription, protein production, metabolism and behavioural data. "The European Bioinformatics Institute made Array Express and the US National Center for Biotechnology Information has made Gene Expression Omnibus and they store transcriptome data," says van Ommen. "That's good but it's not good enough for us. Nobody does just a transcriptome study or just a metabolomics experiment — everybody does it all together."

Ordovas agrees: "When I began studying lipids I only looked at the biochemistry. We all used to be like rhinoceros poachers who took the horn and left the carcass, but now we have more tools and collaborators and everyone extracts information from all the data in a study."

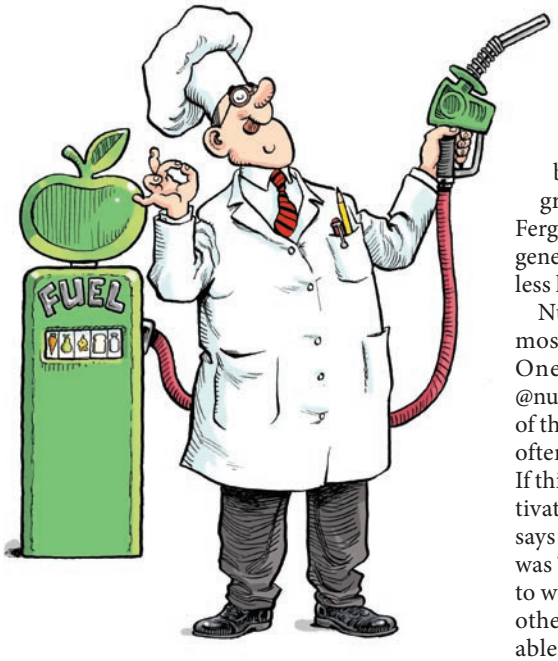
MAKING TEAMWORK PAY OFF

This type of 'extensive phenotyping', quantifying all relevant parameters, is already paying off. A NuGO study led by Gertruud Bakker found that an experimental anti-inflammatory diet in 36 healthy but overweight men increased the concentration of adiponectin, an anti-inflammatory protein, in the bloodstream. By monitoring hundreds of other metabolism-related proteins and metabolites of blood cells and adipose tissue, the team identified more than 500 other diet-driven changes. These included improving the ratio of omega-3 to omega-6 anti-inflammatory precursors in blood plasma and lowering levels of oxidative stress-causing prostaglandin in urine. If the team had used only single-metabolite methods, van Ommen says, they "would only have detected an effect on adiponectin."

Selecting collaborators at first was "like early dating situations".

Adapting pharmaceutical technologies to food isn't the only challenge for researchers: "it's also how you deal with all that data," says Evolos. Some computer models aim to describe observations whereas others try to replicate or predict. "We are trying to integrate those two approaches," says Evelo. This could help researchers working on different facets of the same problem to better understand one another's results, forge new collaborations, and help trace biological problems from the point where food molecules interact with the transcriptome to the symptoms that are presented in a doctor's examination room.

As a proof of principle, the NuGO team used dbNP to track the development of human-like insulin resistance. Evelo and colleagues fed mice a high-fat diet and performed genome-wide transcriptome analysis, tissue sampling, plasma sampling and proteome analysis. They observed that the first signs emerged in a type of fat tissue. This finding neatly explains previous studies that suggested the ratio of saturated to unsaturated fatty acids affects whether a person develops insulin resistance.



In addition to the database, there are a slew of new meetings (see Meeting of minds, page S4). Ferguson established an annual retreat to help New Zealand's nutrition and genomics researchers, from academia and industry, find common ground. "I feel that the slight tension between different priorities [in these groups] has actually been a benefit," says Ferguson. One resulting food developed from genetic research on Crohn's disease is a bread less likely to inflame an irritable bowel.

Nutrigenomics researchers also make the most of social networking to stay in touch. One researcher uses the Twitter handle @nutrigenomics; Ordovas and Jim Kaput, head of the FDA's personalized medicine division, often make Skype calls during the weekend. If this side of big science sounds a bit like cultivating a long-distance relationship — it is, says Ferguson. Selecting collaborators at first was "like early dating situations: did we want to work together? Did we want to work with other partners?" Now that funding is available, there are many more people expressing an interest. Ferguson and collaborators must now ask the hard questions of 'what's your skill set?' and 'what can you contribute?' before inviting would-be partners on board.

The near-term future of nutrigenomics is almost certain: researchers are already hustling to persuade government and funding bodies to finance follow-up studies on the latest research by asking the same questions but on a more ambitious scale — testing hypotheses derived from cell cultures in animals and humans.

LONGER TO WAIT

Some researchers question how useful individual nutrition advice will be in the near term. "Personalized nutrition advice may not be helpful to the general public if they don't know their own genetics," says Albert Koulman, an analytical chemist at the Medical Research Council in Cambridge, UK. But consumer genomic analysis provokes more questions, such as who pays, who gets the results and whether it affects health insurance rates. "There's much more than just the biology, there's the business side and the ethics. We're still just scouting scenarios," says van Ommen.

Commercial pet food today may be a preview of the kind of food categories humans might find in future markets, according to Kenneth Kornman, head of InterLeukin Genetics. "Pet foods I get for my dog are age-categorized, or categorized by sensitivities such as gastrointestinal problems," he says. Dietary needs for individuals also change over the course of their lifetime and from one group of people to the next.

Food manufacturers could one day offer the same choices pet food makers do today — with the additional cost of ensuring that the food is safe for human consumption. There is a big cost to launching such a food, notes Kornman. "You'd need to have a reasonable idea that you'll earn it back." Yet few companies know how to market genetically customized nutrition to customers or how to successfully patent a diet consisting of widely available foods, he says.

Instead, nutrigenomics researchers face the challenge of identifying and measuring a much more subtle state than disease: health. "Optimal health is much more than the absence of disease," says van Ommen, "so we need a different set of biomarkers, not of disease, but of health."

Measuring that will require understanding more than just the chemistry of our food or the on–off switches of our genes. "We've started to better appreciate the fact that it's not just the diet and it's not just the genetic factors but it is an interaction of the two that permits a metabolic change that gets translated in a complex disease over time," says Kornman. It may be a tricky tune to follow, but nutrigenomics researchers are all ears. ■

Lucas Laursen *is a journalist based in Madrid.*

I. HOOTON/SCIENCE PHOTO LIBRARY

DEVELOPMENT

Mother's milk: A rich opportunity

Research on the contents of milk and how breast-feeding benefits a growing child is surprising scientists.

BY ANNA PETHERICK

Should you ever need to mix formula milk for a tammar wallaby, you will face a complicated recipe. During its 300 days in the pouch between birth and weaning, the baby wallaby, or joey, drinks different milk almost on a weekly basis.

Early on, the joey needs colostrum, which is packed with antibodies. After 60 days, the formula should be rich in asparagine-containing peptides, which are thought to help brain development. Ninety days later, the baby wallaby will need a dose of sulphur-containing amino acids, such as cysteine and methionine, which will cause hair follicles and nails to grow.

For healthy development, the number of calories contained in the milk must also rise, such that the joey is weaned from milk that is four and a half times as energy rich as the liquid it first drank. This compositional sequence appears to be entirely dictated by the mother's body. And, bizarrely, her teats can function independently, with each baby wallaby effectively stuck to one teat for the first 200 days in the pouch. In fact, a joey may have a pouch mate of a different age that feeds on an adjacent teat — and which receives milk of a different composition, appropriate for its age[1].

Unlike a wallaby's, human milk does not change so radically over time because the developmental signals, which wallabies transfer in milk, can be delivered through the human placenta. The major constituents of human milk — the fat, protein and carbohydrate — vary little over the course of lactation. But a closer analysis reveals important time-dependent variation in the complement of bioactive ingredients in human milk — the molecules and cells that have biological functions beyond fuelling metabolism and providing the raw materials for infant growth. Finding what these ingredients are and what they do drives much of today's lactation research.

Human milk recipe for newborns DAYS 1–3

Colostrum

An immunological delight!

- ✓ High in short-chain HMOs*
- ✓ High in immunomodulatory IL-10
- ✓ Low in fat
- ✓ Low in caseins

Key ingredients

Whey protein, immunoglobins (particularly IgA), lactoferrin, vitamins A and E, carotenoids and cytokines (especially IL-1β, IL-6 and TNF-α)

*Human milk oligosaccharides

A MAMMALIAN MIXTURE

Until recently, the study of human lactation was conceived mainly from the perspective of public health. Now the trend is to approach the subject from an evolutionary standpoint. This perspective presumes that an infant should breastfeed as much as possible to maximize its chances of survival, whereas a mother must balance her current metabolic investment in milk production with her potential investment in future offspring.

For example, evolutionary theory suggests that mothers should invest more in feeding sons because a successful son can produce many more offspring than a daughter. Several recent studies support this view by identifying clear differences in the breast milk consumed by males and females. In humans, for example, baby boys receive milk that has substantially more fat and protein than the milk girls get[2].

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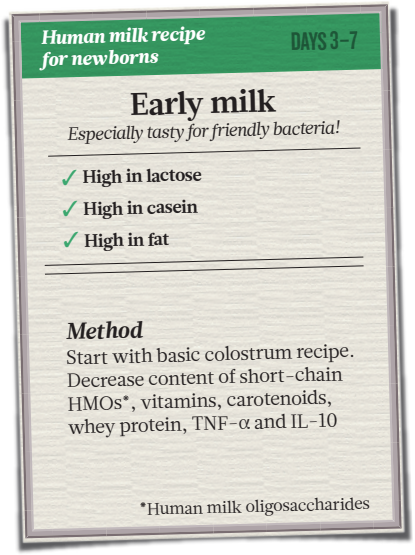
In rhesus macaques, sons drink milk with a higher concentration of cortisol, a hormone that modulates ▶

metabolism, even though their mothers have no more cortisol circulating in their blood than when nursing a daughter. It is unclear whether this cortisol-related sex difference has a function. But there are clues: young male macaques that consume milk containing high levels of the hormone develop bold behaviour, whereas cortisol in milk appears to have no influence on female macaque infants[3]. Whether this has a parallel in humans is yet to be determined.

A second major shift in human lactation research entails the incorporation of new tools to answer traditional questions — such as comparing the effects of breast and formula feeding — and to grapple with evolutionary and functional issues. Human milk is dilute compared to the milk of other placental mammals, but it does contain some surprising ingredients. Advances in high-throughput mass spectrometry, for example, have revealed the existence of more than 200 human milk oligosaccharides (HMOs). Calito Lebrilla, an analytical chemist at the University of California, Davis, has found that mothers seem to produce individual complements of about 100 HMOs — but no one has figured out why different mothers produce different sets of HMOs, or even if it is the same complement of HMOs for each child.

Although they are carbohydrates, HMOs do not appear to nourish infants. Instead they feed certain gut bacteria, giving them a competitive edge over other species. "When a child is born its gut is rapidly populated by pathogenic bacteria," says Lebrilla. "However as the child is fed human milk the population changes to beneficial species." *Bifidobacterium infantis*, which protects against diarrhoea, is particularly efficient at metabolizing the small-mass HMOs that are abundant in early lactation[4]. So breast milk gives *B. infantis* an advantage over other species in establishing a gut population. "The mother is therefore 'selecting' specific bacteria to grow in the infant's gut by her HMOs," says Lebrilla.



Furthermore, some HMOs can inhibit harmful bacteria and viruses directly. For example, certain HMOs block the binding of *Campylobacter jejuni*, the most common cause of bacterial diarrhoea, to intestinal mucosa, and thereby inhibiting pathogenesis[5].

Human milk also delivers some microbes directly to the gut. Breast milk is laced with several species of lactic acid bacteria from the mother's intestine that are thought to travel to her mammary glands inside white blood cells. Most of these species inhibit pathogenic bacteria by secreting hydrogen peroxide and compounds called bacteriocins.

The past decade has seen a large extension in the list of immunological factors detected in human milk. Breast milk was long thought to provide only passive immunity to infants, through maternal antibodies in the form of secretory immunoglobulin A. However, the newly identified crop of immune-regulatory proteins could be prompting and modulating development of the infant's own immune system. Of particular interest are cytokines, which orchestrate the immune system by signalling between its cells.

There is even evidence that breast milk influences gene expression in infant gut cells. In a pilot study, Sharon Donovan, a paediatric nutritionist at the University of Illinois, and Robert Chapkin, a biochemist at Texas A&M University, extracted RNA from exfoliated intestinal cells from several 3-month-old infants. They assessed the statistical difference in RNA expression between breast- and formula-fed infants. Several of the genes that varied were identified as putative master genes, which control the expression of other genes. Most of these genes encode transcription factors associated with angiogenesis and wound repair — including *EPAS1*, a gene that

BRAINY BABIES

Does breast milk make you smarter?

Between late 2002 and the spring of 2005, 13,889 Belarusian children of about six years of age took an IQ test and had their reading and writing skills evaluated by teachers. The mothers of about half of them had been encouraged to breastfeed under a World Health Organization (WHO) programme called the Baby-Friendly Hospital Initiative. As a result, these mothers were seven times more likely to have exclusively breastfed until their child was 3-months old.

Results of this study, called Promotion of Breastfeeding Intervention Trial (PROBIT), showed that the 6-year-olds whose mothers were part of the WHO initiative had better academic ratings from their teachers and IQ scores on average 5.9 points higher[10]. "PROBIT found lots of health benefits in the first year of life," says Michael Kramer, an epidemiologist at McGill University in Montreal, Canada, "but over the longer term the only difference was cognitive ability."

No one is quite sure what causes this intelligence boost. But one 2007 study by

Tests point to higher IQ in breast-fed children.

T. HALL/PHOTOLIBRARY.COM

Duke University psychologist Avshalom Caspi has identified a candidate: a gene that appears to mediate the effects of human milk on brain development[11]. Caspi and colleagues trawled the Kyoto Encyclopaedia of Genes and Genomes (KEGG) database for genes involved in the metabolism of long-chain polyunsaturated fatty acids. These acids are linked to several aspects of neuron development. Two such fats — docosahexaenoic acid (DHA) and arachidonic acid (AA) — are present in human breast milk, but not in cows' milk or most infant formulas.

The KEGG search identified a gene on chromosome 11, called *FADS2*, which is both regulated by dietary AA and DHA and also encodes an enzyme that catalyses metabolism of these two acids. One specific variant of the *FADS2* gene was present in more than 90% of the cohort in the study. Researchers found that only the breastfed babies who had this specific *FADS2* variant exhibited an IQ advantage. The research implies that fatty acid metabolism could be part of the missing link between breastfeeding and IQ. This *FADS2* variant was estimated to account for a difference of 4.1 IQ points, which goes a long way towards explaining the 5.9 IQ points difference found in the PROBIT trial.

is transcribed three times as much in the gut cells of breastfed infants[6].

Donovan and Chapkin's study is the first evidence that breast milk — rich in natural bacteria — affects infant gene expression, and Donovan cautions about over-interpreting their findings. This is, however, likely to be an expanding area of research as probiotics become more commonly used as ingredients in formula milk. "We have no idea how these are potentially affecting gene expression," says Donovan.

Over the years, the 'breast versus formula' debate has become polarized, and several researchers contacted for this article complained that either breastfeeding advocacy groups or formula companies had exaggerated their findings in the past. Donovan's recent gene expression study was sponsored by a formula milk manufacturer, but she is applying for US National Institutes of Health funding for further studies to avoid the criticism that comes with being commercially funded.

HEALTH CONTROVERSIES

Researchers have tried to disentangle the effects of feeding an infant formula rather than breast milk. "The vast majority of studies tend to gravitate towards breast milk as better, rather than equal, but the evidence varies in quality," says Jonathan Wells, who studies human ecology at University College London. "Many of the accepted benefits of human milk relate to avoiding pathogens." And while these pathogens might be less dangerous to a baby in a more medically advanced society than in a developing one, breast milk still offers advantages to all infants. Breastfeeding has consistently been found to protect against necrotizing enterocolitis (in which portions of the bowel tissue die) in pre-term infants, and against diarrhoea and ear infections in full-term infants.

Impacts on health in later life stand out less clearly in the data, although associations between formula feeding and type 2 diabetes and inflammatory bowel disease have been observed. Some meta-analyses report that breastfeeding reduces the chance that a child will be obese at school age by about 20%. But these results are not conclusive. The largest breastfeeding trial, Promotion of Breastfeeding Intervention Trial (PROBIT), found no difference in the plumpness of two groups of six-and-a-half-year-old Belarusian children, where one group had been breastfed for much longer before the introduction of formula milk[7]. There was also no difference between these groups in the prevalence of asthma or allergies[8]. PROBIT did, however, show an intriguing link between breastfeeding and intelligence (see Brainy babies, page S6).

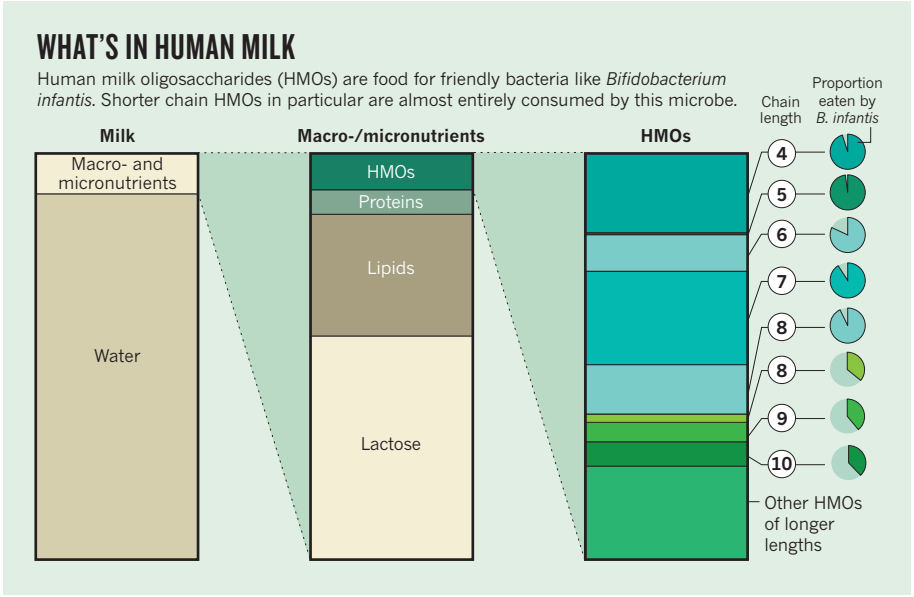
The breastfeeding–IQ association had been reported before, but what made PROBIT's results important was the size of its dataset. It is critical to have a very large sample size in order to eliminate confounding factors. Qualities such as obesity and IQ often vary across rich countries in similar patterns to the tendency of mothers to breastfeed. In developed countries, wealthier women are more likely to breast-feed — but they are also generally slimmer, better educated and spend more time talking to their babies.

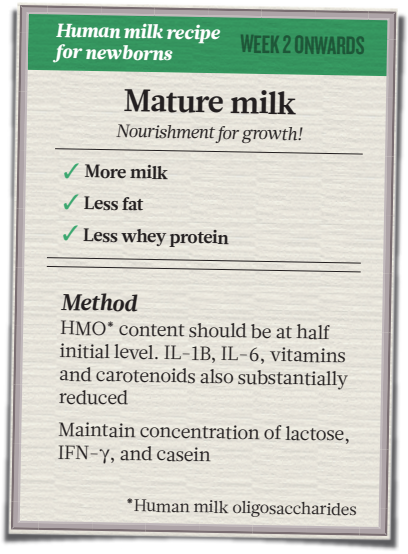
It might be that certain ingredients in formula milk are responsible for later weight issues. Results from the European Childhood Obesity Project supports the 'early protein hypothesis', which holds that higher levels of protein found in standard infant formulas programme the body to become fatter in later years[9]. The project randomized 1,000 European infants to receive either formula of high-protein concentration (standard formula), formula of low-protein concentration (similar to the protein content of human milk), or breast milk. The result: unlike the high-protein group, the low-protein group grew no tubbier than the breastfed control group.

The diverse ingredients of an infant's first meal have an impact on its development, and no matter how much we tinker with the composition of formula milk it will always lack many of the trace constituents of human milk. As research identifies these substances, it increasingly seems they serve a role beyond direct nutritional benefit: that of communicating information to the infant about the environment and even the social structure around the mother, which affects the richness of her diet and her level of physical activity and therefore also affects her milk.

Wells believes that very young humans should be thought of as having to adapt to the mother's surroundings, rather than to the wider world. Indeed, the fact there are so many bioactive molecules in breast milk means that breastfeeding is an activity that empowers mothers. He adds, "The more we learn about the details of breast milk the more we realize that males have a little chance to influence their offspring by non-genetic pathways. Mothers have a very rich opportunity." ■

Anna Petherick *is a journalist in Buenos Aires.*





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EVOLUTION

The first supper

Diet-directed evolution shaped our brains, but whether it was meat or tubers, or their preparation, that spurred our divergence from other primates remains a matter of hot debate.

BY MICHAEL EISENSTEIN

Given the millions of years since our ancestors parted ways, it's unsurprising that a chimpanzee's idea of a good meal differs from our own. "When I visited our study site in Uganda, I followed a chimp in the forest for a day and tried to eat everything it ate," recalls Svante Pääbo, an evolutionary geneticist at the Max Planck Institute in Leipzig, Germany. "It's too disgusting and not digestible — you can't really do it."

Part of the reason is genetics. In 2008, Pääbo and colleagues found evidence for accelerated evolution of both the regulatory and coding sequences of diet-related genes shared by chimpanzees and humans[1]. Many anthropologists now believe that radical changes in diet may have been a major driver of hominin evolution and possibly even the primary factor that propelled our genus *Homo* forward by enabling us to survive and thrive.

One evolutionary milestone was encephalization: an enlargement of the brain estimated to have begun roughly 1.8 million years ago when *Homo habilis* transitioned to *Homo erectus*. What powered this growth spurt remains a subject of ongoing debate.

MEAT AND POTATOES

A big brain is a huge investment in metabolic terms. One model advanced in the mid-1990s, the expensive tissue hypothesis, suggests our ancestors settled that bill by gaining access to more nutrient-rich diets, which spurred brain growth while reducing gut size. Scientists have suggested that the wealth of vitamins, proteins and fats in meat was a major boon and there is evidence our ancestors used stone tools to carve up their food as early as 2.5 million years ago. An article published in *Nature* this year reported the find of 3.4 million year-oldfossil bones scarred by cutting tools, pushing the date back further still to australopithecines.

"There's fairly decent evidence that meat was likely a piece of the diet of australopithecines," says Josh Snodgrass, an anthropologist at the University of Oregon, "but they were probably eating diets that were much more plant-based." Given the richness of nutrients in meat, Snodgrass believes that even minor changes would have had a big impact on caloric intake and contends that use of more sophisticated tools may have increased consumption of meat in early hominins. "Access to high-quality animal foods was probably at least one of the major driving factors in allowing [encephalization] to happen," he says.

On the other hand, the pursuit of a steak dinner is not without hazards, according to David Braun, an archaeologist at the University of Cape Town in South Africa. "There are multiple consequences of making that shift," he says. "There are costs of predator-prey interaction, of entering into a niche that hominins aren't necessarily all that well-adapted to, and all kinds of parasitological costs."

Dartmouth College anthropologist Nathaniel Dominy favours the view that our ancestors might have put their tools to better use in unearthing root vegetables. He has observed how modern hunter-gatherers survive in an African savannah-like environment that may not be radically dissimilar from where *H. erectus* flourished. He suggests that tubers offered an essential buffer against the vicissitudes of the hunter lifestyle. "Modern hunter-gatherers have language, technology and iron-tipped spears, yet they still struggle to get enough meat to survive," he says. "It's hard to imagine a bunch of hominins without those accoutrements getting a lot of meat." Tubers were abundant and may have provided the staple nutrients needed to make brain growth adaptive when easy access to meat was no sure thing.

However, efficient tuber digestion depends on another major technological advance — cooking. "Most tubers absolutely require roasting," says Dominy. Harvard University anthropologist Richard Wrangham believes this is not a problem. In 1999, he published a controversial article promoting his hypothesis that controlled fire and cooking became a component of the hominin toolbox as early as two

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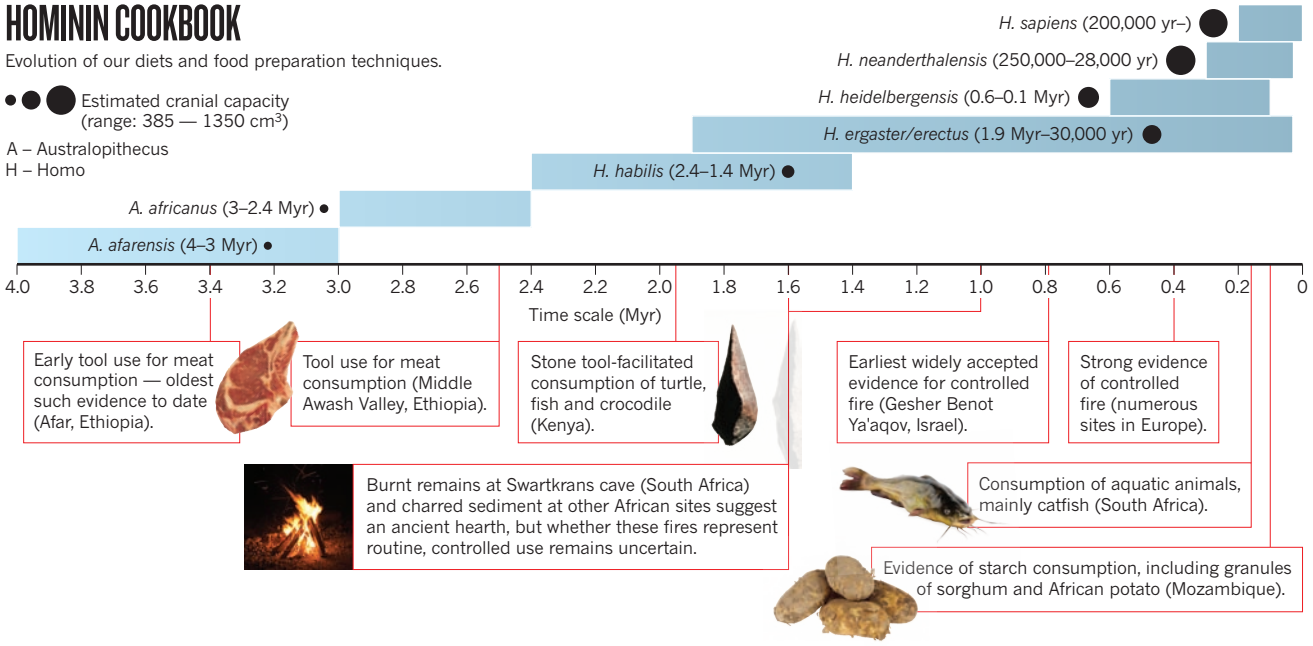

COMSTOCK IMAGES; D. STELLER /ISTOCKPHOTO; J. READER/SCIENCE PHOTO LIBRARY; UP THE RESOLUTION; A. DICKOV/ISTOCKPHOTO

million years ago. Wrangham has since developed this concept to explain how our ancestors maximized the nutritional benefits of tubers, meat and other foodstuffs. "It has not been appreciated by most people until recently that cooking has a large effect on net energy gain," says Wrangham. "Normally it's considered necessary because it enlarges the possible diet and makes food safer, but energy is such a key variable for evolutionary adaptation."

Preliminary analyses by Wrangham and colleagues suggest that cooking may have made proteins and starches more digestible while simultaneously reducing the cost to the immune system of fending off parasites or bacterial infection.

THE HARD FACTS

Many anthropologists remain wary of the evidence gap in Wrangham's hypothesis. The earliest sign of controlled fire comes from Israel, dating back some 800,000 years — considerably shorter than 2 million years. Nevertheless, Braun is hesitant to rule out Wrangham's theory, pointing out that remains of cooking fires can be ephemeral. The evidence found at the Israeli site is particularly unusual. "Gesher Benot Ya'aqov is the kind of place archaeologists dream of," he says. "Wood is preserved there, as are all kinds of activities that aren't preserved elsewhere."

Braun has encountered similar challenges: a recent study by his team at a 1.95 million year old site in Turkana, Kenya, found remains of bones and stone tools indicating that predecessors of *H. erectus* may have routinely eaten fish and other marine life[2]. If this represents a true dietary pattern, then 'brain food' may have lived up to its name by providing an abundant source of the polyunsaturated fatty acids that fuel the growth of the cerebral cortex. Nevertheless, an early role for aquatic animals in the hominin diet remains controversial as archaeological evidence points to seafood only becoming a regular item on the menu between 150,000 and 200,000 years ago. This could be explained by the challenges of actually finding evidence of these foods being prepared. "The preservation that happened at that particular site, I think, is unusually good," says Braun. "We usually use marks on bone surfaces as a determining factor of whether something is part of the diet [and] those don't preserve really well for aquatic animals."

Unfortunately, any efforts to link food choice to human evolution will continue to depend on what can be unearthed at such sites: evidence from the genetic record is likely to be harder to find. Pääbo and colleagues assembled a draft of the Neanderthal genome[3]. This offers a wealth of information on human evolution over the past 50,000 years. However, there is an expiration date for such analyses. "Even in the permafrost, which is probably ideal, [the limit is] somewhere on this side of a million years — and it's much more realistic to say half a million years, maximum," says Pääbo. As such, any hope of obtaining usable genomic data from our early African ancestors is a pipe dream, and attempts to characterize hominin genetic evolution generally focus on our closest extant kin — the chimpanzee and bonobo.

The pursuit of a steak dinner is not without hazards.

Some of the best evidence might be found lining the fossilized jawbones of our ancestors. Peter Ungar, a paleoanthropologist at the University of Arkansas, has been using digital analysis to chart the 'landscapes' of ancient teeth down to the subtle abrasions that cover the chewing surfaces. "Those scratches are the actual result of a hominin passing food across its teeth, and we can relate that to what the animal was adapted to doing," he says.

Based on a growing collection of both *H. habilis* and *H. erectus* samples, Ungar sees a striking transition to teeth that are thinly enamelled and highly textured, which are clues to a diversification in diet. "If our *Homo* ancestors were processing their food outside of the mouth more with tools, then you're not going to get the same selective pressures to maintain big, thickly enamelled, flat teeth," he says. "Teeth with thinner enamel and more relief are actually better for shearing and grinding tougher foods, like meat and leaves." He suggests that although individual *H. erectus* may not have necessarily indulged in a diverse diet, they developed a capacity to rely on a broad array of 'fallback foods' — a skill that would have proved useful in the rapidly changing climate of the early Palaeolithic, and enabled humanity to settle far beyond the continent of Africa.

Braun considers this a reasonable theory, but he also appreciates the need for further investigation into the nutritional building blocks of this increasingly diverse diet. "For every 10 years of field work, we answer one or two questions," he says. "It's going to require a lot more boots on the ground." In the meantime, anthropologists and archaeologists will have to continue to content themselves with reconstructing the Palaeolithic buffet one course at a time. ■

Michael Eisenstein *is a journalist in Philadelphia*

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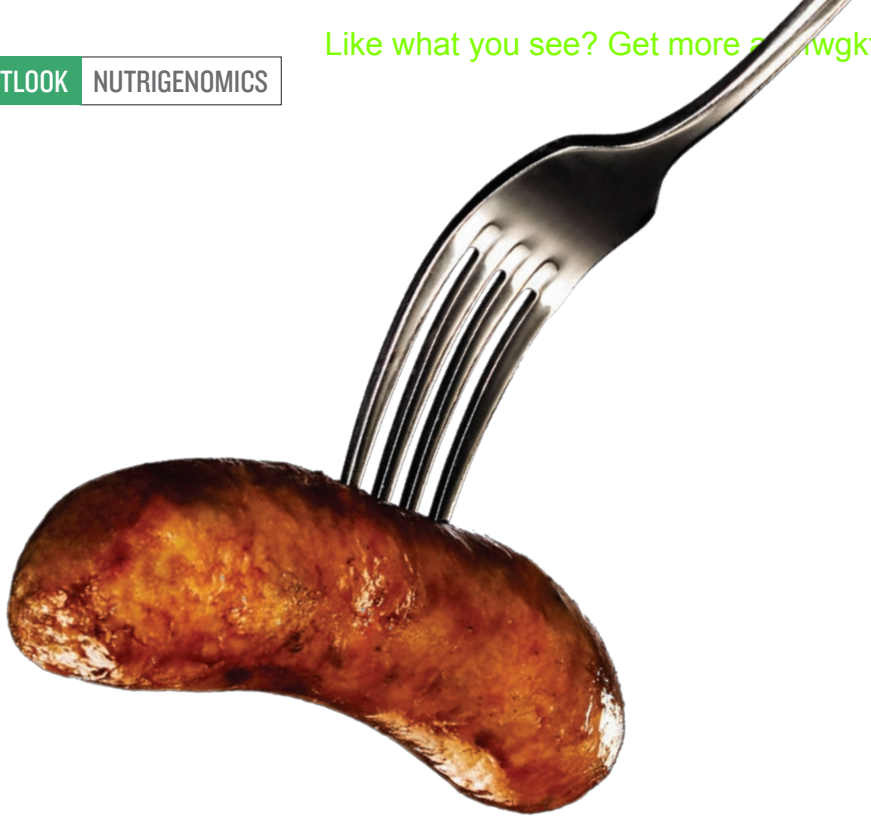

PETER THIEDEKE/ALAMY

HEALTH

Edible advice

Diet-related illnesses are some of the biggest killers today. Can we tailor our food intake to prevent these diseases? Large international projects are underway to find out.

BY FAROOQ AHMED

'Too much tea can treble cancer risk in women'. 'Tea could cut risk of ovarian cancer'. Just two examples of the frequent contradictory newspaper headlines that confuse the public about the health benefits — or risks — of food and confound genuine nutrition-related research.

For some diseases such as diabetes the link with food is subtle. "Although we know that dietary factors are related to the risk of diabetes, there are a lot of inconsistencies between studies in terms of what precise micronutrients or macronutrients associate with the disease. We're quite limited in terms of the data," explains Nick Wareham, head of the epidemiology unit at the UK's Medical Research Council.

Using new tools and methodologies, ambitious projects are underway to make up this shortfall. One such effort, which Wareham coordinates, is InterAct — a multinational study to define how diet and lifestyle influence risk of type 2 diabetes. This disorder of blood glucose regulation is a growing problem in Europe, afflicting nearly 40% of the population at some point in their lifetime. InterAct estimates that the diabetes accounts for as much as 10% of health care costs in Europe.

Through endeavours such as InterAct, researchers are starting to expose the complex interplay of genetics, diet and disease, and bring order to the confusing array of nutritional information.

InterAct began in 2006 as part of the European Community's sixth Framework Programme. It has a budget of €10 million and involves more than 12,000 patients recently diagnosed with diabetes across 10 countries — nine in Europe plus India. Such a broad cohort is important. "Sometimes variation within a country is not so great," says Wareham. "International efforts give you heterogeneity in the lifestyles of patients, especially in the diet, and that's a major advantage." This diversity provides scientists with more variables to study as they attempt to untangle what factors are responsible for causing disease.

THE BIGGER THE BETTER

This research is part of the largest diet and disease study ever undertaken: the European Prospective Investigation into Cancer and Nutrition (EPIC). Initiated in 1992, EPIC has recruited more than half a million people. Participants are physically examined at one of 23 centres, complete lifestyle surveys including detailed diet questionnaires, and have their blood tested. Their DNA is scanned for disease-related genes using techniques that can detect hundreds of thousands of genetic variants in large numbers of individuals.

"Large-scale projects can really be a catalyst to bring together multiple centres to share instruments," says Wareham. "InterAct has benefited greatly from the huge EPIC cohort and access to those technologies."

Another large-scale study, a parallel to InterAct though not part of EPIC, is Interheart — which examined the link between dietary patterns and heart-attack risk. Between 1999 and 2003, the Canadian-led study recruited 5,761 patients and 10,646 control subjects, living in 52 countries, across six continents. Using questionnaires, physical examinations, and blood analysis, the teams compiled data on people including demography, diet, anthropometric measurements such as body mass index and biomarker levels including cholesterol and lipoproteins.

Interheart researchers concluded that the globalization of a Western pattern diet — high in animal products, fried foods, and salty snacks — is responsible for a third of the risk of heart attack worldwide[1]. A 'prudent' diet rich in fruits and vegetables reduced risk regardless of location. Prior to Interheart, few epidemiological studies linked dietary patterns in ethnically diverse populations and cultures to disease. Research like this "is crucial if we truly want to understand these diseases, because they manifest differently in European and other populations," says nutritional geneticist Jim Kaput, who also serves as director of the Division of Personalized Nutrition and Medicine at the US Food and Drug Administration.

CROSSED PATHWAYS

InterAct and Interheart both demonstrate that the metabolic pathways at the epicentre of dietary-related illnesses, such as diabetes and cardiovascular disease, are strongly related. Research on one can uncover clues to the other. "Factors like blood pressure, cholesterol and triglyceride levels, which are predictive of coronary heart disease, are also associated with diabetes," notes Wareham.

Leafy vegetables, such as lettuce and spinach, are core components of the prudent diet as identified by the Interheart study. These vegetables are enriched in polyunsaturated fatty acids (PUFAs) — essential macronutrients also found in some types of fishes, nuts and cheese. Two types of PUFAs in particular, omega-3 and omega-6, are powerful dietary components because they can change gene expression, both directly and indirectly. PUFAs "act more like hormones than like typical food," says nutrition scientist Donald Jump of Oregon State University, who studies these macronutrients.

For example, PUFAs have two ways to

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modulate gene activity and lower the levels of fatty acids and triglycerides in the liver: they can bind and activate a family of transcription factors called peroxisome-proliferator-activated receptors (PPARs) to speed up the breakdown of fatty acids; PUFAs can also deplete another transcription factor, sterol regulatory element binding protein-1 — thereby curtailing fatty-acid synthesis. This two-pronged attack provides a significant health benefit. "Along with cholesterol," explains Jump, "elevated triglycerides are a common target for the management of atherosclerosis, cardiovascular disease and stroke."

Although most research on PUFAs has focused on their connection to cardiovascular disease, by manipulating an enzyme involved in fatty-acid metabolism Jump's team has demonstrated that PUFAs can also balance blood glucose levels, suggesting a potential treatment for type 2 diabetes[2].

"Dietary omega-3s are not usually thought of as a treatment for elevated blood sugar," says Jump. Yet by studying diet and its effect on metabolic pathways, these types of links are being uncovered.

After diet and metabolism, a third element in development of diet-related disease is the genome. "In humans, genetic background plays a role in either responding well, or not, to particular nutrients," says Jump. Adding global genetic diversity to the mix greatly increases the complexity of the research.

The International HapMap Project, a database of genetic variation, began in 2002 and stores data from Canada, China, Japan, Nigeria, the United Kingdom and the United States. So far, the project has identified tens of millions of single nucleotide polymorphisms (SNPs) associated with both disease and drug response. SNPs in PPAR genes that are regulated by PUFAs affect, among other things, the ability to lose weight, a crucial step to controlling diabetes. One SNP in particular has been found to account for 7% of the variation in people's weight loss[3].

In 2010, InterAct recruited the ten-thousandth person to their search for markers that reveal the roles of obesity and exercise in the risk of developing diabetes. Analyses of their genomes will be published in 2011, and Wareham is confident this approach will uncover new interactions. "We're using a discovery approach on a mass scale," he says. "We don't focus so much on the expression of particular genes, but on the interplay between innate susceptibility and dietary and lifestyle factors."

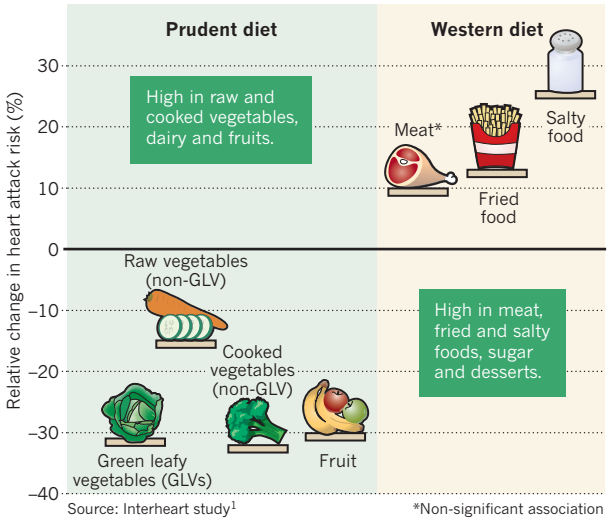
Such a link would fill a gap in our knowledge. "Hypothetically," muses Wareham, "the genome-wide influence of dietary and lifestyle factors may account for the heritability we have seen in diabetes that remains unaccounted for on the basis of simple genetic variation."

Subtle changes are lost in the background noise of standard single-gene studies. But they do have an impact — not least of all for our definition of disease and the way that clinical trials are designed. "Geneticists," says Kaput, "have been treating all of us like coins when it comes to deciding whether we have a disease: heads or tails — are you or are you not diabetic? That's how many studies are designed."

There is a need for new ways to interpret disease that recognize the contribution of genes

RISKY FOODS

Diets high in certain food types carry an elevated risk of heart attack.



Source: Interheart study[1] *Non-significant association

DEVELOPING WORLD NUTRIGENOMICS

Reversing the health and nutrition relationship.

As globalization is exporting fatty fast-food diets around the world, malnutrition is rampant in many developing countries. According to Médecins Sans Frontières, malnutrition causes 60% of deaths in children under the age of five in developing nations. New technologies that provide nourishment while treating diseases could save millions of lives.

That's the stark reality that led Raymond Rodriguez to launch the Global HealthShare Initiative (GHSI). "We started to look at health disparities in racial, ethnic and economically disadvantaged communities," explains Rodriguez, director of the Center of Excellence for Nutritional Genomics at the University of California, Davis. "Often the people who need food and drugs the most are the last to get them."

Lack of proper nutrition opens the door to disease. "Malnourished individuals have reduced immunity, and thus vaccines are less effective," says Somen Nandi of GHSI. Nandi is leading efforts to develop an international network of researchers, investors, non-governmental organizations and drug makers to combat diseases with nutrition-based therapeutics. "We're merging the concepts of nutrition and immunity," says Nandi.

AAMIR QURESHI/AFP/GETTY IMAGES

Malnutrition impairs vaccine efficacy.

One tangible benefit of this new way of thinking is a novel rice-based matrix in development as a delivery base for a vaccine against cholera and diarrhoea. This could help sustain the malnourished and bolster their immunity, while immunizing against the diseases.

GHSI's vaccine development projects also recognize the economic factors involved. Rodriguez's and Nandi's ambitious goal is to help create sustainable economies in countries where diarrhoeal diseases are prevalent, such as Bangladesh. They hope to identify and develop sites in resource-limited countries where therapeutics can be formulated, manufactured and distributed.

"We think that GHSI will create an opportunity for the four billion people who are not a part of the global economy to enjoy better health and a better standard of living," says Rodriguez. Thus the fruits of nutrigenomics research could not only help Westerners cope with a diet of excess, but also bring better lives to the impoverished people in developing nations.

and metabolic pathways. Metabolites are small molecules — amino acids, vitamins and other chemicals — in circulation and influenced by both genes and nutrient intake. Metabolomics, or the study of these metabolites, offers perhaps the best opportunity to observe these interactions in a minimally invasive manner.

Metabolic phenotypes can be very finely segregated, as demonstrated in an analysis of urinary metabolite patterns found in thousands of individuals in China, Japan, the United Kingdom and the United States. Not only did East Asians have a different pattern of metabolites from Western populations, but individuals from northern China could be differentiated from those in southern China. Both Chinese groups were distinct from Japanese, who were in turn different from Japanese Americans[4]. People who consumed a lot of meat, as is common in Western diets, had elevated levels of biomarkers indicative of high blood pressure compared with people who have a primarily vegetarian diet.

InterAct is also searching for novel biomarkers that accumulate as an individual's risk of diabetes rises. When combined with epidemiological studies, this type of metabolic phenotyping could lead to the identification of biological red flags for individuals, even before disease manifests. Biomarker metabolites might also be therapeutic targets one day.

BREAKING DOWN SILOS

While large-scale scientific projects such as InterAct and Interheart have had success, barriers still exist to international collaborations. Researchers occasionally encounter a lack of willingness or an inability to share information. "It has sometimes been a challenge to convince colleagues who run the individual centres that by working together we end up with better science," says Wareham.

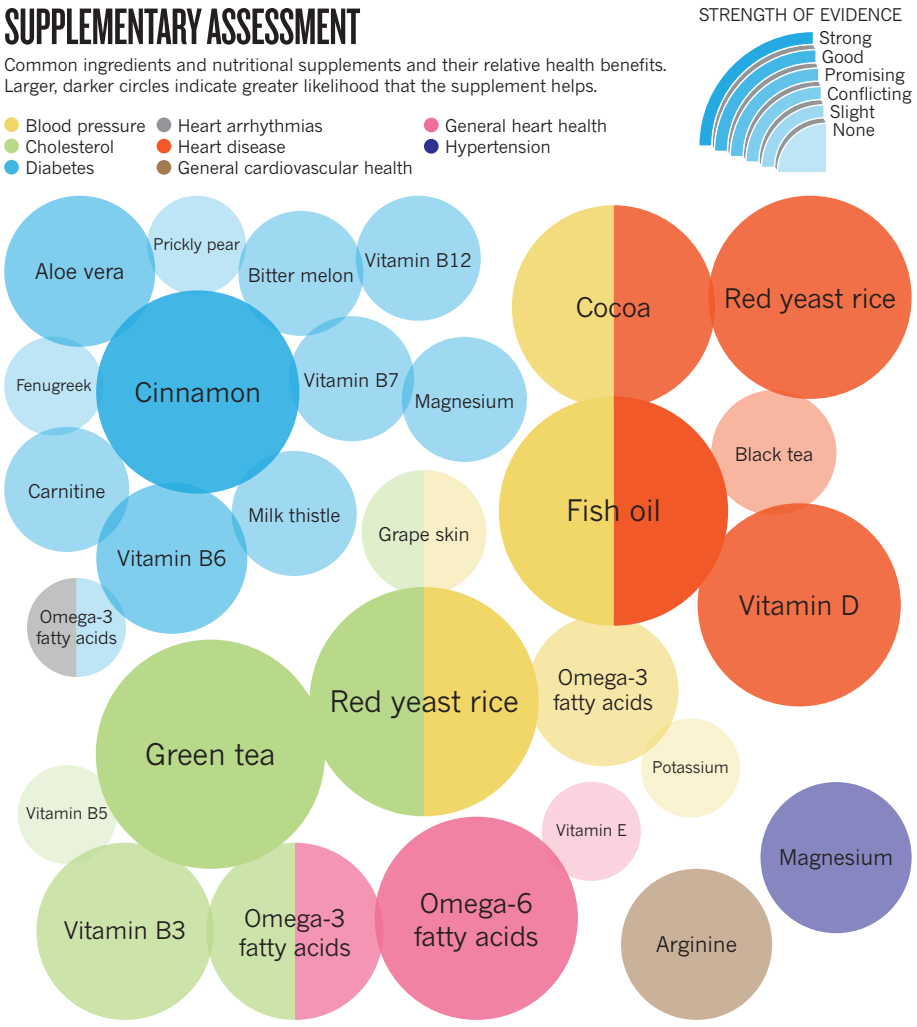
Kaput agrees. He suggests that biologists take a page from the physicists' handbook. "They built the Large Hadron Collider, thereby working across disciplines", he says, "but we still haven't made the silos go away in the biological sciences community."

Wareham has faith in the technology-driven approach that encourages and facilitates collaboration. These major projects, he says, can bring different disciplines closer together — as they have in the genetic HapMap project. "The ability to measure multiple SNPs at very low cost on a mass scale revolutionized that field, and I think that's where we're headed for other risk factors such as diet and nutrition," contends Wareham.

Such a large-scale, system-wide approach is being used by Kaput and FDA chemist Carolyn Wise. They are considering early environmental influences, micronutrient availability, metabolic and regulatory pathways and genome-wide association maps as they try to define combined genetic–metabolic types for people in terms of obesity and type 2 diabetes[5]. This approach can be emulated and scaled in other studies to help researchers work together and share information as they try to make sense of huge amounts of data.

SUPPLEMENTARY ASSESSMENT

Common ingredients and nutritional supplements and their relative health benefits. Larger, darker circles indicate greater likelihood that the supplement helps.



Adapted from www.informationisbeautiful.net/play/snake-oil-supplements/

PREVENTION IS BETTER THAN CURE

As these nutrigenomic studies begin to classify individuals into specific groups based on the interplay of their lifestyle, metabolic pathways and genetic variants, tailored diets may become early therapeutic interventions. Personalized diets might even guide people genetically at risk for diabetes, but not yet in a pre-diabetic state, to help them avoid developing the disorder by fine-tuning what they eat. A well-regulated ounce of prevention could obviate the need for a cure.

However, the development of personalized diets has been prematurely promised before. In the early 2000s, a slew of companies claimed to be able to provide personalized nutritional advice based on genetic tests. An investigation by the US Government Accountability Office in 2006 found that these companies "misled consumers" and provided only generalized advice. The US Senate Special Committee on Aging convened a hearing that further criticized these direct-to-consumer genetic tests. Unable to secure funding, several of these companies went bust.

Here, says Kaput, is where the FDA's Division of Personalized Nutrition and Medicine is ahead of the curve. "Right now, we don't have a product to regulate. We're not sure where the field is going necessarily, but when products come in for possible FDA regulatory activities, we will have the research background to help the regulatory centres make their evaluation."

Teasing out the relationship between food and disease is a tricky task, one that involves tens of thousands of people and encompasses hundreds of nutritional and genetic factors. It is not likely to provide simple or quick fixes either, meaning that for now at least the 'tea causes cancer' stories can safely be ignored. ■

Farooq Ahmed *is a science writer in New York.*

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DIVERSITY

Of beans and genes

Several human genes involved in digestion have diverged along cultural lines. Research suggests these adaptations influence the range of foods tolerated and even certain diseases.

BY MICHAEL EISENSTEIN

Nathaniel Dominy was surprised to find that diet-related genes, like people, sometimes simply repeat themselves to get a point across rather than change the message.

In 2007 while most evolutionary biologists were looking for evidence of selection in the form of genetic mutations, Dominy and colleagues learned that people with high-starch diets had additional copies of the gene coding salivary amylase and that these repeats increased production of the carbohydrate-processing enzyme. "Few would have expected at the time that [these repeats] could have any effect at all — and they had a big effect," recalls Dominy, an anthropologist at Dartmouth College in New Hampshire.

This discovery also offered proof to the growing number of evolutionary geneticists who believe that culture-specific factors, such as diet, have had as powerful an effect on human evolution as more obvious externalities like climate and habitat — with some even suggesting that these factors could have accelerated the overall evolutionary pace. "I don't think we have the data yet to make those claims," cautions Mark Stoneking, a population geneticist at the Max Planck Institute in Leipzig, Germany. "[But] there certainly has been recent evolution in modern humans because of responses to natural selection, and culture may very well be playing a role in a lot of those."

THE GENETICS OF LUNCH

Even if you can enjoy a cold glass of milk without then feeling sick to your stomach, chances are you know somebody who can't. In fact, adult lactose intolerance is the biologically 'normal' state of affairs. "The general pattern in mammals is to lose lactase expression after weaning," explains Dallas Swallow, a geneticist at University College London.

Nevertheless, adults with 'lactase persistence' are widespread in many parts of the world. For example, lactase persistence is characteristic of 89%–96% of Scandinavian and British people, is widespread among pastoralist cultures in Africa and the Middle East, but appears in only 1% of Chinese individuals.

Although one single nucleotide polymorphism (SNP) affecting lactase gene expression accounts for the vast majority of European instances, this trait seems to have arisen independently in different regions of Africa as a result of several distinct yet tightly-clustered variations within a regulatory segment of the lactase gene. "That's convergent or parallel evolution," says Swallow. "The same phenotype is being selected, with different mutations causing that phenotype."

Each of these variants is thought to have emerged within the last 10,000 years, roughly coinciding with the emergence of agriculture and dairy farming, and conferring obvious advantages on those cultures. "Milk is nutritionally good, and if you don't have lactase you can't digest the main carbohydrates in it: you might get diarrhoea or flatulence, and you've lost a source of food, water and calcium," says Swallow. "In the context of African tribes, the most plausible thing is that it was a source of clean, nutritious liquid."

Most geneticists cite lactase persistence as a leading example of recent human evolution driven by shifts in culture and diet. "This happens to be a 'low-hanging fruit'," says Sarah Tishkoff, a geneticist at the University of Pennsylvania. "It's a Mendelian trait and it left a really strong selection signature." Identifying other, equally clear examples has proven challenging, although the subsequent amylase breakthrough by Dominy and colleagues suggests that other ▶

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such traces are there to be found.

Today, many people enjoy starch-rich diets as a matter of choice, but for early humans, tubers and other starchy plants might have been an essential staple in lean times (see The first supper, page S8). "Amylase is the only enzyme that can hydrolyze starch," says Dominy. "If you can produce a lot of amylase, you have a big advantage in the sense that you can extract and assimilate carbohydrates almost instantaneously at the level of the mouth."

The number of copies of the salivary amylase gene, *AMY1*, was already known to vary among individuals. In partnership with Anne Stone and then graduate student George Perry at Arizona State University, Dominy demonstrated that not only does this copy number directly correlate with enzyme levels, but the average copy number within a population also correlates with the starch content of their traditional diet. For example, the Japanese routinely consume large amounts of rice and other starch, whereas the Yakut, a Siberian hunting and fishing culture, have a diet based on fish and meat; these differences are reflected at the level of the *AMY1* gene in Japanese and Yakut populations. "Even though they are closely related genetically, and geographically not separated by a great distance, there's a difference in the number of copies in these two populations on average," says Dominy.

SUFFERING FOR HEALTH

The shift from an active foraging lifestyle to a more sedentary agricultural existence also appears to have introduced selective pressures, as populations struggled to survive nutritional deficiencies. Some intriguing but enigmatic signs of lifestyle-specific adaptation have been detected in the gene encoding N-acetyltransferase 2 (NAT2), an enzyme that is best known for its role in drug metabolism, but which also contributes to the processing of toxins ingested from plants and well-cooked meat. In a series of recent studies, geneticist Lluis Quintana-Murci of the Institut Pasteur and colleagues investigated the extent by which different populations express NAT2 variants that acetylate — and thereby help break down — target molecules quickly or slowly. "We showed that most hunter-gatherer populations present fast-acetylation alleles," he says, "whereas the slower acetylators are very common in farmer-descended populations, like with most Europeans and particularly in the Middle East." NAT2 is also associated with the metabolism of folate, the natural form of folic acid, normally obtained from leafy greens or animal liver. Quintana-Murci's team proposed a model in which the sharp drop in folate intake associated with a shift to a grain- and cereal-rich diet favoured the emergence of alleles that suppress use of folate reserves, although he emphasizes that this is purely speculative until further data are available.

Both versions of the NAT2 enzyme carry certain disadvantages, as acetylation can actually enhance rather than reduce the toxicity of certain compounds: fast acetylation is linked with colon and lung cancers, whereas slow acetylation is associated with prostate and bladder cancers. Therefore, any nutritional benefits are likely to be closely balanced against the potentially harmful outcomes of NAT2 variation.

There are a number of other instances where selective pressures appear to have resulted in a trade-off. For example, although the twenty-odd T2R proteins involved in bitter taste perception represent a potent early warning system for harmful compounds, the genes encoding these factors also exhibit a striking level of variability (see More than meets the mouth, page S18). Unusual patterns of distribution have been observed for several variants that could alter the sensitivity of the mouth to bitter chemicals. "It's clear that there are ethnic differences in the composition of T2R haplotypes," says Wolfgang Meyerhof, a geneticist at the German Institute of Human Nutrition in Nuthetal.

Most traits are complicated and multifactorial in nature.

In a study of taste variation in central African populations by Meyerhof and colleagues, one low-sensitivity variant of the T2R16 bitter receptor, which normally responds to cyanogenic glycosides found in the starchy tuber cassava, was found to be unexpectedly common. These glycosides are metabolized in the gut to release toxic cyanide. The researchers speculated that the health costs of consuming potentially toxic compounds must be balanced by some sort of positive selection, perhaps arising from enhanced resistance against the malarial parasites that are widespread in this region, to sustain this allele in the population.

In fact, there are several instances where the benefits of lowering pathogen susceptibility are apparently sufficient to select for otherwise deleterious alleles. "Infectious disease is probably one of the strongest selective forces in the past or ever," says Tishkoff. She points to the example of glucose-6-phosphate dehydrogenase (G6PD) deficiency, a widespread enzymopathy associated with blood cell defects and potentially severe toxic reaction to foods including the fava bean (shown in main image, page S13). The gene variants associated with G6PD deficiency, also known as 'favism', are widespread in several ethnic groups that routinely eat these beans. Their prevalence, in spite of the near-term dietary and health costs, could result from the protection these variants confer against malaria.

As with lactase persistence, the strong adaptive advantages of this phenotype are demonstrated by its independent appearance in diverse populations; distinct G6PD-deficient alleles have emerged in Africa, the Mediterranean and the Middle East. More recently, Quintana-Murci and colleagues determined that an allele of this gene is prevalent in Southeast Asia, which results in only moderate enzyme deficiency, appears to protect against *Plasmodium vivax* — an unexpected finding, as *P. vivax* is seldom lethal and was presumed to represent a much less potent force for short-term human evolution than its highly dangerous relative *P. falciparum*. "It's about the consequences," says Quintana-Murci. "*P. vivax* could be important in childhood, or in women who are pregnant and infected — maybe they won't die, but their babies are born with low birth weight, which eventually weakens them and raises their chances of dying."

However, not all phenotypes can be directly linked to genetic variation. As years of genome-wide association studies have demonstrated, most traits are considerably more complicated and multifactorial in nature, and tracking

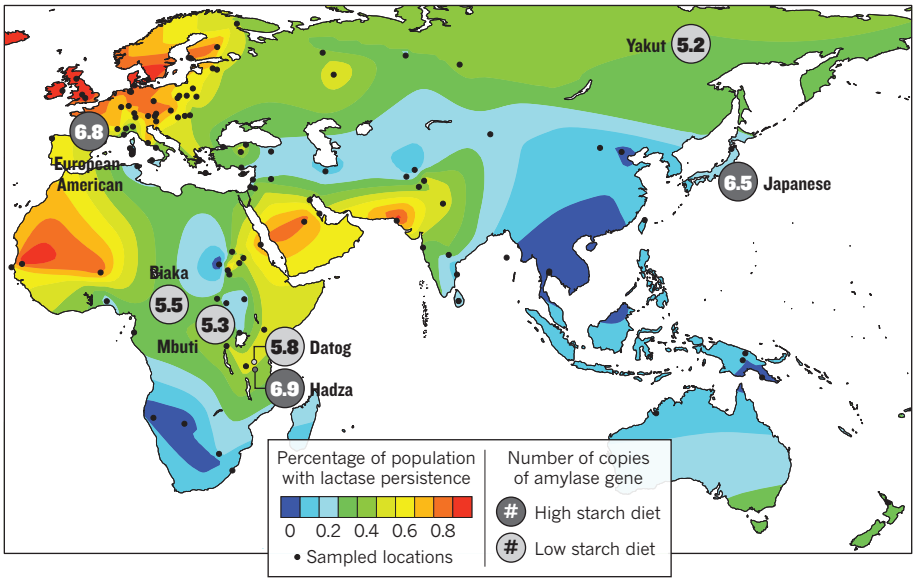
ERNST TOBISCH/STILL PICTURES/PHOTOLIBRARY.COM

Milk provides many nutrients to those who can tolerate it (see map on opposite page).

MILK AND STARCH CONSUMPTION

Global distribution of genes related to ability to digest starchy foods and lactose in milk.

SOURCE: REF. 3, 4.



down these complex changes will require alternative allele-hunting tactics.

A TANGLED WEB

University of Chicago geneticist Anna Di Rienzo recently tried to identify allelic variants that differ in frequency among populations residing in similar geographic regions or ecosystems but who have distinct diets or modes of subsistence, such as farming or foraging. Through this approach, her team uncovered various hints of genetic adaptation in carbohydrate metabolism and folate production, associated with the adoption of diets based on roots and tubers. Conversely, cultures with a cereal-rich diet were more likely to produce a truncated, hyperactive version of PLPR2 — an enzyme responsible for breaking down plant glycolipids — than their non-cereal-consuming counterparts. "There is a consistent frequency shift between populations," says Di Rienzo. "The stop codon [in *PLPR2*] occurs always at higher frequencies in populations that have a cereal-rich diet compared to populations that don't and yet live in the same geographic region."

Yet it remains a challenge to piece together such minor genetic variations scattered throughout the genome. "These aren't mutations that will knock you dead," says Di Rienzo. "They make subtle changes to gene function or expression, and detecting those subtle changes can really be quite hard."

For more recent adaptations, the mutations can also be very rare, making it difficult to detect clear patterns. Even when the data seem to suggest the presence of selective pressure behind a given variant, it is essential to have a solid understanding of the cultural history of the region to eliminate demographic biases. "If a lot of the individuals you've tested have the same great-grandparents, it's quite a different story from if they were relatively unrelated," explains Swallow.

Most importantly, studies need to demonstrate clear functional contributions from a particular variant or subset of variants, and arrive at plausible reasons for why these changes are adaptive in some cases but not in others. "We want to know what the biology is that's being affected by these unusual patterns," says Stoneking. "How many of them are real, and how many are false positives, and what are the underlying stories? That's sort of where the field is a bit stuck at the moment."

IT TAKES A COMMUNITY

Clearly, scouring for signals of recent evolution amid the tens of thousands of interconnected human genes and regulatory regions can be compared to finding the proverbial needle in the haystack — but what if that haystack is far bigger than most people think?

Jeremy Nicholson, a biological chemist at Imperial College London, points out the tremendous diversity of the intestinal microbial flora, citing a report in 2010 which showed that Europeans each carry a complement of at least 160 bacterial species, with more than 536,000 bacterial genes between them — well over 20-times the human gene count[1]. "It actually should be thought of as a multicellular organism with a very large genome," he adds.

Even with our limited understanding of the microbial communities that thrive in our digestive tract and elsewhere in the body, it's increasingly clear that their net genomic output is inextricably linked with our own metabolic function, and the composition and activity of these communities is a direct by-product of our environment, culture and diet. "The gut microbial community can be viewed as a metabolic organ — an organ within an organ; they sense, adjust to, and process components of our diet, and their metabolic products profoundly influence our physiology," says Jeffrey Gordon, a microbiologist at Washington University in St Louis, Missouri. "It's like bringing a set of utensils to a dinner party that the host does not have."

Nicholson has already found some compelling evidence that genes expressed by the gut flora have effects that reach far beyond the digestive tract. "We've found deep compartmental connections between microbial status and bile acid metabolism," he says, "[And] there are some staggering connectivities between blood pressure and gut microbial metabolites."

Research from Gordon's lab has shown there are differences in the sets of bacterial species that reside in the guts of individuals, even identical twins. "Certainly less than 10% — and it might even be less than 2%—of the bugs that are in you are also in me," says Nicholson. Gordon and others are confident that the impact of cultural variation is at least as strong.

Our understanding of the genetic basis of even relatively well-characterized phenomena pertaining to dietary variation, like lactase persistence, could be confounded by the impact of these commensals. "There are Chinese students who come to the West who can drink quite a lot of milk even though they come from a genetic background where they're not lactase persistent," says Swallow. "We think that's due to adaptation of the intestinal flora." It also appears possible that the considerably smaller, but potentially equally diverse, microbial communities in our mouths may play an important role in the early stages of meal digestion, as indicated by a recent study that suggests oral bacteria may facilitate the processing of wheat gluten.

One of the most striking findings comes from a recent study by a team at France's Centre National de la Recherche Scientifique, presenting strong evidence that Japanese individuals can digest seaweed carbohydrates more efficiently[2]. This was made possible by an ancestral gene transfer event from kelp-borne bacteria that endowed their gut flora with the capacity to produce porphyranase and agarase enzymes. This adaptation is seemingly absent in North Americans who have not historically consumed raw kelp. Microbe-watchers like Nicholson suggest that this study could be a strong indicator of the future, as the research community begins to come to terms with the extent to which human genetic effects on diet might be overwhelmed by the bacteria we carry. "It's a piece of genius," he says. "It's something I use in my slide presentations now to worry geneticists." ■

Michael Eisenstein *is a journalist in Philadelphia.*

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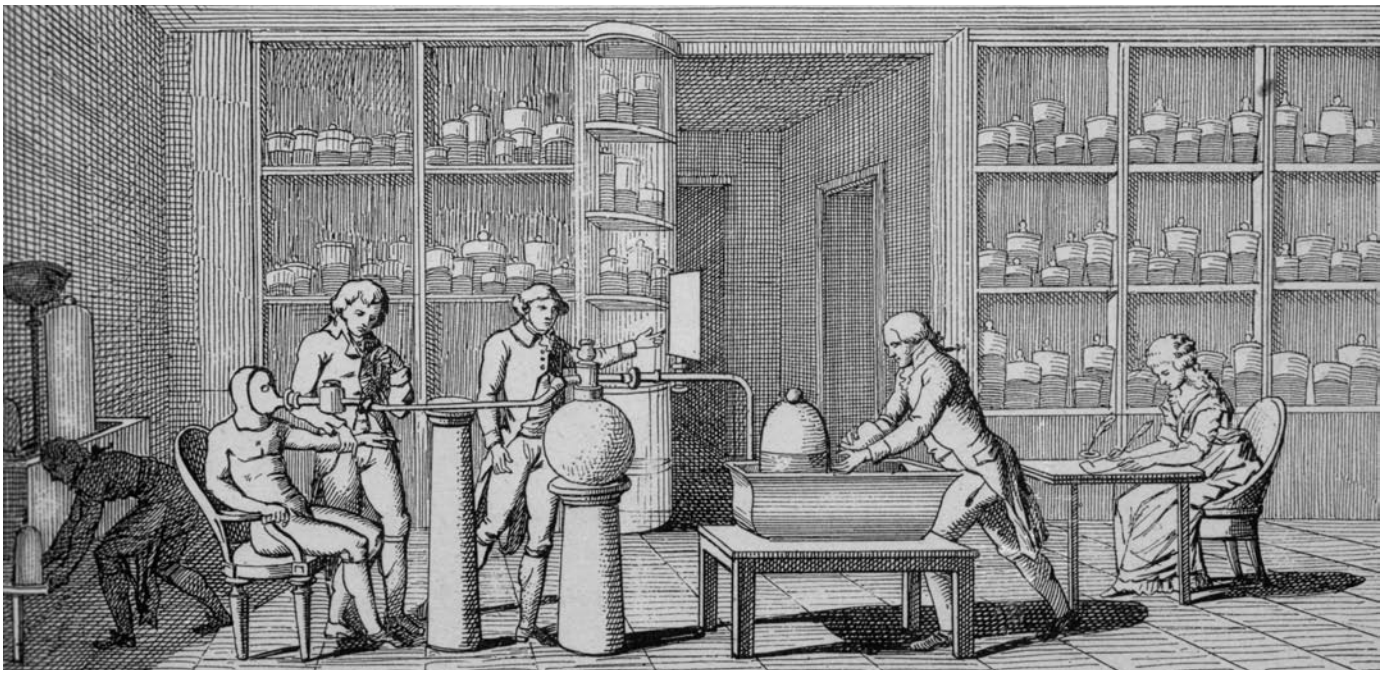


BETTMANN/CORBIS

Eighteenth century chemist Antoine Lavoisier investigates whether exhaled breath is analogous to the fumes of a combustion engine.

HISTORY

The changing notion of food

The pioneers of nutrition research determined the energy content of food and also helped to overturn misconceptions about various diseases that plagued humankind.

BY NED STAFFORD

Nutrigenomics — and the rest of modern nutrition science — stands on foundations laid in the late eighteenth century.

That is not to say that nobody had taken an interest before then in how food works. The ancient civilizations of Egypt, Greece, Rome, Persia, China and India were aware of a link between food and health. "They all had their food rules, many of which are still valid today," says Claus Leitzmann, a human nutritionist at the University of Giessen in Germany. "The ancient Egyptians used garlic medicinally."

Some of our food-truths hark back millennia. The ancient Greek physician Hippocrates recommended that food should be thoroughly chewed before swallowing, and consumed in moderation to maintain good health. In the Middle Ages, the German nun and Christian mystic Hildegard of Bingen "knew a lot about food", says Leitzmann. "She made some very intelligent recommendations," such as eating cooked rather than raw foods.

But before the eighteenth century there was little scientific investigation into the composition of food or how the body processes it. The researchers of the time were "dependent on experimental observation", says Leitzmann. Their method was 'feed and watch'. It was French chemist Antoine Lavoisier, regarded as the father of modern chemistry, who first conducted the research that led to today's science of nutrigenomics.

FOOD AS FUEL

Lavoisier was one of the first scientists to design laboratory equipment to test what happens to food after it is swallowed. Before his work, scientists knew that the weight of ingested food exceeded the weight of excreted faeces and urine. They attributed this loss to perspiration. But Lavoisier believed that food was fuel and that the body, like the fuel-burning engines being developed at the time, must expel carbon dioxide as a product of combustion. He suspected that exhaled carbon dioxide accounted for this lost matter.

To test his theory, in the early 1780s Lavoisier invented a new type of device — the ice calorimeter. It was composed of an outer shell packed with ice, to maintain a constant temperature of 0 °C, encasing a chamber housing a guinea pig. The animal's body heat melted the ice. By weighing water flowing out of the calorimeter, Lavoisier was able to estimate metabolic heat and compare it with the heat produced by a lit candle or burning charcoal.

His theory proved correct. Lavoisier declared: "respiratory gas exchange is a combustion like that of a candle burning."

In today's calorie-counting world, this does not sound like much of a revelation. But at the time it was a breakthrough. "It was theoretically important to realize that the body needed energy to function and that one major function of food is to supply it," says Elizabeth Neswald, a science historian at Brock University in Ontario, Canada. "It was a basis for determining what someone needs to survive; what leads to weight gain, what leads to weight loss, what enables physical labour and what the relationship between food and physical labour is."

Lavoisier's research also emphasized the importance of food composition and of realizing that faeces, urine, perspiration and respiration are an essential part of the equation.

"Early nutrition scientists spent a large part of their time inspecting other people's excrement."

"These early nutrition scientists spent a large part of their time — or their assistants' time — inspecting and analysing other

people's excrement," says Neswald. "In nutrition experiments, it was vital to assess the differences between input and output — food going in and all products coming out."

This method, known as 'balance trials', was pioneered in the 1830s by French chemist Jean-Baptiste Boussingault. He conducted balance trials for nitrogen — a constituent element of proteins — by comparing the nitrogen content of hay, oats and potatoes fed to cows and horses with the animals' excrement and, in the case of cows, milk. He showed that animal feed contained sufficient nitrogen to meet bodily requirements, ending speculation that additional nitrogen was obtained from the atmosphere.

MACRONUTRIENT EXPLORATION

By the mid-nineteenth century, scientists had learned that the primary elements in food are carbon, nitrogen, hydrogen and oxygen, and had divided food constituents into four main types: carbohydrates, fats, protein and water. Yet the chemical make-up of the first three classes was unknown.

It was a German chemist, Justus von Liebig, who made the next leap forward. The precocious von Liebig (appointed professor at the University of Giessen at age 21) invented the 'kaliapparat', a special piece of glassware for analysis of carbon in organic compounds.

Von Liebig's laboratory, arguably the first teaching laboratory, attracted scientists from around the world. He helped train a generation of nutritional researchers whose work would carry on into the early twentieth century. In the 1860s, for example, two of von Liebig's protégés — physiologist Carl von Voit and chemist Max Joseph von Pettenkofer — obtained funding from the Bavarian government to build a state-of-the-art respiration chamber large enough to hold a person. The chamber could measure the daily balances of both carbon and nitrogen and thereby estimate human protein requirements.

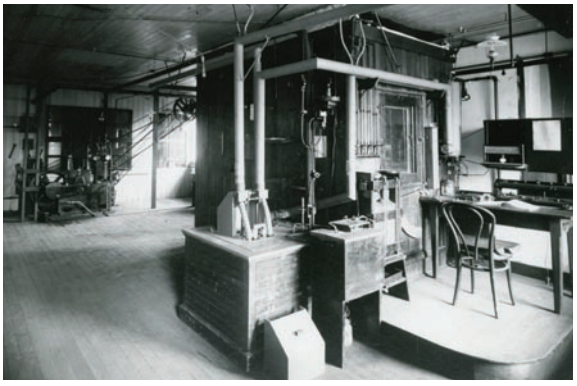
Neswald notes that most of the nutrition research of this period focused not on the health of individuals, but rather on finding the cheapest, easiest methods to feed "institutionalized and impoverished populations" to prevent food riots. Von Voit, says Neswald, visited prisons and workhouses "to assess what people were fed and what their state of health was, with the aim of providing dietary guidelines".

The concept of food as fuel, which contains important dietary components, was further refined in the United States. Agricultural chemist Wilbur Olin Atwater had spent time in von Voit's laboratory as a postdoc, returning to the United States in 1871 to spearhead nutrition science. Atwater spent five years in the 1890s building a respiration calorimeter larger than von Voit's and able to hold humans for longer than a day. His measurements were so precise that his energy equivalents for protein, fat and carbohydrate are still used today. Atwater was first to adopt the word 'calorie' as an energy unit for food. (A calorie of food energy is actually equivalent to 1000 calories of thermal energy.)

SMALLER AND SMALLER

Scientists soon began to realize that in addition to supplying energy and macronutrients, food also played a more subtle role in health and disease. Japanese physician Takaki Kanehiro, who studied in the 1870s at St Thomas's Hospital Medical School in London, was a rare exception to the nineteenth century German dominance of nutrition. "He was the first person to show that beriberi arises from malnutrition," says Katsuhiko Yokoi, a human nutritionist at Seitoku University in Japan. Previously beri-beri was thought to be an infectious disease.

By the early twentieth century, other scientists around the world had begun to explore links between nutritional deficiencies and other ailments, including rickets and scurvy. Unable to explain these afflictions in terms of fats, protein or carbohydrates, some scientists began to suspect the existence of another class of food ingredients.



Atwater-Rosa calorimeter used to measure human energy demands.

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It was Polish biochemist Casimir Funk who in 1912, while studying beriberi, isolated thiamine, the nutrient that protects against this disease. He called the substance a 'vital amine', which soon became 'vitamin'.

The battle against scurvy is an example of science later refining a nutrition-related disease association. In the mid-eighteenth century, Scottish naval physician James Lind found that scurvy could be treated or prevented by eating citrus fruits. But he incorrectly thought that sea air was to blame for the disease. Other erroneous suggestions followed: in 1846, for example, Scottish toxicologist Robert Christison hypothesized that scurvy was caused by protein deficiency. Scurvy continued to be a sporadic problem into the early twentieth century. It was not until 1932 that US biochemist Charles Glen King showed that scurvy was caused by a deficiency of the newly discovered vitamin C.

Animal research led to further vitamin and disease-related discoveries. US biochemist Elmer Verner McCollum learned German so he could read the works of past nutrition researchers, which inspired him to experiment on rats. At the University of Wisconsin, where McCollum initially worked, research protocols stipulated the use of cows as animal models. But McCollum convinced his superiors to let him try smaller animals. He bought 12 albino rats from a pet store and established the first colony of rats for nutritional experimentation in the United States. In 1913, his studies with these rats led him to identify the first fat-soluble vitamin, vitamin A, and later showed that it is vitamin D — and not vitamin A as some thought — that prevents rickets.

Proving the link between micronutrients and disease didn't come easily. US Public Health Service worker and epidemiologist Joseph Goldberger theorized that pellagra, then a major disease causing diarrhoea, dermatitis, dementia and death, was diet-related and not, as prevailing medical opinion held at the time, an infectious disease. In 1916, to prove his point, Goldberger and his assistant subjected themselves to a series of tests — they injected each other with blood from a pellagra sufferer, swabbed out the secretions of an pellagra-infected person's nose and throat and rubbed them into their own, and swallowed capsules containing scabs of pellagra sufferers' rashes. And yet despite such gross exposure, they did not develop pellagra. However, Goldberger was unable to find the diet-related cause. It was another two decades before American biochemist Conrad Elvehjem realized that pellagra was caused by a deficiency of niacin (vitamin B3).

So many micronutrients had been discovered by 1944 that some believed the field of nutrition had been fully defined with little else to discover. But while the constituent parts of food might have been teased out, their impact on the body was only starting to be appreciated.

From Lavoisier, through von Liebig, to scientists today such as Jose Ordovas (see Big science at the table, page S2), nutrition research has focused on smaller and smaller elements. As scientists have probed deeper into biochemical mechanisms of bodily absorption and function — unlocking mysteries as they go — they have also triggered new questions, until we get to 'how do our genes interact with the food we eat?' And that's the question we are still trying to answer today. ■

Ned Stafford *is a science writer in Hamburg.*

RADIUS IMAGES/CORBIS

TASTE

More than meets the mouth

Certain things taste differently to different people. Why is this, and does this affect our choice of food?

BY MICHAEL EISENSTEIN

Nearly 80 years after DuPont chemists stumbled across evidence of genetic variation in perception of the bitter-tasting compound phenylthiocarbamide (PTC), Danielle Reed's team at the Monell Chemical Senses Center in Philadelphia, Pennsylvania, made a similarly serendipitous discovery.

Reed was approached by a lab technician worried she made a mistake with a experimental quinine preparation. "She said, 'I think I made the solutions wrong — here, taste this,'" recalls Reed, who then tasted the bitter compound. "I'm like, 'ugh, it seems fine to me.' But she said, 'It tastes like water to me.'"

This strange observation eventually led to the discovery of a genetic locus that affects our tongue's ability to detect bitterness in quinine — a big step on the road to understanding how people differ from one another in terms of taste, and how these differences shape what we like to eat.

A BITTER TASTE

Bitter is one of the five primary tastes — along with sweet, sour, salty and the savoury umami — that compose the gustatory system. Of these, bitter is perhaps the best characterized in terms of the influence of genetic variability on taste.

In humans, the cells responsible for bitter taste perception express 25 receptors (T2Rs) that vary in the chemicals they recognize but which appear to perform a common role in preventing people from eating toxic compounds. Accordingly, some scientists are convinced that humans evolved taste to detect harmful substances. "A newborn baby is born loving sweet and hating bitter — no experience required," says Linda Bartoshuk, director of human research at the Center for Smell and Taste at the University of Florida.

Insensitive variants have been identified for several bitter receptor genes and are common in the general population. For example, mutations in T2R38 render individuals incapable of tasting PTC or the related compound 6-n-propylthiouracil (PROP).

Such limited sensitivity can be an asset as many nutritious vegetables, including broccoli and sprouts, also produce bitter tasting glucosinolates. These compounds include goitrin, a thyroid toxin in large doses but which may protect against cancer in lower doses.

There are obvious nutritional advantages in mitigating the urge to avoid sprouts, and the adaptive value of this reduced sensitivity allele is evident in its global distribution alongside the more common sensitive version. "The ratio of the alleles varies depending on where you go," says Paul Breslin, a taste perception researcher at Monell, "but you see that both have been maintained in almost every population you look at anywhere on Earth."

Yet efforts to firmly link individual genetic variations with altered food preferences have not been easy. Several studies have revealed geographic or ethnic differences in the distribution of taste receptor variants that may have arisen from selective pressures (see Of beans and genes, page S13), but their effects on diet — and association with overall health — are controversial. "I'm a PTC non-taster: I can't taste goitrin in vegetables very well. But I think this has very little to do with how much broccoli I choose to eat on a daily basis," says Reed.

Attempts to establish similar correlations between disease and taste have proven equally problematic. For example, there is no clear link between sensitivity to sweet tastes and predisposition to obesity, diabetes or other diseases related to excess consumption of sugars.

Some of the strongest connections identified relate to alcohol preference. In one study, Bartoshuk partnered with Yale University geneticist Ken Kidd to examine how bitter taste shapes alcohol perception within a cohort of students. "There was a clear relationship between sensitivity and whether ethanol is perceived as bitter and harsh or slightly sweet," says Kidd. "Among those who were homozygous for the high-sensitivity [bitterness allele], nobody drank very much." Other studies at Monell have hinted at a parallel role for sweetness receptor variation, where sensitivity to, and preference for, sweet tastes is seemingly correlated with alcohol consumption. However, Kidd and others point out that this variability

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must be considered alongside the numerous other brain and metabolic factors involved in drinking alcohol.

Collectively, these data raise a question: given the front-line role of taste perception in food consumption, and the clear advantages of quickly recognizing good and bad food sources, why is it so hard to associate genetic differences in taste function with dietary behaviour?

NAME THAT TASTE

A large part of this problem arises from challenges in experimentally linking the highly subjective experience of taste with biological mechanisms. But gaps also remain in our understanding of the basic machinery of taste perception. This past spring, Charles Zuker's team at Columbia University, New York, validated the involvement of epithelial sodium channel ENaC as a component of sodium chloride salt perception in mice. Other salt receptors remain at large. "People describe potassium chloride as being kind of brackish tasting, maybe kind of metallic, like a dirty salt solution. It's clearly salty," says Breslin. "That can't be through an ENaC, because those channels pass potassium ions very poorly."

Furthermore, even though researchers have known the cells responsible for sour taste since 2006, a definitive receptor has yet to be identified. This is partly because of the complex nature of oral response to acid, where taste effects overlap with somatosensory sensations, a category of perceptual information that encompasses non-taste qualities such as temperature, texture or spiciness.

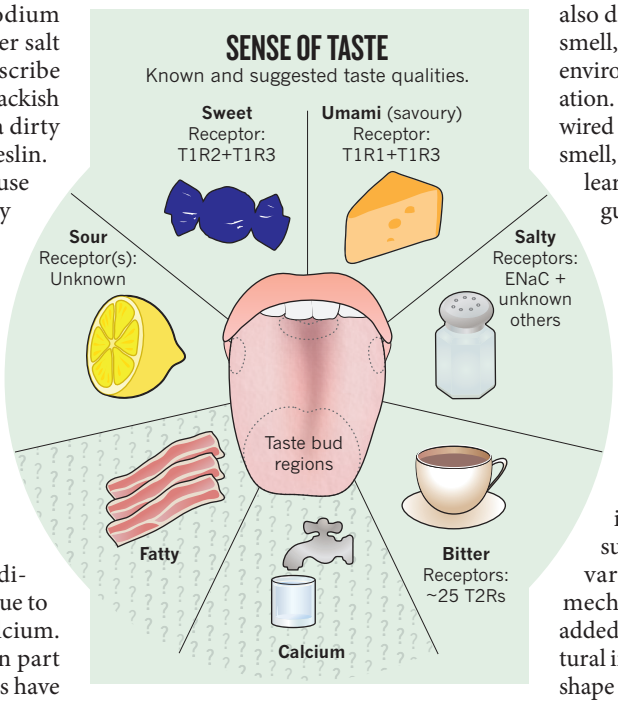
Preliminary reports also hint at additional taste qualities, enabling the tongue to recognize things like fatty acids or calcium. But there is little consensus on this, in part because no dedicated taste-quality cells have been identified and also because candidate receptors only partially account for our ability to distinguish these putative tastes. Some scientists are also sceptical because humans lack a lexicon to describe these qualities. "Just take a little canola oil and taste it — it doesn't really have a taste," says Bartoshuk. "My guess is that the real sensory input from fat is tactile — fat is gooey and oily and viscous and creamy."

Most investigators remain open to the possibility that there's more to the mouth than just the 'basic five'. A 2009 study by Zuker's team identified a protein expressed in sour cells that apparently contributes — in conjunction with somatosensory receptors — to the discrimination of a 'carbonation taste', and they are on the hunt for mechanisms that monitor other undiscovered qualities. "If you take an animal and label all the sweet, sour, bitter, salty and umami cells, there are still plenty of cells left," he says. "What we're doing now is looking for things that are uniquely found in those [other] cells."

A GUT FEELING

Taste doesn't end at the back of the tongue. Many of the same taste receptor genes expressed in taste buds are expressed throughout the digestive system and in other tissues. Preliminary investigations suggest that these non-oral receptors help regulate appetite and metabolism. "What better way to do so than having the very same receptors reporting back from the gastrointestinal tract?" asks Zuker.

There is already strong evidence that taste receptors in the mouth help steer organisms towards the nutrients that the body needs most. "If you offer malnourished kids soups that are either plain, ordinary stocks or stocks that have been fortified, they generally prefer soups that are amino acid-fortified over everything else, including very tasty high-calorie soups," says Breslin. "This is in young kids, who have no idea what's going on. This suggests that somehow there's this 'wisdom of the body'."



Evidence suggests that at least some of this activity may arise from metabolic signals triggered by taste receptor activation. "Taste cells express all sorts of different peptide hormones that are used in other areas of the body for regulating satiety or blood glucose," says Steven Munger, a neurobiologist at the University of Maryland.

Several studies in the past few years suggest that these receptors also direct the secretion of metabolic hormones in the lower digestive tract in response to sweet, bitter or umami stimuli; for example, intestinal sweetness receptor signalling may help regulate glucose absorption from the blood. Munger adds that his own investigations of genes associated with diabetes among Amish people have been confounded by these gut receptors and the ambiguity of their function. "We did see an association with variation in a particular bitter receptor and the ability of non-diabetic individuals to regulate their blood glucose," he says. However, it remains unclear whether this association arises from the effects of receptor variation on tongue-level taste preference and food selection or whether the difference lies in how the gut reacts to particular foods.

UNWIRING FLAVOUR

The biggest outstanding issue for many taste scientists is understanding how the various raw chemical sensations that transmit taste are incorporated into a more nuanced and sophisticated sense of flavour. Perception at this level also depends on signals received by sense of smell, which exhibits far greater complexity, environmental adaptability and personal variation. "You've got one sense, taste, that's hard-wired for affect," says Bartoshuk, "and another, smell, where the affect is extremely labile and learned very quickly and can also be extinguished."

Equally important is how the brain decides whether or not it likes what it senses. Alexander Bachmanov, a geneticist at Monell, cites the example of sweet-liking mice developed in his lab. "Through selective breeding, we have created mice with the same genotype for sweet taste receptors, but some are very avid consumers of sweeteners while others consume them in very modest amounts," he says, and suggests that this behaviour arises from variations in more central neurological mechanisms related to taste response. This added complexity leaves a lot of room for cultural influences and environmental factors to shape how we assign reward value to a flavour and might in turn affect the contribution of more subtle genome-level factors. As such, inherited differences in taste receptor expression or function alone are probably insufficient to explain how many of us overcome our innate aversion to bitterness and sourness to thoroughly enjoy a steaming demitasse of espresso or a bracing gin and tonic.

Nevertheless, there is evidence that genetic changes can modulate the response of this normally hard-wired sensory system. Zuker concludes that meaningful progress in untangling the neural processes behind food choice will require a solid understanding of what happens when meal meets mouth. "Before we can understand how the brain knows," he says, "we need to figure out how the tongue knows." ■

Michael Eisenstein *is a journalist in Philadelphia.*

AD WINDIG/MAI/HOLLANDSE HOOGTE/EYEVINE

Children wait to be fed during the Dutch Hongerwinter of 1944–1945.

EPIGENETICS

Tales of adversity

Genetic studies of people conceived during famine reveals that prenatal malnutrition lingers long after the event.

BY FAROOQ AHMED

It is well established that a pregnant woman's habits affect the health of her unborn child, but the extent of the impact is less well known. Recent studies of tragic historical events, namely the Dutch Hongerwinter and the Great Chinese Famine, have begun to highlight the trans-generational relationship between food and genes.

The Hongerwinter (hunger winter) began late in 1944 towards the end of the Second World War. Food supplies in the northern and western regions of Nazi-occupied Holland became increasingly limited as the Germans halted overland transport of goods into Amsterdam and nearby cities.

Exacerbating this blockade, the harsh winter froze canals — cutting off a vital supply route. Rations in cities dropped to as few as 500 calories per day, less than a quarter of the recommended intake, until the country was liberated in May 1945, but not before 18,000 people starved to death.

Many children conceived during the Hongerwinter were small and underweight. What's more, certain health problems have persisted long into their adult lives. Compared to their siblings conceived before or after the famine, the Hongerwinter children are at increased risk for obesity, for example. A propensity for obesity was also found in children of the 1968–1970 Biafra famine in a recent study in Nigeria.

The Great Chinese Famine, from 1958 to 1961, was caused by a combination of leader Mao Zedong's agricultural policies during the Great Leap Forward, widespread mismanagement and severe weather. Tens of millions of people died. Studies of Chinese born during this period link prenatal famine exposure to an increased risk of schizophrenia — a link also found in the Dutch Hongerwinter cohort.

"These extreme events offer special opportunities for research in humans that you might not otherwise have," says Lambert Lumey, an epidemiologist at Columbia University, New York, who is studying the effects of the Hongerwinter. There are obvious ethical issues and long time spans involved that make recreating the circumstances of famine impossible. "These events are crucial to helping us develop and discover underlying disease mechanisms," says Lumey.

TELL-TALE DNA

Scientists have discovered that certain genes of children conceived during a prolonged period of starvation receive special epigenetic 'tags' through a process called methylation — a gene modification that typically deactivates a gene, but does not alter the genetic code. Methylation is part of normal development, but patterns vary across individuals.

Nearly six decades after the famine, Lumey and colleagues isolated DNA from Hongerwinter individuals. They found a below-average methylation of the insulin-like growth factor II gene *(IGF2)*, which codes for a growth hormone critical to gestation. Decreasing the methylation of *IGF2* should increase the expression of the hormone. In contrast, later studies in this cohort found increased methylation of five other genes, among them genes associated with cholesterol transport and ageing, as well as the gene that produces IL-10, which has been linked with schizophrenia.

The mechanisms of these epigenetic changes and whether they have a bearing on disease remain unclear. "In humans, these are the $100,000 questions," says epigeneticist Robert Waterland from Baylor College of Medicine in Texas.

Lumey hopes to study the children of the 'tagged' individuals to see if these changes persist into the next generation. Epigenetic information is almost fully reset in very early development, so the outcome, he says, is difficult to predict. "This is an important question regardless of what the data will later show."

Nevertheless, studies on these extreme events "provide the first convincing evidence that early nutritional exposure causes a persistent change in epigenetic regulation in humans," notes Waterland. "It's a proof of principle."

Lumey is now looking to high-throughput sequencing methods to measure genome-wide DNA methylation. "We expect that this will tell us whether there also are more epigenetic differences between prenatally exposed individuals and their unexposed siblings, than the ones we found studying candidate loci," says epigeneticist Bastiaan Heijmans of Leiden University in the Netherlands, who works with Lumey. If these modifications are indeed widespread throughout the genome, the cumulative effect of famine-induced epigenetic alterations might play a substantial role in disease progression.

Other research has shown that less-extreme diets also affect methylation patterns and disease susceptibility. For example, folic acid is an important supplement for pregnant women to help prevent neural tube defects in developing embryos. It has been shown to increase the methylation of *IGF2,* hinting that it works through an epigenetic mechanism.

Nevertheless, studying such catastrophes provides researchers with valuable information that is not otherwise available, revealing that the aftermath of famine and prenatal malnutrition lasts long after help arrives with life-saving food. ■

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DAVID PARKINS

TECHNOLOGY

A flavour of the future

Health biomarkers, smart technology and social networks are hastening an era of nutrition tailored to your individual needs but relying on information generated by the crowd.

A man steps out of a health clinic after his monthly nutritional profile. He slides a ring onto his finger and the injection-free technology transmits a read-out of his blood constituents to a central server. Skimming the data sent to his smart phone, he looks at the recommendation for his evening snack — something with a little more selenium: brazil nuts, perhaps. He considers his diet for the coming week — logged with his refrigerator — and confirms an updated home-delivery shopping list. Finally, he tots up his credits for sharing this personal health data with a population-wide genome study—redeemable against the cost of his health insurance and nutritional supplements. It's a familiar sight to his girlfriend. "We're having dinner at my parents' tomorrow. Don't you dare let the FatNav tell you what to eat, or me what to drink."

There are signs that this future is fast approaching. Domestic sleep and weight monitors can transmit results using WiFi; fridges are in development that log what you've eaten; and dinner parties are complicated by food intolerance and fad diets. Already, pin-prick blood test results for diabetes can be uploaded online. Websites such as patientslikeme.org offer tips on drug and nutritional supplement regimens. And at SNPedia.com and DIYGenomics.org, people can share their entire genomic data to pool resources and provide more personal guidance on health issues.

Can all these platforms create genetics-based nutrition advice? Will this affect our definition of health, or the distinction between food and drugs? And how personalized will our diets become?

NOT IN SICKNESS BUT IN HEALTH

Many researchers think that personalized nutrition must begin with a new suite of biomarkers: ones that measure health rather than disease. But what does that mean? "Here we are in the twenty-first century and we don't have a definition of health other than 'the absence of disease'," says Siân Astley, a nutrition researcher at the Institute of Food Research, UK. "Health is about much more."

Astley says that to comprehend what bioactive food compounds are doing we first have to understand what's going on in the body before it becomes ill. "Our difficulty is that the only biomarkers we have are for when the disease process has already started."

'Omics' sciences, such as transcriptomics, proteomics and metabolomics, study many thousands of putative biomarkers in a process called 'extensive phenotyping'. "We now have examples where the protein fingerprint in tissues can indicate precancerous changes long before symptoms appear," says Astley. "The protein fingerprint offers us early diagnosis as well as an insight into potential changes that might be elicited by feeding people a different diet."

Astley also works for the Nutrigenomics Organisation (NuGO), an EU-funded project involving 23 universities and research institutes. NuGO researchers believe that to find these health biomarkers, testing conditions will need a rethink. For example, although we are all in a state of homeostatic equilibrium, the 'normal' levels of metabolites, including glucose, plasma proteins, cytokines and signalling molecules, vary from person to ▶

person. Challenging that state with exercise or new foods, and then measuring changes in metabolites as the body recovers, reveals more about its reaction to bioactive compounds than simply measuring metabolites in a resting state.

THE DEVIL IN THE DETAILS

Extensive phenotyping is a big job and costs big money. Resource-limited researchers have two options: measure many people in lesser detail, or a smaller number in greater detail. Large population studies have more statistical power, but as the ultimate goal is personalized nutrition, an investigation of the individual will provide more in-depth information.

It's a conundrum facing Mike Gibney, director of University College Dublin's Institute of Food and Health. "Too many people in a study smooths out the data and is too expensive in an era when so many measurements are needed," he says. Gibney contends we are in transition towards personalized nutrition and advocates temporarily abandoning the 'individual' mantra. Instead, people should be grouped into broader categories based on biomarkers that indicate, for example, how efficiently different sugars or proteins are metabolized. "I'm taking my research in the direction of clusters," he says. "I believe it's a half-way house." These wider groupings have the advantages of consisting of larger populations and can act as a proof of concept.

"Finally we have the proof in the pudding — genetic variety in dietary advice is relevant."

Results are emerging that support the notion of these clusters. Kenneth Kornman is founder of InterLeukin Genetics (ILG), a Massachusetts-based company developing tests for genes that affect food metabolism based on single nucleotide polymorphisms (SNPs). Kornman recently reanalysed a 2007 study by Christopher Gardner and colleagues at Stanford University. In Gardner's study, 311 women were randomized to four different diets, which varied in the content of carbohydrates. After 12 months, women on the low-carb, high-protein Atkins diet had lost the most weight.

Kornman's reanalysis involved placing 101 of the women (those available for the follow-up study) into one of three groups categorized by three SNPs related to the metabolism of dietary fats and carbohydrates. Women in the 'fat-sensitive' group shared a SNP that meant they gained more weight from a high-fat diet than did women in the 'carbohydrate-sensitive' group, and vice versa. The third group was sensitive to neither fat or carbohydrate. "Our company screened the published evidence on more than 200 SNPs and determined that these three were the only ones that met our criteria," says Kornman. The criteria were that each SNP should have at least three validating clinical studies, should be functional (directly linked to biological or clinical effects) and linked to body weight.

Kornman found that women on a diet that matched their genotype lost two-to-three times more weight than those on an unmatched diet. The study, sponsored by ILG, was presented at the 2010 Joint Conference of the American Heart Association in San Francisco. "The scientists in the audience were shocked," recalls Ben van Ommen, director at the Netherlands Organisation for Applied Scientific Research and NuGO, who had invited Kornman to speak. Kornman, he says, "has been scrutinized by the audience and he's survived. Finally we have the proof in the pudding — genetic variety in dietary advice is relevant".

FOOD TRIBES

Moving towards the more personalized end of the nutrition spectrum will require millions more data points from many diverse groups. One way to collect information from disparate populations is to use crowd-sourcing technologies. Many people who have discovered some or all of their genetic information are sharing or offering it for analysis using websites such as SNPedia, DIYgenomics and Harvard Medical School's Personal Genome Project. As genome testing becomes cheaper, more data will become available to use in this way.

Founders of personal genome information-sharing websites, such as DIYGenomics' Melanie Swan, say they can facilitate this data-gathering process by offering a new way to conduct science that appeals to the subjects. "We aim to give individuals the opportunity to participate in citizen science research studies," says Swan. "The whole point is to experiment and find out what works best for you."

A typical experiment might investigate vitamin supplements and their precursors. Participants would consent to taking regular supplements, pay for their own genetic sequencing test, submit regular tests to an approved laboratory, and upload results to the website. Combining data from all participants paints a picture of the relationship between certain genes and the impact of a vitamin or vitamin precursor on health. DIYGenomics' first study — submitted to a peer-review journal — is a proof-of-concept, extending existing research on gene mutations and vitamin B deficiency. Another study on ageing is designed and set to recruit participants.

This new approach to research blurs the distinction between study organizer and participant. "We all design the study and we all participate. We have our own consenting process too," says Swan, adding that she sees a 'citizen ethicist' version of the Hippocratic oath evolving to accommodate new ways of conducting research.

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Some people see personal genomics as a logical follow-on to social networking and a valuable asset. "There is definitely potential in a citizen science approach," says Marina Levina, a communication researcher at The University of Memphis. Levina, however, adds a few caveats. "Citizen science implies that conventional science has failed us in some ways, whereas I would argue that guidelines and restrictions that perhaps slow down conventional science are there because of valid ethical issues."

There are other potential pitfalls. Genetic testing companies that provide genome-sharing websites have been criticized for offering inconsistent results and flimsy diagnoses regarding genetic propensity to disease. There are signs that the US Food and Drug Administration is moving to clip their wings, perhaps by enforcing tougher regulation. This echoes ongoing changes to regulation of the nutritional supplements industry in the United States and Europe, which is to be treated more like the pharmaceutical industry.

Genes are not the only important considerations when developing tailored nutritional advice. The nascent science of epigenetics, which describes how and when genes are turned on and off in the body, promises to both complicate and frustrate the road to personalized nutrition.

ILG's Kornman says epigenetics is the elephant in the room when it comes to determining optimal diet: "There is growing evidence that prenatal nutrition and environmental effects have a life long and maybe multi-generational effect in terms of fetal development and early childhood nutrition." Even if we can decode the genetic recipe of the diet–health relationship, without a greater knowledge of the epigenetic modifications put in place early in life — or in a mother's or perhaps grandmother's life — this recipe still might not taste right.

What's more, can we ever over-ride our love for sweet, fatty and salty food? "People are perverse about dietary choice," says Tom Sanders, head of nutrition and dietetics at King's College London. "They tend to offset what they perceive as good food with bad food." Put another way, we are bad at eating good food, and good at eating bad food.

Nutrigenomics may well change our definition of health and disease; blur the distinction between food and drugs; between experimenter and experimentee; and demonstrate new models of the scientific method driven by food tribes, citizen scientists and online social networks. The paradox is that as our lifestyles become ever more individualized, it could be the crowd that delivers the best advice for healthy eating. ■

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LETTER

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Chronic high-fat diet in fathers programs β-cell dysfunction in female rat offspring

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The global prevalence of obesity is increasing across most ages in both sexes. This is contributing to the early emergence of type 2 diabetes and its related epidemic[1,2]. Having either parent obese is an independent risk factor for childhood obesity[3]. Although the detrimental impacts of diet-induced maternal obesity on adiposity and metabolism in offspring are well established[4], the extent of any contribution of obese fathers is unclear, particularly the role of non-genetic factors in the causal pathway. Here we show that paternal high-fat-diet (HFD) exposure programs β-cell 'dysfunction' in rat F_1 female offspring. Chronic HFD consumption in Sprague–Dawley fathers induced increased body weight, adiposity, impaired glucose tolerance and insulin sensitivity. Relative to controls, their female offspring had an early onset of impaired insulin secretion and glucose tolerance that worsened with time, and normal adiposity. Paternal HFD altered the expression of 642 pancreatic islet genes in adult female offspring ($P < 0.01$); genes belonged to 13 functional clusters, including cation and ATP binding, cytoskeleton and intracellular transport. Broader pathway analysis of 2,492 genes differentially expressed ($P < 0.05$) demonstrated involvement of calcium-, MAPK- and Wnt-signalling pathways, apoptosis and the cell cycle. Hypomethylation of the *Il13ra2* gene, which showed the highest fold difference in expression (1.76-fold increase), was demonstrated. This is the first report in mammals of non-genetic, intergenerational transmission of metabolic sequelae of a HFD from father to offspring.

Increasing evidence indicates an important biological role of fathers in obesity and metabolic programming of their offspring[5,6]. Most human obesity seems to be related to complex gene–environment interactions[7]. Although some alleles associated with obesity are inherited solely from the father[8,9], parental environmental exposures can also affect offspring phenotype[10], with the potential to contribute to the rapid increase in obesity. Susceptibility of the metabolic phenotype to environmentally initiated change also extends into early life through developmental plasticity[10]. In humans, it is difficult to separate the effects of paternal genetic makeup from those of the father's environmental exposures on offspring, including variations in paternal nutrition, metabolic and hormonal status, or obesity itself[11]. In mice, however, males whose mothers consumed a HFD were heavier, diabetic and insulin resistant, and produced second-generation offspring who were insulin resistant, although not obese[12]. Whether this is a consequence of paternal *in utero* exposure or their adult sequelae of obesity and diabetes is unclear. In mice, a HFD also alters testicular gene expression[13]. Obesity affects sperm concentration, motility and morphology, and increases sperm DNA damage in humans[14]. Collectively, this indicates that fathers can initiate intergenerational transmission of obesity/metabolic diseases, induced indirectly or directly, such as through exposure to a HFD.

To test this hypothesis we mated male Sprague–Dawley founder rats fed either a HFD or a control diet (Table 1), with females consuming a control diet (Supplementary Table 1). As expected, HFD males had increased body weight, energy intake, adiposity and plasma leptin and liver mass (Fig. 1a–c and Table 1), but reduced skeletal muscle mass relative to body weight ($P = 0.017$). The HFD males were also glucose

Table 1 | Hormonal and metabolic parameters and pancreas morphology

Group and parameter	Control	HFD	*P* value
Fathers	$n = 8$	$n = 9$	
Body weight (g)	550 ± 13	705 ± 17	<0.0005
Length (cm)	26.8 ± 0.3	27.8 ± 0.3	0.017
Liver (g)	15.16 ± 0.43	19.51 ± 1.23	0.006
BAT (mg)	0.462 ± 0.026	0.779 ± 0.100	0.013
Mesenteric WAT (g)	4.76 ± 0.35	12.43 ± 1.23	<0.0005
Retroperitoneal WAT (g)	8.85 ± 0.60	30.85 ± 3.09	<0.0005
Gonadal WAT (g)	7.56 ± 0.32	20.83 ± 1.10	<0.0005
Sum of WAT (g)	21.17 ± 0.75	64.10 ± 4.84	<0.0005
Leptin (ng ml^{-1})	2.70 ± 0.30	13.26 ± 1.99	<0.0005
Glucose (mM)	4.71 ± 0.08	5.43 ± 0.16	0.002
Insulin (ng ml^{-1})	0.18 ± 0.02	0.47 ± 0.07	0.002
HOMA-IR	0.88 ± 0.11	2.29 ± 0.18	<0.0005
Female offspring	$n = 8$	$n = 9$	
Body weight (g)	253 ± 8	260 ± 5	0.92
Length (cm)	22.6 ± 0.2	22.3 ± 0.1	0.28
Liver (g)	7.15 ± 0.19	7.34 ± 0.16	0.46
BAT (mg)	0.19 ± 0.02	0.21 ± 0.01	0.43
Selected skeletal muscle mass (mg)	0.78 ± 0.02	0.77 ± 0.03	0.66
Mesenteric WAT (g)	2.14 ± 0.16	2.28 ± 0.20	0.59
Retroperitoneal WAT (g)	2.50 ± 0.37	2.81 ± 0.36	0.55
Gonadal WAT (g)	2.58 ± 0.10	2.80 ± 0.48	0.67
Sum of WAT (g)	6.76 ± 0.32	7.87 ± 0.89	0.28
Leptin (ng ml^{-1})	0.89 ± 0.09	1.06 ± 0.16	0.38
Triglyceride (mM)	0.92 ± 0.14	0.78 ± 0.12	0.46
NEFA (mEq l^{-1})	2.22 ± 0.12	2.59 ± 0.33	0.32
Pancreas morphology	$n = 7$	$n = 7$	
Total islet area (percentage pancreas area)	1.17 ± 0.09	0.90 ± 0.08	0.040
Per cent small islet (0–5,000 μm^2)	71.81 ± 0.82	76.08 ± 1.58	0.034
Per cent medium islet (5,001–10,000 μm^2)	10.04 ± 0.98	9.08 ± 0.79	0.46
Per cent large islet (>10,000 μm^2)	18.14 ± 0.48	14.84 ± 1.26	0.031
Total no. islet per mm^2 pancreas	1.56 ± 0.11	1.51 ± 0.13	0.80
No. small islet per mm^2 pancreas	1.12 ± 0.08	1.15 ± 0.11	0.80
No. medium islet per mm^2 pancreas	0.16 ± 0.02	0.14 ± 0.01	0.35
No. large islet per mm^2 pancreas	0.28 ± 0.02	0.23 ± 0.02	0.13
Total β-cell area (percentage pancreas area)	0.72 ± 0.06	0.58 ± 0.05	0.09

HFD, high fat diet. BW, body weight. BAT, brown adipose tissue. WAT, white adipose tissue. Sum of WAT, sum of mesenteric, retroperitoneal and gonadal WAT. Selected skeletal muscle mass, sum of anterior tibialis, extensor digital longus and soleus. HOMA-IR, homeostasis model assessment = fasting insulin (ng ml^{-1}) × fasting glucose (mM) / 22.5 × 0.0417. All results are expressed as mean ± s.e.m.

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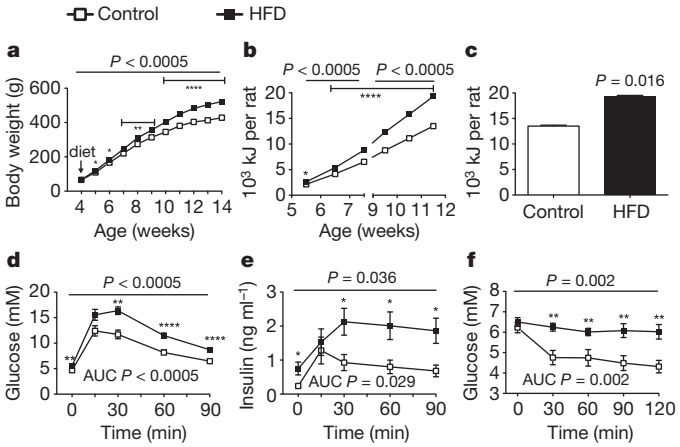

Figure 1 | HFD leads to adiposity, glucose intolerance and insulin resistance in fathers. **a**, Body-weight trajectories (control, HFD: $n = 8$ and 9, respectively). **b**, Cumulative energy intake ($n = 4$ and 5, respectively). **c**, Total energy intake ($n = 4$ and 5, respectively). **d**, Blood glucose during glucose tolerance test ($n = 8$ and 9, respectively). **e**, Plasma insulin during glucose tolerance test ($n = 7$ and 9, respectively). **f**, Blood glucose during insulin tolerance test (1 U kg^{-1}) ($n = 7$ and 9, respectively). Data are expressed as mean ± s.e.m. $*P < 0.05$, $**P < 0.01$, $****P < 0.0005$, versus control. P values for significant differences between male founder groups in repeated-measure analysis are shown at top of panel.

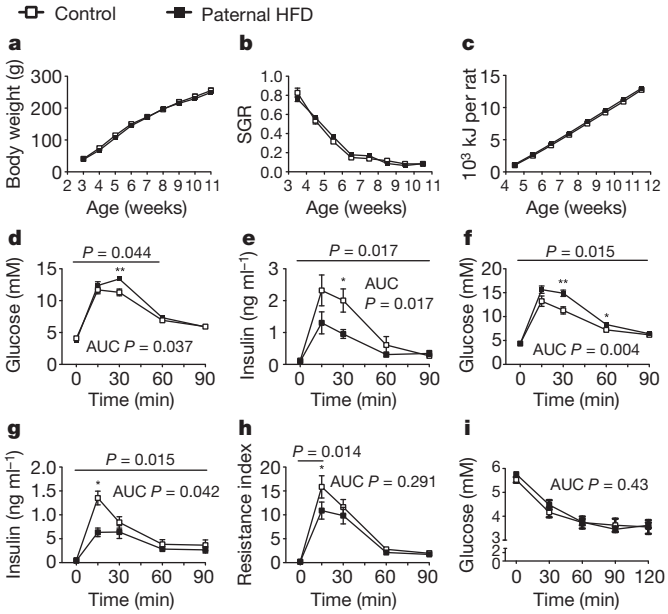

Figure 2 | Female offspring demonstrate impaired glucose tolerance and insulin secretion to a glucose challenge. **a**, Body weight (control, HFD: $n = 8$ and 9, respectively). **b**, Specific growth rate (SGR, change in body weight/body weight; $n = 8$ and 9, respectively). **c**, Cumulative energy intake ($n = 9$ and 7, respectively). **d**–**g** Blood glucose (**d**) and plasma insulin (**e**) during a glucose tolerance test at 6 weeks ($n = 8$ and 8, respectively) and 12 weeks (**f**, **g**) ($n = 5$ and 7, respectively). **h**, Insulin resistance index (glucose (mM) × insulin (ng ml^{-1}) × 0.0417/22.5) at 12 weeks. **i**, Blood glucose during an insulin tolerance test (0.5 U kg^{-1}) at 11 weeks ($n = 8$ and 9, respectively). Data are mean ± s.e.m. $*P < 0.05$, $**P < 0.01$, versus control. Significant differences between groups shown at top of panel.

intolerant and insulin resistant, showing raised blood glucose and plasma insulin at fasting and during a glucose tolerance test (Fig. 1d, e). The homeostasis model assessment of insulin resistance index (HOMA-IR; Table 1) was increased and the insulin-tolerance-test response blunted (Fig. 1f). Paternal HFD did not alter litter size or sex ratios.

In humans, paternal obesity is associated with low birth weight in offspring[6]. Here, day-1 body weight of female offspring of HFD fathers tended to be reduced (6.61 ± 0.15 versus 7.08 ± 0.26 g in controls; $n = 9$ and 8, respectively; $P = 0.07$); males (7.40 ± 0.21 versus 7.30 ± 0.20 g in controls; $n = 9$ and 8, respectively; $P = 0.74$). In girls, adiposity[15] and insulin resistance[16] closely resembled that of their obese fathers. As a pilot study identified significant impairment of glucose tolerance in female but not male offspring (S.F.N. and M.J.M., unpublished data), we further assessed females after weaning onto a control diet. A paternal HFD did not alter body weight, specific growth rate, energy intake (Fig. 2a–c) or energy efficiency (not shown) in female offspring.

In humans, paternal adiposity predicted that of their pre-menarcheal daughters[15]. Here, paternal HFD did not alter adiposity, muscle mass, fasting plasma leptin, triglyceride or non-esterified fatty acid (NEFA) concentrations in adult female offspring (Table 1). Either obesity may emerge later or it may not progress through the paternal lineage in rodents, as reported for those with undernourished[17] and HFD-fed[12] grandmothers.

Next we assessed glucose tolerance and its two key determinants, insulin secretion and sensitivity, in the female rat offspring. A paternal HFD did not alter fasting blood glucose (Fig. 2d, f) or plasma insulin (Fig. 2e, g) in female offspring, but increased the blood glucose rise (peak 13.6 ± 0.3 versus 12.3 ± 0.4 mM; $P = 0.043$) and reduced insulin secretion (peak 1.4 ± 0.3 versus 2.7 ± 0.4 ng ml^{-1}; $P = 0.016$) during a glucose tolerance test at 6 weeks (Fig. 2d, e). A similar pattern was observed at 12 weeks (Fig. 2f, g), but with a further impairment of glucose tolerance evidenced by a larger glucose peak (+10% to +23% versus control) and increased the area under the glucose curve during the glucose tolerance test, $AUC_{glucose}$ (+9% to +19%) in paternal HFD offspring. Insulin secretion during the first 30 min after glucose (insulinogenic index[18], $AUC_{insulin(0–30\,min)}/AUC_{glucose(0–30\,min)}$) was halved in offspring of HFD fathers (38.7 ± 5.8 versus 86.8 ± 7.3 ng mmol^{-1}; $P = 0.004$); but their insulin resistance index and response during the insulin tolerance test were unaltered (Fig. 2h, i).

We then examined islet and β-cell abundance, and performed genome-wide microarray analysis of isolated islets to explore the mechanisms of impaired insulin secretion. A paternal HFD reduced relative islet area (−23%; $P = 0.04$), mainly owing to reduced large islets (−18%; $P = 0.031$) and tended to reduce β-cell area ($P = 0.09$; Table 1) in offspring, implying impaired β-cell replication. We also observed an increase in small islets (+6%; $P = 0.034$; Table 1) in the offspring of HFD fathers, indicating a compensatory response to maintain normal β-cell mass. We propose that limited β-cell reserve in the female offspring of HFD fathers is sufficient to maintain normal fasting glucose and insulin levels, but inadequate to preserve glucose-stimulated insulin secretion and glucose tolerance.

A paternal HFD altered the expression of 77 genes (21 upregulated, 56 downregulated, $P < 0.001$; Supplementary Table 2) in adult female offspring; 642 genes at $P < 0.01$ had enriched gene ontology terms belonging to regulatory pathways associated with insulin and glucose metabolism, that is, cation and ATP binding, cytostructure and intracellular transport (Supplementary Fig. 1). Broader Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of 2,492 genes ($P < 0.05$) revealed the involvement of calcium-, MAPK- and Wnt-signalling, apoptosis and the cell cycle (Table 2). Molecular networks were also identified, including direct interactions between members of Jak–Stat and MAPK signalling (Supplementary Figs 2 and 3 and Supplementary Table 3) and other functionally enriched pathways (Supplementary Table 3). Overall, these molecular findings are consistent with the alterations in pancreas morphology and indicate impaired insulin-granule exocytosis[19,20]. The greatest fold difference in gene expression was observed in *Il13ra2*, part of the Jak–Stat signalling pathway (Table 2). *Il13ra2* is expressed in and modulates growth and invasion of various pancreatic cancer cell lines[21] and is upregulated by TNF-α (*Tnf*)[22]. Quantitative polymerase chain reaction with

Table 2 | Differentially expressed islet genes ($P < 0.05$) of female offspring in functionally enriched pathways

Gene symbol	RefSeq	Probe-set ID	Mean		HFD versus control	
			HFD* ($n = 5$)	Control* ($n = 6$)	Fold change†	*P* value
Ca signalling (KEGG 04020)						
Adrb1 (d)	NM_012701	10716262	5.51	5.70	−1.14	0.035
Chrm1 (d)	NM_080773	10713581	6.01	6.17	−1.12	0.041
Htr6 (d)	NM_024365	10882383	5.50	5.69	−1.14	0.014
Htr7 (d)	NM_022938	10729825	5.12	5.37	−1.19	0.004
Adcy2 (d)	NM_031007	10793338	5.58	5.71	−1.09	0.024
Cacna1e (d)	NM_019294	10768765	4.85	5.01	−1.11	0.026
Ryr1 (d)	ENSRNOT00000027893	10720308	5.65	5.78	−1.10	0.020
Camk2a (d)	NM_012920	10802026	5.47	5.58	−1.07	0.036
Pde1b (d)	NM_022710	10899676	5.88	6.13	−1.19	0.039
Sphk1 (d)	NM_133386	10739796	5.38	5.59	−1.15	0.022
Grin1 (u)	NM_017010	10843400	7.16	6.92	1.18	0.006
Vdac3 (u)	NM_031355	10789127	10.66	10.52	1.11	0.010
Phkg2 (u)	NM_080584	10711127	8.15	8.06	1.07	0.019
Pde1c (u)	NM_031078	10862700	7.00	6.68	1.26	0.007
MAPK signalling (KEGG 04010)						
Cacna1e (d)	NM_019294	10768765	4.85	5.01	−1.11	0.026
Cacna2d3 (d)	NM_175595	10789819	5.14	5.26	−1.09	0.032
Cacna2d4 (d)	ENSRNOT00000010746	10858218	5.19	5.32	−1.09	0.014
Tnf (d)	NM_012675	10828021	5.21	5.50	−1.22	0.019
Fos (d)	NM_022197	10886031	7.91	8.82	−1.87	0.014
Ptpn7 (d)	NM_145683	10764196	5.28	5.44	−1.12	0.004
Map3k4 (d)	NM_001107456	10717995	6.24	6.53	−1.22	0.005
Rap1a (u)	NM_001005765	10825727	8.81	8.69	1.09	0.046
Rasa1 (u)	NM_013135	10820245	9.33	9.25	1.06	0.016
Map2k4 (u)	NM_001030023	10743668	8.35	8.22	1.09	0.002
Crk (u)	NM_019302	10736033	9.25	9.19	1.04	0.047
Casp3 (u)	NM_012922	10791652	8.60	8.53	1.05	0.050
Daxx (u)	NM_080891	10831792	7.14	7.00	1.10	0.031
Il1rl1 (u)	NM_001127689	10922857	5.60	4.79	1.75	0.025
Mknk2 (u)	NM_001011985	10893744	7.72	7.55	1.13	0.050
Wnt signalling (KEGG 04310)						
Wnt9a (d)	NM_001105783	10733966	5.83	6.03	−1.15	0.003
Wnt9b (d)	NM_001107055	10748113	6.19	6.39	−1.15	0.001
Fzd9 (d)	NM_153305	10757698	6.02	6.15	−1.09	0.014
Ctnnb1 (u)	NM_053357	10914371	10.07	9.96	1.08	0.049
Ppp2r5a (u)	NM_001107891	10770721	9.52	9.36	1.12	0.036
Skp1 (u)	NM_001007608	10733430	10.81	10.64	1.12	0.024
Cul1 (u)	NM_001108627	10855163	10.03	9.96	1.05	0.041
Rock1 (u)	NM_031098	10803158	9.29	9.20	1.06	0.016
Apoptosis (KEGG 04210)						
Tnf (d)	NM_012675	10828021	5.21	5.50	−1.22	0.019
Bcl2l1 (d)	NM_031535	10850826	7.01	7.07	−1.04	0.045
Il1rl1 (u)	NM_001127689	10922857	5.60	4.79	1.75	0.025
Prkar2a (u)	NM_019264	10913228	8.64	8.51	1.09	0.046
Casp3 (u)	NM_012922	10791652	8.60	8.53	1.05	0.050
Xiap (u)	AF304334	10921195	9.98	9.85	1.10	0.020
Aifm1 (u)	NM_031356	10939595	7.98	7.83	1.11	0.029
Cell cycle (KEGG 04110)						
Orc1l (d)	NM_177931	10870791	4.47	4.72	−1.19	0.015
Smc1b (d)	NM_001130498	10905944	3.77	4.00	−1.18	0.032
Skp1 (u)	NM_001007608	10733430	10.81	10.64	1.12	0.024
Cul1 (u)	NM_001108627	10855163	10.03	9.96	1.05	0.041
Prkdc (u)	NM_001108327	10755897	8.37	8.29	1.05	0.025
Stag1 (u)	NM_001108179	10912525	8.06	7.89	1.12	0.003
E2f3 (u)	NM_001137626	10798213	5.85	5.59	1.20	0.017
Jak–Stat signalling (KEGG 04630)						
Mpl (d)	ENSRNOT00000042602	10879267	4.65	4.99	−1.27	0.001
Stat1 (d)	ENSRNOT00000052121	10927873	7.18	7.43	−1.19	0.006
Ifnb1 (d)	NM_019127	10877952	4.10	4.31	−1.16	0.012
Jak3 (d)	NM_012855	10787364	6.56	6.77	−1.15	0.035
Il9 (d)	NM_001105747	10793945	5.73	5.92	−1.14	0.022
Ifna1 (d)	NM_001014786	10877972	5.56	5.73	−1.13	0.013
Ifna1 (d)	NM_001014786	10877968	5.37	5.52	−1.11	0.032
Socs3 (d)	NM_053565	10749372	6.08	6.24	−1.11	0.033
Il23a (d)	NM_130410	10899749	6.11	6.22	−1.08	0.012
Bcl2l1 (d)	NM_031535	10850826	7.01	7.07	−1.04	0.045
Il13ra2 (u)	NM_133538	10937279	3.77	2.95	1.76	0.018
Il13ra2 (u)	NM_133538	10937292	3.97	3.16	1.75	0.033

U, upregulated gene; d, downregulated gene.
* Values represent fluorescent intensity of probe-set and are presented in log 2 space.
† Fold change is gene expression in offspring of HFD father relative to control.

reverse transcription (RT–PCR) confirmed upregulation of messenger RNA expression ($n = 5$ per group) of *Il13ra2* (+6.3; $P < 0.05$) and *Ikbke* (+2.9; $P < 0.01$) and a decrease in *Fos* (−4.0; $P < 0.05$) in the islets of offspring of HFD fathers. To determine if epigenetic mechanisms could contribute to the altered *Il13ra2* expression, we performed bisulphite sequencing of a region proximal to the transcription start site.

Methylation at cytosine −960 of *Il13ra2* was reduced in HFD offspring (8.9 ± 2.2%) compared to controls (33.6 ± 4.0%, $P < 0.001$). Cytosine −960 was found to be located in a putative binding site for the T-cell factor-1A and NF-X, the latter being a methylated DNA-binding protein[23]. This epigenetic modification of *Il13ra2*, a gene that is part of key molecular networks (Table 2, Supplementary Table 3 and Supplementary Fig. 4), indicates that a paternal HFD alters offspring islet function, in part by affecting the epigenome of offspring.

In humans, paternal insulin resistance/diabetes is inversely associated with offspring birthweight[24,25] and increases subsequent risk of diabetes[24]. Although genetic factors may contribute[26], our findings show that paternal exposure to a HFD can induce a similar phenotype in offspring, identifying an additional and influential pathway. Notably, the impaired glucose tolerance and insulin secretion, in the absence of obesity, in these female offspring indicate that a paternal HFD acts to particularly target the endocrine pancreas and β-cells early in offspring. Whether similar defects emerge in male offspring remains to be determined.

Paternal lifestyle and particular environmental factors can affect spermatogenesis at the level of germ and Sertoli cells[27] and the composition of seminal fluid[28]. Increased testicular temperature resulting from fat accumulation and increased dietary fat and by-products of cell metabolism can be directly genotoxic to germ cells within the mature testis, leading to increased DNA damage through oxidative injury[29]. Furthermore, hyperleptinaemia, hyperinsulinaemia and the relative hypogonadotrophic hypogonadism in obese males may consequently affect spermatogenesis[30]. A HFD may also interfere with Sertoli-cell proliferation, and the integrity of the blood–testis barrier, thus affecting DNA reprogramming of the gamete[29]. A critical unanswered question, given the rising obesity epidemic in children, is whether early onset and prolonged HFD exposure may also affect gametogenesis and thereby offspring.

To our knowledge, this is the first direct demonstration in any species that a paternal environmental exposure, HFD consumption, can induce intergenerational transmission of impaired glucose–insulin homeostasis in their female offspring. The underlying mechanisms seem to include epigenetic modifications, the functional implications of which remain to be elucidated. These findings extend the concept of developmental and adaptive plasticity to include a paternal role in the early life origins of disease and amplification of the diabetes epidemic.

METHODS SUMMARY

Animal experiments. Litters from eight control and nine HFD fathers were included; one animal per litter was used for each test. Experimental protocols were approved by the Animal Care and Ethics Committee, University of New South Wales. **Microarray gene expression analysis.** Total islet mRNA was extracted using miRNeasy Mini kits (Qiagen). Samples from six control and five HFD offspring, each from different fathers, with RNA integrity number (RIN, Agilent) ≥7.5 were selected for transcriptomics using Affymetrix GeneChip Rat Gene ST 1.0 arrays. **Statistical analyses.** Phenotype data were analysed using SPSS 16.0 after log transformation or square-root transformation unless raw data were normally distributed. Single time measurements were analysed by two-tailed Student's *t*-test or Mann–Whitney *U* test, and time-courses were analysed by repeated-measures ANOVA.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions S.F.N. and M.J.M. designed the study. S.F.N. performed animal work, histology, islet harvest and RNA extraction, data analysis and wrote the manuscript. M.J.M. supervised the project and wrote the manuscript. R.C.Y.L. conducted microarray data analysis. D.R.L. assisted with islet harvest. R.B. conducted bisulphite sequencing and DNA methylation analysis. J.A.O. conducted ingenuity analysis and wrote the manuscript. All authors contributed to data interpretation, reviewed the manuscript and approved the final version.

Author Information Gene expression data have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus (GEO; http://www.ncbi.nim.nih.gov/geo) and are accessible using GEO series accession number GSE19877. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of this article at www.nature.com/nature. Correspondence and requests for materials should be addressed to M.J.M. (m.morris@unsw.edu.au).

METHODS

Animal care. Sprague–Dawley rats from the Animal Resource Centre were housed at 22 ± 2 °C, on a 12:12 h light:dark cycle. Male F_0 founders were assigned to a HFD (SF01-025, SF03-020; 40.7%, 43% energy as fat; Speciality Feeds) or control (Gordon's Stockfeeds) diet at 4 weeks of age. At 13 weeks, HFD males were 22% heavier (522 ± 11 versus 428 ± 13 g, $P = 0.008$); mating with females consuming control diet commenced at 14 weeks. During mating, one male and one female were housed together, with free access to control diet from 0800–1800 h, for 8 consecutive days. Males returned to their cages overnight to continue their assigned diets, whereas females consumed control diet throughout mating, gestation and lactation. Male and female founders were killed in the fasting state shortly after litters were harvested. Females mated with the two paternal groups did not differ in body weight, adiposity, fasting blood glucose, insulin and HOMA-IR (Supplementary Table 1). Only litter sizes between 9–16 were included and litters were standardized to 12 pups at day 1 within father groups, to control for intrauterine and postnatal nutrition. Phenotypic data (body weight, specific growth rate, glucose tolerance, insulin tolerance, post mortem) from one offspring per father, chosen at random, were generated. At week 13, animals were killed and islets were generated from 5 HFD and 6 control offspring, each from a different father. Littermates were killed for pancreas histology and post-mortem analysis at week 14.

Body weight and energy intake were monitored weekly, the latter by collecting and weighing food remaining after 24 h. Energy intake was averaged across animals housed with 3–4 per cage to reduce stress. Specific growth rate (SGR; weight gain between two time points divided by previous body weight[31]) and energy efficiency (weight gain divided by energy intake between the two time points) were calculated.

Blood glucose (Accu check Advantage glucometer; Roche), plasma leptin and insulin (Linco radioimmunoassay), plasma NEFA (Wako) and triglyceride (Roche colorimetric enzymatic assay) were determined.

Glucose and insulin tolerance tests. Glucose tolerance test was performed after a 15-h overnight fast and insulin tolerance test was performed 2 h after food removal. Glucose (2 g kg^{-1} body weight) and insulin (Actrapid, Novo Nordisk; 1 U kg^{-1} for fathers and 0.5 U kg^{-1} for offspring based on their predicted insulin resistance) were administered intraperitoneally. Separate cohorts of littermates underwent a glucose tolerance test for blood glucose and plasma insulin measures at 6 weeks of age, to reduce stress associated with blood sampling.

Immunohistochemistry and morphometric analysis. Three fixed pancreas sections (5 μm) per animal per test, 200 μm apart, were stained with polyclonal guinea-pig anti-swine insulin primary antibody followed by goat anti-rabbit immunoglobulin secondary antibody (DAKO). Adjacent sections were stained with haematoxylin and eosin. All slides were scanned using Aperio ScanScope XT Slide Scanner. Pancreas, islet and β-cell areas were determined using ImageJ 1.40 software (http://rsb.info.nih.gov/ij/). Islets were classified into small (1–5,000 μm^2), medium (5,001–10,000 μm^2) and large (>10,000 μm^2), respectively[32].

Islet isolation. Islets were harvested by standard techniques with cannulation of the pancreatic duct of anaesthetized rats[33–35] after an overnight fast.

Islets transcriptomics. Affymetrix probe-set data were normalized using the robust multi-array average (RMA) method[36], which can yield attenuated estimates of differential expression for genes at low expression levels, albeit with high precision. Gene expression levels were compared using one-way ANOVA. This yielded 77, 642 and 2,492 differentially expressed genes at unadjusted $P < 0.001$, $P < 0.01$ and $P < 0.05$ levels, respectively. Differentially expressed genes ($P < 0.01$) were functionally annotated according to gene ontology terms and enriched terms were calculated using DAVID[37,38] (Supplementary Fig. 1). In addition, we hierarchically clustered[39] differentially expressed genes based on Euclidean distance to look for possible co-regulated pathways affecting islet metabolism. We also mapped differentially expressed genes at $P < 0.05$ to KEGG[40].

Quantitative RT–PCR. Total islet RNA (one offspring per father; $n = 5$, HFD; $n = 5$, control), extracted using miRNeasy Mini kit (Qiagen), was used as a template for complementary DNA synthesis, using SuperScript III first strand synthesis (Invitrogen) with random hexamers. mRNA expression was determined using quantitative RT–PCR (Stratagene Mx3000P, Agilent) using primer sequences summarized in Supplementary Table 4 and Platinum SYBR Green SuperMix UDG (Invitrogen), normalized against β actin.

Molecular network generation using Ingenuity pathways analysis. Networks were generated through Ingenuity pathways analysis (Ingenuity Systems, http://www.ingenuity.com). Briefly, differentially expressed genes at $P < 0.01$ or $P < 0.05$ and corresponding fold changes were used; the number of networks and eligible molecules per network is limited to 25 and 35, respectively. Networks were algorithmically generated based on their connectivity and ranked by score (negative exponent of the right-tailed Fisher's exact test result). Molecules are represented as nodes, and the biological relationship between two nodes as an edge (line). Nodes are displayed using various shapes that represent the functional class of the gene product, whereas edges describe the nature of the relationship between the nodes, as defined in Ingenuity Systems.

DNA methylation analysis by bisulphite sequencing. Bisulphite treatment was performed as described[41]. One microgram of NaOH-denaturated DNA was embedded in 2% low-melting-point agarose solution; bisulphite solution (Sigma) was added, followed by 4 h of incubation at 50 °C under light exclusion. Treatment was terminated by equilibration against Tris-EDTA and 0.2 M NaOH, DNA was washed with distilled H_2O. *Il13ra2*, forward primer TAAATTAAAA TTTTAAAAATTGAAAAGTAT, reverse primer AAATAAAAAAAACTCATA AAATCAAC. The obtained PCR fragments were purified using MinElute Gel Extraction Kit (Qiagen) and cloned into PCR-TOPO vector using TOPO TA Cloning kit (Invitrogen). Individual clones were grown and plasmids purified using PureLink Miniprep kit (Invitrogen). For each animal eight to nine clones were sequenced using T7 promoter primer on an ABI 3730xl DNA Analyser platform at the Ramaciotti Center. Results were analysed using Methtools 2.0[42].

Statistical analysis. Results are expressed as mean ± s.e.m. $P < 0.05$ was considered statistically significant.

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ORIGINAL ARTICLE

Dairy intake associates with the *IGF* rs680 polymorphism to height variation in periadolescent children

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Background/Objectives: Height is a classic polygenic trait, with a number of genes underlying its variation. We evaluated the prospect of gene-to-diet interactions in a children's cohort, for the insulin-like growth factor II (*IGF*) *rs680* polymorphism and height variation.
Subjects/Methods: We screened 795 periadolescent children (424 girls) aged 10–11 years old from the Gene and Diet Attica Investigation (GENDAI) pediatric cohort for the *IGF* rs680 polymorphism (rs680).
Results: Children homozygous for the common allele (GG) were taller (148.9 ± 7.9 cm) compared with those with the A allele (148.1 ± 7.9 cm), after adjusting for age, sex and dairy intake ($\beta \pm$ s.e.: 2.1 ± 0.95, $P = 0.026$). A trend for rs680 × dairy intake interaction was also revealed ($P = 0.09$). Stratification by *IGF* rs680 genotype revealed positive significant ($P = 0.014$) association between dairy product intake and height in A-allele children adjusted for the same confounders. A daily increase of four dairy servings was associated with a 0.4 cm increase in height. On grouping dairy intake into low (1.9 ± 0.7 servings per day) and high dairy product consumption (4.4 ± 1.5 servings per day), children with the A allele who were high dairy product consumers were taller compared with the low dairy product consumers (148.8 ± 7.9 vs 147.4 ± 7.7 cm, respectively, $P = 0.05$).
Conclusions: A higher consumption of dairy products is associated with increased height depending on the rs680 IGF2 genotype.
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Keywords: height; polymorphism; *IGF*; children; diet; dairy products

Introduction

Adult height is one of the classical complex human traits reflecting the combined influence of multiple genetic factors, with up to 90% of the variation within a population being explained by genetic variation (Silventoinen *et al.*, 2003; Mullis 2005; Macgregor *et al.*, 2006; Perola *et al.*, 2007). At the same time, as evidenced in industrialized societies over the previous century, environmental factors and in particular dietary habits are widely accepted to affect significantly a person's height (Ogden *et al.*, 2004; Rosenfeld, 2007).

Despite height's strong heritability, until very recently, there was limited success in identifying specific genetic variants in the general population. Rare cases associated with extreme stature could not explain the normal variation in the general population (Palmert and Hirschhorn, 2003). Recently, genome-wide association (GWA) studies have identified several loci that were strongly associated with variation with height (Perola *et al.*, 2007; Weedon *et al.*, 2007; Gudbjartsson *et al.*, 2008; Lettre *et al.*, 2008; Sanna *et al.*, 2008). Initially, common variants in the *HMGA2* oncogene on chromosome 12 were found to explain 0.3% of the population variation in height (Weedon *et al.*, 2007), whereas others (Perola *et al.*, 2007; Sanna *et al.*, 2008) reported evidence for major quantitative trait loci (QTLs) on 8q21.3 and common variants in the osteoarthritis-associated locus *GDF5 UQCC* on chromosome 20. More recently (Gudbjartsson *et al.*, 2008; Lettre *et al.*, 2008; Weedon *et al.*, 2008), new sequence variants at >30 loci were found

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to be associated significantly with adult human height. A large proportion of the loci contain genes that were previously known to be involved in growth and mitosis. This is coherent with previous studies and suggests that genetic differences in adult human height stem from variations in local regulation of the skeletal development, rather than systemic regulation.

The insulin-like growth factors (IGFs) are powerful mitogenic agents that stimulate cell differentiation and inhibit apoptosis (Walenkamp and Wit, 2007). *IGF2*, located on chromosome 11p15.5, also known as somatomedin A, is a single-chain polypeptide that shares an amino acid sequence homology of about 47% with insulin and 31% with relaxin. Collectively these molecules comprise the insulin family of polypeptide growth factors (O'Dell and Day, 1998). The *IGF2* gene is exclusively paternally expressed and maternally imprinted, and has a key role in fetal growth and development (Reik and Walter, 2001). In humans, *IGF2* underexpression caused by methylation defects results in the low birth weight Silver–Russell syndrome (Gicquel *et al*., 2005), and conversely, loss-of-imprinting leading to *IGF2* overexpression results in the Beckwith–Wiedemann somatic overgrowth syndrome (Engel *et al*., 2000). A single nucleotide polymorphism (SNP) in the 3 untranslated region of the *IGF2* gene (rs680) has been associated with body mass index (BMI) (O'Dell *et al*., 1997), including suggestive evidence from a genome scan for obesity (Rice *et al*., 2002), whereas others (Heude *et al*., 2007) were unable to replicate these results. GWA meta-analyses on height have not directly identified this locus as influencing the adult height or BMI. Nevertheless, it was recently reported (Perola *et al*., 2007) that glypican 3 (*GPC3*), a gene that causes the Simpson–Golabi–Behmel syndrome, a condition characterized by pre- and postnatal overgrowth, may interact with the *IGF2*, thus regulating growth.

With regard to dietary intake, increased milk consumption has been reported to raise IGF1 levels in pediatric cohorts, such as in adolescent girls (Cadogan *et al*., 1997) and 8-year-old boys (Hoppe *et al*., 2004). In this study, we investigated the prospect of gene-to-diet interactions in a children's cohort (Papoutsakis *et al*., 2007) for the *IGF2 rs680* polymorphism, revealing significant associations between dairy product intake and height.

Materials and methods

Subjects

The GENDAI target population comprised children attending the fifth and sixth grades and living in the Attica region of Greece (Papoutsakis *et al*., 2007). From November 2005 to June 2006, 1138 periadolescent children (53% girls; mean age: 11.2 ± 0.7 years) were recruited from randomly selected elementary schools of Attica. This research study was approved by the Institutional Review Board of Harokopio University, the Greek Ministry of Education and the Ethics Committee of Harokopio University. A written consent was obtained from the parents of the participants.

Dietary assessment

Dietary information was collected by two nonconsecutive 24-h recalls (Frank *et al*., 1977). The second dietary recall was always conducted on a different day of the week, allowing 3–10 days after the first recall to calculate the usual nutrient intake. The staff reviewed all 24-h recalls during regular weekly meetings to resolve issues on missing foods or unrealistic quantities reported. The 24-h recalls were analyzed using the Nutritionist Pro software, version 2.2 (Axxya Systems-Nutritionist Pro Inc, Stafford, TX, USA). The Nutritionist Pro food database was expanded by adding the analyses of traditional Greek foods and recipes (Kafatos *et al*., 2000), and nutrient information of locally processed food items as shared by the industry. The frequency of several food groups' consumption was also approximately quantified in terms of the number of servings per day. The food groups assessed were the core food groups of the traditional Greek diet (dairy products, fruits, vegetables, cereals, potatoes, legumes, red meat and products, poultry, fish and nuts), as well as other food groups that are prevalent in children's diet. Dairy products were the dietary predictor variable of interest. Dairy products were defined as the sum of mean daily servings of all types of milk, cheese and yoghurt.

Physical activity assessment

Coinciding with the dates of the dietary recalls, students completed a physical activity checklist recall twice (Sallis *et al*., 1996). This instrument inquired about the student's time spent on mild, moderate and strenuous exercise, and sedentary pursuits (such as time spent viewing TV or playing computer/video games) during the previous 24 h.

Demographic and clinical assessment

Socio-demographic characteristics of the child as well as his/her family, in terms of age, employment, income, years of education, body weight and height of parents and siblings of the participant, and medical history of the family, were also recorded. Arterial blood pressure was measured twice using an appropriate cuff size, with the subject in sitting position after at least 5 min rest. Sexual maturity status was assessed by a self-evaluation of the subject (Bonat *et al*., 2002) in the presence of the team's pediatrician according to Tanner's criteria for breast, pubic hair and genital development.

Anthropometry and body composition

Physical measurements of weight and height were obtained in light clothing without shoes, and BMI was computed as weight (kg)/height^2 (m^2). Children were classified as normal weight, overweight and obese according to the previously

proposed cut-off points for childhood overweight and obesity adopted by the International Obesity Task Force (IOTF) (Cole *et al*., 2000). In addition, central adiposity was assessed by measuring the waist and hip circumference and calculating the ratio (WHR). A set of Lange skinfold callipers (Cambridge Scientific Instruments, Cambridge, MA, USA) was applied to obtain two repeated measurements of triceps, and subscapular skinfolds for the right side of the body to a precision of 0.2 mm. The mean of the two measurements was calculated. The body fat percentage was estimated using the sex- and age-specific Slaughter equations (Slaughter *et al*., 1988).

Blood sampling

A volume of 20 ml of venous blood was collected after an overnight fast (⩾10 h). The samples were placed in plain tubes as well as ethylenediaminetetraacetic acid (EDTA)-containing tubes as separate aliquots, one for serum and one for plasma. Plasma was immediately separated by centrifugation (1800 *g*, 10 min, 4 °C) and all aliquots were stored at −80 °C until assayed. DNA extraction was performed by the salting-out procedure (Miller *et al*., 1988). *IGF2* rs680 polymorphism was performed by the TaqMan assay. Briefly, 5 ng/μl of each sample of DNA was used for the PCR. The forward and reverse primers, and fluorescently labelled VIC and FAM probes were ordered from Applied Biosystems (Foster City, California, USA; www.appliedbiosystems.com). A master mix was prepared in a bigue (Sterilin, London, UK) using the following reagents: Sigma Jump Start Taq Ready Mix for High Throughput # D644Z-(2000RXN) (Sigma-Aldrich, Gillingham, Dorset, UK). A volume of 5 μl of the master mix was added to each of the 384 wells of the reaction plate using a Finnpipette multichannel dispenser (Life Sciences, Basingstoke, UK). An optical adhesive film (Applied Biosystems) was used to seal the plate. The plate was then centrifuged at 1000 r.p.m. for 1 min. Each plate was then processed according to a universal thermal cycling protocol determined by ABI on Thermo Hybaid (Basingstoke, Hampshire, UK) 384 well-heated blocks. After PCR amplification, allelic discrimination was determined using a 7900HT Fast Real-Time PCR System and Sequence Detection Software version 2.1 (Applied Biosystems).

Genotypes were determined and confirmed by two experienced technicians blinded to all study data. For 343 subjects, DNA samples were of limited amount or quality. Thus, we present the data on 795 children (424 girls and 371 boys) for whom complete information and high-quality genotype data were available.

Statistical methods

Continuous variables were presented as mean values ± s.d., whereas categorical variables were presented as frequencies or proportions (%). The normal distribution of the investigated variables was assessed using the Kolmogorov–Smirnov criterion. However, in tables, untransformed means are presented. Distributions of frequencies of categorical variables were analyzed using the χ^2 test of independence. The association of genotypes with height was tested by applying a linear regression model, after controlling for the effects of several potential confounders, such as age, gender and diary intake, which are known from the available literature for their effects on height. The selection of each confounder was based on Pearson's coefficient ($P<0.05$). Interaction terms between genotypes and dairy intake were also tested through linear regression analysis. All statistical analyses were performed using SPSS 13.0 for Windows (SPSS Inc., Chicago, IL, USA).

Results

The overall frequency of the G allele was 72%, while that of the minor A allele was 28%. The genotype frequencies were 51.9% for the GG, 39.9% for the GA and 8.2% for the AA genotype, and they were in Hardy–Weinberg equilibrium ($P=0.94$). Owing to the limited number of AA homozygotes, all analyses were undertaken by merging GA and AA into one group. The distribution of Tanner stage did not differ ($P=0.65$) between GG and A-allele subjects. Table 1 shows the anthropometric and dietary parameters depending on the *IGF* rs680 genotype. A trend for tallness was found in children homozygous for the common allele (GG), compared with A-allele carriers (148.9 ± 7.9 vs 148.1 ± 7.9 cm, respectively; $P=0.19$). When the macronutrient and food group intake was evaluated by the *IGF2* genotype, no statistically significant difference for any of the parameters evaluated was detected. Multivariate analysis showed that, after adjustment for age, sex and dairy intake, the *IGF2* rs680 genotype was a statistically significant predictor of height

Table 1 Anthropometric, biochemical and dietary parameters of the study participants stratified by the *IGF* rs680 genotype (data are presented as means ± s.d.)

	IGF *rs680 genotype*		
	GG	*GA + ÁÁ*	P-*value*
N	412	383	
Age	11.15 ± 0.62	11.13 ± 0.65	0.69
Male/female	188/224	183/200	0.54
Anthropometric variables			
Weight (kg)	44.3 ± 9.6	44.3 ± 9.8	0.94
Height (cm)	148.9 ± 7.9	148.1 ± 7.9	0.19
BMI (kg/m^2)	20 ± 3.5	20.3 ± 3.7	0.23
Waist-to-hip ratio	0.80 ± 0.05	0.81 ± 0.09	0.45
Dietary variables			
Energy (kcal)	1848.3 ± 566.5	1889.8 ± 575.4	0.30
Fats (g)	81.7 ± 28.9	83.6 ± 29.5	0.19
Carbohydrates (g)	210.4 ± 72	213.6 ± 71.3	0.53
Proteins (g)	69.3 ± 22.3	70.2 ± 26.9	0.63
Dairy products (servings per day)	3.0 ± 1.4	3.2 ± 1.7	0.30

($P=0.026$) (Table 2), while a trend ($P=0.09$) for interaction with dairy intake was also found. Stratification by *IGF2* rs680 genotype revealed a positive significant association between dairy product intake and height only in A-allele carriers, adjusted for the same confounders (standardized $\beta=0.111$, $P=0.014$) (Figure 1). When dairy intake was classified, based on the median value, into two equal groups of low (1.9 ± 0.7 servings per day) and high dairy product intake (4.4 ± 1.5 servings per day), it was found that in A-allele children high dairy consumers were significantly taller ($P=0.05$) than low dairy consumers (148.8 ± 7.9 vs 147.4 ± 7.7 cm, respectively, adjusted for age and sex).

Table 2 Effect of the dairy products intake and *IGF2* rs680 polymorphism on height in children from GENDAI cohort, adjusted by age and sex, using multiple regression model

Independent variables	β ± *s.e.*	P
Age (years)	5.7 ± 0.38	0.0004
Sex	−1.0 ± 0.50	0.041
Dairy products intake (servings per day)	0.45 ± 0.18	0.013
IGF2 rs680 (GG vs GA + AA)	2.1 ± 0.95	0.026
IGF2 rs680 (GG × dairy products intake) vs (GA + AA × dairy products intake)	−0.442 ± 0.26	0.09
Variance explained	0.23	0.0003

B-coefficients are referred in cm variation. *P*-value shown for 'variance explained' is the *P*-value for the full model.

Discussion

To our knowledge, this is the first study reporting a genotype-driven dairy association with height variation in children. The existence of a possible link between human *IGF2*/rs680 polymorphism and body height in childhood is a striking, yet unresolved question. Genotype frequencies in our pediatric cohort were almost identical (51.9 vs 51.7% for GG, 39.9 vs 40.6% for GA and 8.2 vs 7.7% for AA) to those previously described in an adult study (Heude *et al*., 2007). In this investigation, the Norfolk arm of the European Prospective Investigation of Cancer (EPIC), the authors reported no significant associations between rs680 SNP and BMI, whereas they found that the rs680 *IGF2* SNP was independently associated with height. In accordance to their findings, we did not find any association with BMI, although others have shown that the less common rs680 A allele was associated with a lower BMI (Gaunt *et al*., 2001). In the Hertfordshire, UK, population GG homozygotes tended to be

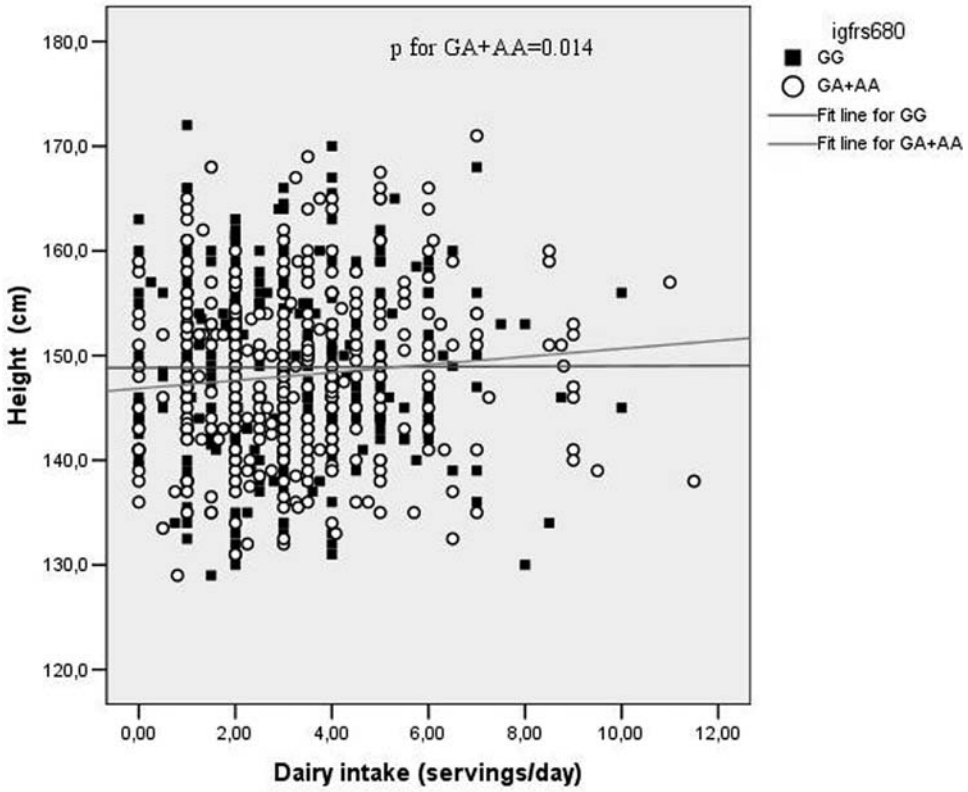

Figure 1 Scatterplot of the observed values (open squares indicate GA + AA subjects; filled squares, GG subjects) and regression lines of predicted values (green line indicates GA + AA subjects; blue lines, GG subjects) of height values by *IGF2* rs680 polymorphism ($n=795$) depending on the dairy product intake (servings per day) (as a continuous variable). The potential confounders were age and sex.

both heavier and taller than AA homozygotes, with heterozygotes (GA) being intermediate (Sayer *et al*., 2002). In the Baltimore Longitudinal Study of Aging, women with the A/A genotype were shorter than G/G carriers, and a similar trend was observed in men (Roth *et al*., 2002).

The mechanism that might explain the relationship of *IGF2* with height resides in early childhood. The rs680 G allele has been associated with significantly higher levels of *IGF2* mRNA compared with the A allele, showing a role for this polymorphism in the transcription of this gene (Vafiadis *et al*., 1998). Children with constitutionally tall stature had significantly higher growth velocity and higher IGF2 levels than children with normal height (Garrone *et al*., 2002). It has been suggested that IGF2 might be responsible for overgrowth of children with constitutionally tall stature, having an increase in activity on target tissues, particularly at the level of cartilaginous and bone tissue.

We found an association between dairy intake and height variation in our pediatric cohort. More precisely, a positive correlation was found between the dairy intake and height in those children who were A-allele carriers for the *IGF2* polymorphism. Results from the NHANES study (Wiley, 2005) indicated that adult height was positively associated with milk consumption at ages 5–12 and 13–17 years, after controlling for sex, education and ethnicity. The strongest evidence that milk and dairy product intake stimulate linear growth comes from observational and intervention studies in developing countries that show considerable effects. In addition, many observational studies from well-nourished populations also show an association between milk intake and growth. These results suggest that milk has a growth-stimulating effect even in situations in which the nutrient intake is adequate. In a randomized, controlled feeding intervention study, increase in milk intake resulted in improved linear growth in younger and already stunted children (Neumann *et al*., 2007). The associations of dietary intakes in prepubertal children with IGF levels and growth have been less often studied, but are of considerable interest. The associations of milk and dairy products with IGF-I are of particular interest in this context, as children are high consumers of these foods.

The study of Silventoinen *et al*. (2000) showed that changing environmental factors affect the heritability of height. The heritability is higher when the standard of living is better. We have recently shown (Yannakoulia *et al*., 2008) that family factors, including divorce, influence the obesity status in the GENDAI population. Compared with children of married parents, those of divorced parents had significantly higher BMI levels even on adjusting for various confounders like socioeconomic and physical activity. Thus, unfavorable family circumstances were associated with children's overweight, as well as with aspects of their eating behavior. When we stratified our sample by the family status, the effect of gene on height was masked in children of divorced parents. On the other hand, in a stable family environment, which seems to be that in which both parents are present, the effect of the *IGF2* gene on height variation remained statistically important (data not shown). Thus, exploring height variants and elucidating the possible interactions with environmental factors such as diet could help us to design effective strategies for the prevention of height-associated diseases.

Conflict of interest

The authors declare no conflict of interest.

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FUNCTIONAL GENOMICS

Vitamin D and disease

A recent study demonstrates the power of combining chromatin immunoprecipitation followed by sequencing (ChIP–seq) with genome-wide association (GWA) study data sets to explore the molecular basis of complex disease.

Ramagopalan and colleagues used ChIP–seq to produce high-resolution maps of the genomic binding of the vitamin D receptor (VDR) — a ligand-activated transcription factor — in human lymphoblastoid cell lines, with and without active ligand. In the presence of ligand, they identified 2,776 binding sites, many of which are in regions associated with active chromatin, consistent with the expected role of VDR at gene regulatory elements.Vitamin D has been linked to several diseases, particularly autoimmune diseases, but the basis for the link is unclear. The authors compared their ChIP–seq data with GWA study data sets for 47 common traits and found significant enrichment of VDR sites in associated genomic intervals for multiple sclerosis and type 1 diabetes, along with other autoimmune diseases, cancers and traits such as height. The results implicated VDR in the control of many genes that were not previously thought to be regulated by this transcription factor. In most cases, the disease-associated SNPs do not disrupt VDR-binding motifs, so further resequencing might be informative for pinpointing which genetic variants alter gene regulation. Interestingly, VDR binding is also enriched in regions with signatures of positive selection, so future studies might be aimed at investigating the role of vitamin D during human evolution.

Mary Muers

ORIGINAL RESEARCH PAPER
Ramagopalan, S. V. *et al.* A ChIP-seq defined genome-wide map of vitamin D receptor binding: associations with disease and evolution. *Genome Res.* 24 Aug 2010 (doi:10.1101/gr.107920.110)
FURTHER READING Hawkins, R. D., Hon, G. C. & Ren, B. Next-generation genomics: an integrative approach. *Nature Rev. Genet.* **11**, 476–486 (2010)

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ORIGINAL ARTICLE

CLOCK gene is implicated in weight reduction in obese patients participating in a dietary programme based on the Mediterranean diet

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Introduction: The success of obesity therapy is dependent on the genetic background of the patient. Circadian Locomotor Output Cycles Kaput (*CLOCK*), one of the transcription factors from the positive limb of the molecular clock, is involved in metabolic alterations.
Objective: To investigate whether five candidate polymorphisms from *CLOCK* were associated with anthropometric, metabolic measures and weight loss in response to a behavioural weight reduction programme based on the Mediterranean diet.
Methods: Five hundred overweight/obese subjects, aged 20–65 years, who attended outpatient clinics specializing in obesity, were studied. Anthropometric, biochemical and dietary intake variables were analysed. Effectiveness of the programme and weight loss progression during 28 weeks of treatment was assessed.
Results: Four of five *CLOCK* SNPs selected were significantly associated with obesity variables ($P<0.05$). The genetic variation in the rs1801260 *CLOCK* was associated with obesity at baseline and also affected weight loss. Patients with the variant allele (G) lost significantly less weight i($P=0.008$) compared with wild type. Repeated measures analysis showed that weight loss over time was significantly different between rs1801260 *CLOCK* variations ($P=0.038$). Carriers of the G allele displayed greater difficulty in losing weight than non-carriers. In this particular polymorphism, the frequency of short-time sleepers ($\leqslant 6$ h per day) was greater in minor allele carriers than in non-carriers (59% vs 41%; $P<0.05$). *CLOCK* polymorphisms were also associated with significant differences in total plasma cholesterol at the completion of dietary treatment ($P<0.05$).
Conclusions: We have replicated previous studies showing a relationship between *CLOCK* gene polymorphisms and obesity. *CLOCK* rs1801260 SNP may predict the outcome of body weight reduction strategies based on low-energy diets.
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Keywords: *CLOCK*; polymorphism; behavioural therapy

Introduction

Weight loss in response to obesity management strategies shows a wide range of interindividual variation that is largely influenced by nutritional, hormonal and psycho-behavioural factors. In addition, the success of obesity therapy may be modulated by the genetic background of the patient,[1] as shown by studies carried out in monozygotic twins; weight loss seems to show substantial heritability. In addition, parental obesity has a role in the likelihood of obesity in the offspring.[1] A number of potential candidate genes has been previously examined for associations with body weight loss and weight loss maintenance.[2–5]

The Human Obesity Gene Map lists about 250 loci possibly involved in the development of obesity.[1] However, results from association studies for most of these loci are inconclusive for obesity and weight loss outcomes. Although some studies find no associations between weight loss and candidate genes,[6] others have shown that changes in fat mass were predicted by polymorphisms at several obesity candidate genes, explaining up to 8.5% of the fat mass variance.[1] Examples of genes related to changes in fat mass include leptin, G protein, ADRB3, PPARγ and perilipin.[2–5]

Recent clinical and epidemiological studies have shown significant relationships between chronobiology and obesity. For example, shift work, sleep deprivation and bright light exposure at night have been associated with increased

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adiposity.[7] Although our understanding of the biological clock model continues to evolve, previous studies have established that Circadian Locomotor Output Cycles Kaput (*CLOCK*), one of the transcription factors from the positive limb of the molecular clock, is involved in altered metabolic function.[8] Animal models have revealed that mice with *Clock* gene disruptions are prone to develop obesity.[9] In 2004, Rudic *et al*. [10] showed that mutations in *Clock* gene were associated with impaired glucose tolerance. More recently, these research efforts have been extended to gain further understanding about the role of *CLOCK* gene variants in human obesity. Thus far, only two studies focused on obesity have shown interesting associations between different *CLOCK* gene polymorphisms and body mass index (BMI).[11,12] However, no study has yet reported relationships between *CLOCK* gene polymorphisms and weight loss in response to a weight reduction programme.

On the basis of these previous studies and on a number of remaining unexplored questions, we investigated whether five candidate polymorphisms from *CLOCK* were associated with anthropometric, metabolic measures and weight loss in response to a behavioural weight reduction programme based on the Mediterranean diet in an obese population from southeastern Spain.

Subjects and methods

Subjects

We recruited overweight or obese subjects (BMI $>25\,kg\,m^{-2}$ and $<40\,kg\,m^{-2}$) within the age range of 20–65 years, ($n=500$) who attended during 2008 five outpatient obesity clinics in the city of Murcia, located in southeastern Spain. Patients receiving thermogenic or lipogenic drugs, or those diagnosed with diabetes mellitus, chronic renal failure, hepatic diseases or cancer were excluded from the study (11%). The final sample consisted of 454 individuals.

All procedures were in accordance with good clinical practice. Written consent was obtained from each patient before participation and the study principles were approved by the Research Ethics Committee of the Virgen de la Arrixaca Hospital. Patient data were codified to guarantee anonymity.

Characteristics of the treatment

The characteristics of the weight reduction programme have been described elsewhere.[13,14] Briefly, during the initial 4 months, subjects attended a weekly 60-min therapy session in support groups ($n=10$), followed by a 5-month maintenance period. Sessions were conducted by a nutritionist. Treatment was based on the following:

Dietary treatment. Dietary individual energy requirements were assessed by calculating (1) resting energy expenditure (REE) according to the Harris-Benedict formula and (2) total energy expenditure (TEE) according to the type and duration of physical activity. Next, about 2.6 MJ per day were subtracted from the TEE. The final dietary energy content ranged from 1200–1800 kcal per day for women and 1500–2000 kcal per day for men to induce an approximate loss of 0.5–1 kg per week. The recommendations were consistent with the Mediterranean type of diet [14] and the macronutrient distribution followed the recommendations of the Spanish Society of Community Nutrition.[15]

Nutritional education was given during group therapy sessions to help subjects plan their own menus and to educate subjects to adopt appropriate lifetime eating habits.

Physical activity emphasised individual goals of 15–30 min or more of moderate intensity physical activity, at least 2 or 3 times a week. Patients were encouraged to use a pedometer to reach at least 10 000 steps per day.

Behavioural techniques included stimulus control, self-monitoring, positive reinforcement and cognitive behavioural therapy.

Anthropometric measurements

Subjects were weighed in barefoot wearing light clothes, with a digital scale to the nearest 0.1 kg, at the same time each day. Height was measured using a Harpenden digital stadiometer (rank 0.7–2.05). The subject was positioned upright, relaxed and with the head in the Frankfurt plane. BMI was calculated according to these measurements as weight(kg)/(height(m))^2. Total body fat was measured by bioelectrical impedance using TANITA TBF-300 (TANITA Corporation of America, Arlington Heights, IL, USA) equipment. Body fat distribution was assessed by the measurement of different circumferences: waist circumference, at the level of the umbilicus, and hip circumference, as the widest circumference over the greater trochanters.[16] All measurements were made with a flexible and inextensible tape measure. Waist–hip ratio (WHR) was then calculated.

Biochemical analysis

Plasma concentrations of glucose, total cholesterol, total haemoglobin and uric acid were determined from venous blood samples after overnight fast with commercial kits (Roche Diagnostics GmbH, Mannheim, Germany).

Habitual dietary intake

To evaluate food habits, initial nutrient intake was determined by a 24-h dietary recall. Interviews were conducted from monday to friday, including 24-h recalls of food intake from weekend and weekdays. Total energy intake and macronutrient composition from the initial 24-h recalls were analysed with the nutritional evaluation software program Grunumur[17] on the basis of Spanish food composition tables.[18]

DNA isolation and clock genotyping

We selected tagSNPs as effective proxies for untyped SNPs in strong linkage disequilibrium (LD) by using the Tagger[19]

based on HapMap Caucasian European Utah data (www.hapmap.org) [20] with a minor allele frequency (MAF) $\geqslant 0.10$ and a minimum r^2 of 0.8. Tagger uses an algorithm that selects tagSNPs to construct single- and multi-marker tests to capture alleles of interest based on the computed correlation r^2 between them.

DNA was isolated from blood samples using routine DNA isolation sets (Qiagen). We performed genotyping of *CLOCK* gene polymorphisms using a TaqMan assay with allele-specific probes on the ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA) according to the standardized laboratory protocols.[21]

Bioinformatics analysis

The LD plot between the studied SNPs is shown in Figure 1. SNPs were selected using three criteria: literature reports of genetic associations or biological function of interest, bioinformatics functional assessment and LD structure. In conjunction with the selection of tagSNPs, we also performed bioinformatics analysis of the genomic DNA sequence encompassing different SNPs to ascertain the putative biological consequences of different alleles. SNPs mapping to regions upstream of the transcription start site or within introns were studied with MAPPER[22] to identify the potential allele-specific transcription factor binding sites.

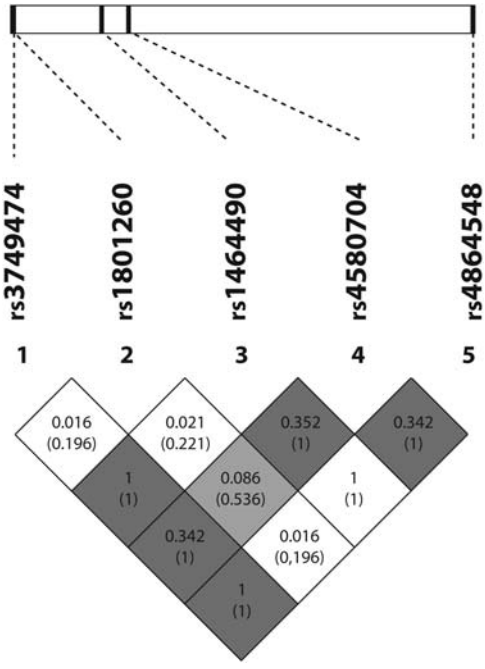

Figure 1 Linkage disequilibrium (LD) plot across the *CLOCK* gene. The horizontal white bar depicts the 113 kb DNA segment of chromosome 4q12 analysed in the sample. The 5 tagSNP locations are indicated by hatch marks. An LD plot is depicted in the bottom part of the figure: each diamond represents the magnitude of LD for a single pair of markers. Black indicates strong LD ($r^2 = 1.0$); white indicates no LD ($r^2 = 0$) and the grey tones indicate intermediate LD. The numbers inside the diamonds indicate the r^2 (D′) value.

No intronic SNPs altered splice acceptor or donor sites or other signals recognised by the splicing machinery such as the poly-pyrimidine tract. Polymorphisms within the 3′-UTR of the mRNA can exert an effect on the folding of the mRNA with concomitant changes in mRNA stability. Potential effects of 3′-UTR SNPs were tested with RNAfold [23] within the Vienna RNA package.

Statistical analysis

We used Pearson's χ^2 and Fisher's tests to test differences in frequencies. To assess the effectiveness of the program, changes in the characteristics of the population at the beginning and at the end of the treatment were assessed with the Student's paired *t*-test. We applied ANOVA and Student's *t*-test to compare crude means across genotype groups. We tested different genetic inheritance models and a dominant model was applied in the final analyses for all SNPs selected, except for rs4580704 that followed a recessive model. We performed multivariate adjustments of the associations by analysis of covariance and estimated adjusted means. We adjusted analyses for sex, age and centre. We also tested the statistical homogeneity of the effects by sex in the corresponding regression model with interaction terms. Statistical analyses were performed using SPSS 15.0 software (SPSS). A two-tailed *P*-value of <0.05 was considered statistically significant.

Results

Characteristics of the population

General characteristics of the population studied, including anthropometric measurements, biochemical variables, dietary intake and physical activity characteristics, are listed in Table 1. The population of 454 subjects (380 women and 67 men) had a mean age 39 ± 12 years and BMI of $31 \pm 5.3\,\mathrm{kg\,m^{-2}}$ (mean ± s.d.). Seventy-five percent of the population was considered sedentary (engaging in less than 1 h of physical activity per week). The mean number of hours of sleep per day was 7 h. Dietary habits obtained by the 24-h recall showed that the percentage of carbohydrate was lower than Spanish recommendations, whereas those of protein and fat were above the recommended levels.

Genotype frequency of clock variants in Garaulet subjects and associations with obesity parameters

SNP rs1464490 was selected because it was the tagSNP for a large LD block (LD1); rs3749474, also from LD1, was selected after bioinformatics functional assessment suggested important changes to mRNA structure; and rs4864584 because it was previously associated with overweight/obesity.[11,12] From different SNPs comprising LD2, selection of rs4580704 was based on its previous relationship with BMI.[12] From LD3, we selected rs1801260 SNP based on the previous reports

Table 1 Characteristics of the population studied

	Total population
N	447
Anthropometric	Mean (s.d.)
Age (years)	39 (12)
Weight (kg)	84.61 (16.72)
BMI (kg m^{-2})	31.3 (5.3)
Body fat (%)	37.5 (7.5)
Hip (cm)	114 (10)
Waist (cm)	102 (14)
WHR	0.89 (0.08)
Plasma values	
Glucose (mg per 100 ml)	90.7 (22.4)
Cholesterol (mg per 100 ml)	173.8 (40.6)
Uric acid (mg per 100 ml)	4.4(1.5)
Haemoglobin (mg per 100 ml)	14.6 (7.1)
Activity	
Sleep (hours per day)	7.1 (1.5)
Exercise (METs)	4140 (4426)
Dietary intake	
Total energy (kcal per day)	1903 (709)
Carbohydrates (%)	39.6 (10.5)
g per day	148.6 (52.7)
Proteins (%)	16.9 (3.6)
g per day	65.6 (26.8)
Fats (%)	43.5 (10.7)
g per day	79.0 (38.3)
CLOCK polymorphism, n (%)	
rs4580704	
GG	65 (14.4)
CG	202 (44.8)
CC	166 (36.8)
rs1801260	
GG	33 (7.7)
AG	170 (37.7)
AA	220 (54.6)
rs3749474	
TT	46 (10.2)
TC	178 (39.5)
CC	204 (45.2)

Abbreviation: WHR, weight–hip ratio.

showing associations between this SNP, sleep alterations[24] and binge eating disorders.[25] Their locations in the *CLOCK* gene, the Hardy-Weinberg equilibrium and minor allele frequency are listed in Table 2. *CLOCK* genotype frequencies did not deviate from the Hardy-Weinberg equilibrium. As SNPs, rs3749474, rs1464490 and rs4864548, were almost in complete LD and displayed a similar pattern of phenotypic associations, only the results for rs3749474 are presented.

We first examined the association between the *CLOCK* SNPs and obesity parameters (Figure 2). We did not detect sex heterogeneity for any of the SNPs examined. Therefore, we present the results for men and women combined. We found significant associations with weight and BMI for SNPs rs3749474 and rs1801260 and with waist for rs3749474. A similar trend was found for rs4580704, although it did not reach statistical significance: weight ($P=0.077$), waist ($P=0.077$) and WHR ($P=0.097$).

For rs3749474, carriers of the minor allele T had significantly higher weight, BMI and waist than CC subjects. Similar results were found for rs1801260 in which minor allele carriers also showed the highest BMI values. These differences remained statistically significant after additional adjustment for sex, age, treatment centre and initial BMI (Figure 2). For rs4580704, the trend was the opposite; subjects homozygous for the minor allele showed lower values for weight, waist and WHR than non-carriers.

Longitudinal data

Characteristics of the population before and after the dietary behavioural treatment are shown in Table 3. All of the anthropometric variables were significantly improved after treatment. Total cholesterol, uric acid and blood pressure were also significantly reduced after treatment.

The mean duration of the treatment was 26 ± 17 weeks. The average weight loss was 8.85 kg. The percentage of weight loss compared with the initial body weight was 10.24 and the rate of weight loss was 450 g per week.

Genetic variation at the *CLOCK* rs1801260 SNP was associated with differences in body weight reduction.

Table 2 Characteristics of the population studied before and after behavioral treatment

Measures	*Total group*	*Total group*	P
	Before treatment (N = *447*)	*After treatment* (N = *447*)	
Weight (kg)	84.60 ± 0.79	75.27 ± 0.72	0.0001
BMI (kg m^{-2})	31.4 ± 0.3	28.1 ± 0.2	0.0001
Hip (cm)	115 ± 0.76	108 ± 0.86	0.0001
Waist (cm)	105 ± 1.17	97 ± 1.22	0.0001
WHR	0.91 ± 0.08	0.89 ± 0.08	0.0001
Glucose (mg per 100 ml)	89.4 ± 2.5	89.0 ± 3.0	0.897
Cholesterol (mg per 100 ml)	180.1 ± 3.78	163.8 ± 4.94	0.002
Uric acid (mg per 100 ml)	4.63 ± 0.15	4.01 ± 0.15	0.001
Diastolic pressure (mm Hg)	70 ± 1.0	66 ± 1.1	0.010
Systolic pressure (mm Hg)	112 ± 1.7	107 ± 1.6	0.005

Abbreviations: BMI, body mass index; WHR: waist–hip ratio. Data are presented as mean ± EEM. Student's *t*-test: $P<0.05$ considered significant.

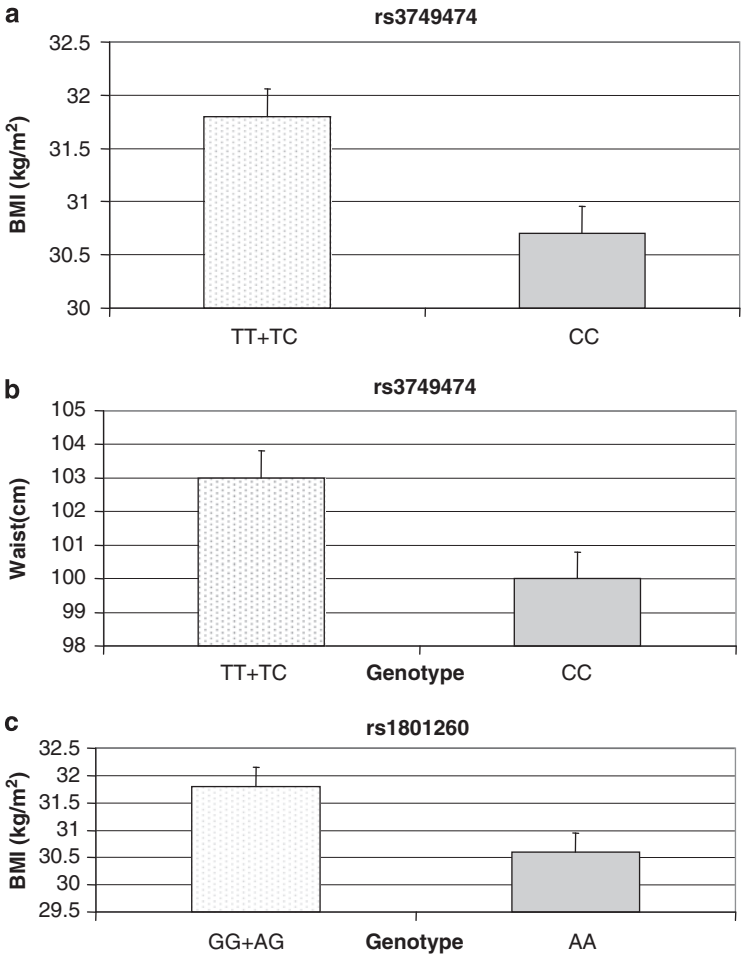

Figure 2 Significant associations between *CLOCK* SNPs and obesity parameters. (**a**) *CLOCK* rs3749474 and BMI; (**b**) *CLOCK* rs3749474 and waist circumference; (**c**) *CLOCK* rs180126 (3111T>C) and BMI.

Table 3 Description of Clock SNPs

Name	*Location*	*HWPval*	*MAF*	*Alleles*	*Minor allele*
rs3749474	3′-UTR	0.444	0.32	C/T	T
rs1801260	3′-UTR	0.984	0.28	A/G	G
rs1464490	Intron 11	0.453	0.42	C/T	T
rs4580704	Intron 9	0.782	0.38	C/G	G
rs4864548	Promoter	0.445	0.41	G/A	A

Abbreviations: HW Pval, Hardy–Weinberg equilibrium expectations *P* values; MAF, minor allele frequency.

Patients with the minor allele were less successful losing weight after adjusting for baseline BMI ($P=0.008$) (Table 4). The difficulty in losing weight was particularly evident after 12 weeks of treatment as evidenced by the analysis of repeated measurements after adjusting for baseline BMI ($P=0.038$). No statistically significant differences were found for the remaining *CLOCK* SNPs analysed ($P>0.05$) (Figure 3 a–c). However, significant associations were found between *CLOCK* polymorphisms and changes in the total plasma cholesterol, even for those SNPS that showed no statistical associations with weight loss, such as rs374947 and rs4580704 (Table 4).

Table 4 Associations of *CLOCK* polymorphisms with changes in weight and metabolic parameters

CLOCK	*Total weight loss (kg)*	P-*value*[b]	*Serum cholesterol changes*	P-*value*
rs4580704				
GG[a]	8.82 ± 5.13	0.877	6.80 ± 14.96	0.026
CG+CC	8.99 ± 5.54		44.61 ± 7.30	
rs1801260				
GG+AG[a]	7.96 ± 0.57	0.008	25.08 ± 9.65	0.064
AA	10.41 ± 0.53		52 ± 9.45	
rs3749474				
TT+TC[a]	9.37 ± 0.56	0.513	49.58 ± 9.54	0.047
CC	8.70 ± 0.62		22.42 ± 9.54	

All data are given as mean ± s.e.m. [a]Recessive model. [b]Adjusted for initial BMI.

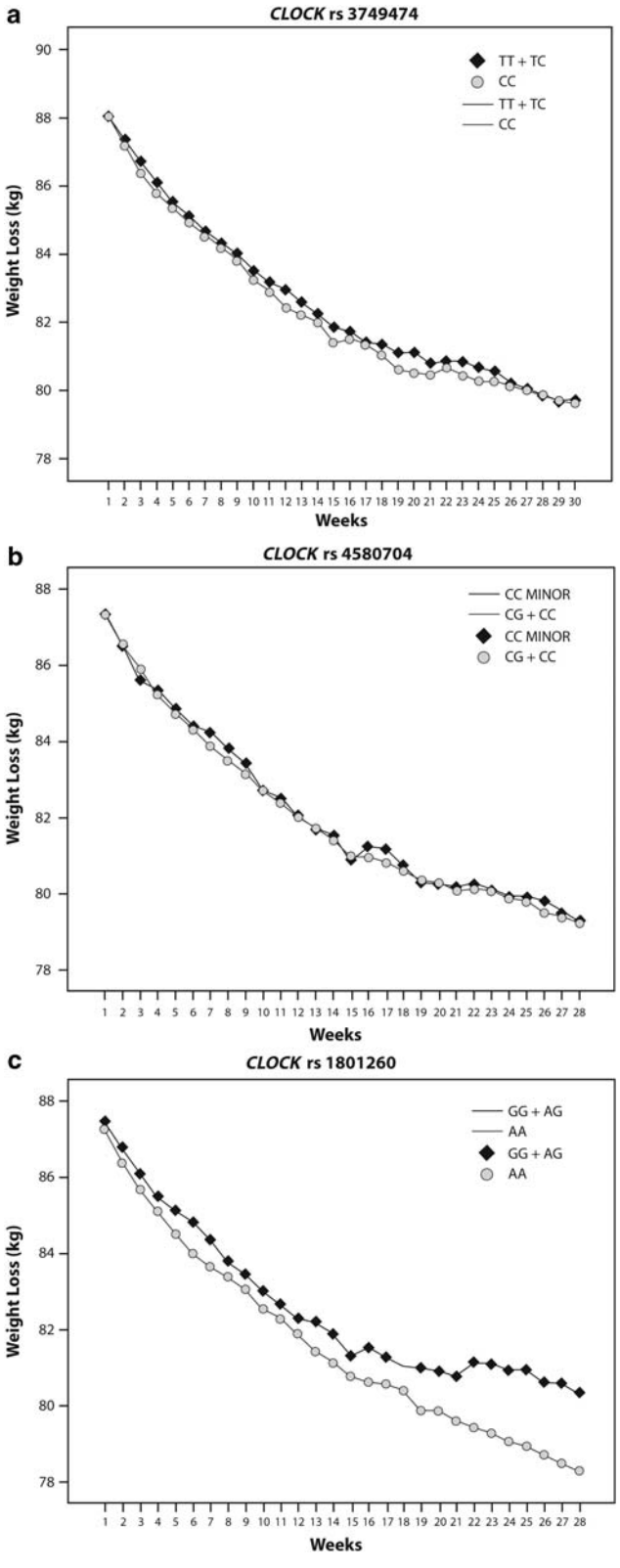

Figure 3 *CLOCK* variants and weight loss evolution during 28 weeks of treatment: (**a**) *CLOCK* rs3749474; (**b**) *CLOCK* rs4580704; (**c**) *CLOCK* rs180126 (3111T>C).

When potential associations between sleep duration and *CLOCK* SNPs were examined, a trend was found for rs 1801260. Subjects carrying the minor allele were 2.10 times more likely to sleep $\leqslant$6 h per day than non-carriers (95% CI 0.95–4.45) ($P=0.060$). Moreover, the frequency of short-time sleepers ($\leqslant$6 h per day) was higher in minor allele carriers than in subjects homozygous for the major allele (59 vs 41%), whereas the opposite was true fore those who slept more than 6 h a day, 41% in minor allele carriers vs 69% in non-carriers ($P<0.05$).

Discussion

In this study, we have confirmed a role of the *CLOCK* gene in body weight regulation. Specifically, four out of five *CLOCK* SNPs genotyped, that is rs3749474, rs4864548, rs1464490 and rs1801260, were significantly associated with obesity parameters, and a trend was found for the fifth one (rs4580704). We have also shown that *CLOCK* rs1801260 was associated with response to a dietary and behavioural weight loss programme. Specifically, carriers of the G variant may be at higher risk for obesity, and they were least responsive to a weight loss intervention.

In this study, we have replicated previous findings showing the association between different *CLOCK* SNPs and obesity, particularly for rs4864548 and rs4580704.[11,12] In addition, we have shown that minor allele carriers carrying at least one copy of the T allele at the *CLOCK* rs3749474 displayed a significantly higher degree of obesity (weight and BMI) and abdominal obesity (waist circumference) than major allele carriers. This SNP was selected because of its location in the 3′-UTR. The mechanism through which this particular SNP is associated with obesity could be attributed to changes in mRNA structure. Indeed, our analysis suggests that this one-base change (T/C) in the *CLOCK* 3′-UTR transforms the structure of this particular region (Figure 2). The 3′-UTR is characterized by sequences such as SECIS or AU-rich elements (AREs), which are recognised by particular proteins that exert an effect on mRNA stability or location in the cell. Although specific binding sites are not elucidated in the analysis presented here, the difference in the predicted allele-specific structures is consistent with the hypothesis that this SNP strongly affects mRNA structure and stability. On the other hand, this SNP is in complete LD with rs4864548 (Figure 1), which is located in the promoter region. Variation in the promoter activity may also explain the results we observed.

These findings support the evolving view that the circadian oscillator has an important role in the development of obesity and metabolic syndrome.[7] Recently, several studies have suggested that the disruption of the circadian system may contribute to obesity.[26] Shift work, sleep deprivation and exposure to bright light at night are related to increased adiposity.[27] Studies performed in animal models

have revealed that mice with disruption of the *Clock* gene are prone to develop obesity and a phenotype resembling MetS.[9] In a similar vein, we have recently provided evidence of *CLOCK* gene expression in human adipose tissue and shown its association with waist circumference and different components of the MetS.[28] The precise mechanisms linking MetS to circadian system disruption are not well known. However, most hypotheses suggest that the internal desynchronization (ID) between different circadian rhythms involved in metabolism is a key factor in the development of MetS.[29]

Our dietary intervention study has revealed that minor allele carriers of rs1801260 at the *CLOCK* locus are more resistant to weight loss and to metabolic changes in response to an energy-restricted diet than AA homozygotes. Previous research supports the hypothesis that individual *CLOCK* genotypes may influence several variables linked with behaviour in both healthy individuals and in those with mental illness.[30] Specifically, for rs1801260, it has been shown that this polymorphism is related to clinical features of mood disorders influencing diurnal preference in healthy humans and that it causes sleep phase delay and insomnia in people with depression and bipolar disorder.[30–32] These alterations could be related not only to obesity but also to weight loss. Along the same lines, we have previously shown that most of the obstacles of weight loss are related to the subject's eating behaviours and psychological characteristics and that these obstacles are directly related to the difficulty in losing weight.[14] Moreover, previous works have related rs1801260 to eating disorders and obesity.[12,25]

This idea is supported by animal findings suggesting a direct involvement of the *Clock* gene in the regulation of body weight, as homozygous *Clock* mutant mice developed obesity, hyperphagia and also suffered from changes in eating behaviour, sleep pattern and mood.[9] Interestingly, we found that minor allele carriers of the rs1801260 variant had a tendency to sleep less than major allele subjects. Our data are consistent with earlier studies associating this SNP with insomnia [30] and also with epidemiological studies that indicate that sleep deprivation may increase the risk of obesity and weight gain.[33] Although the molecular mechanisms underlying the observed results remain unknown, we can hypothesise from this study that carriers of the rs1801260 variant allele might experiment a disruption in the chronobiology system similar to that shown in the *Clock* knockout mouse. Loss of the *Clock* gene in the animal model affects obesity, sleep and eating behaviours that may, in human subjects, consequently influence weight loss as well.

In a previous study, investigators reported that polymorphisms in a panel of obesity-related candidate genes have a minor role, if any, in modulating weight changes induced by a moderately hypo-energetic diet.[6] The investigators concluded that none of the genetic variants they examined had a significant association with weight loss in the intervention. Although the *CLOCK* gene was not included in that study, one important difference between the earlier study and this study was that in the earlier study the duration of interventions was limited to 10 weeks, whereas in this study the loss treatment was followed for 28 weeks. Sorenson *et al.* [6] have suggested that more prolonged interventions are necessary to effectively evaluate the rolls of particular polymorphisms in weight loss, and to detect differences among variants. Indeed, in this study, the most important differences between rs1801260 variants in weight loss were identified since the third month of treatment. Other studies that have identified associations between genetic variants and weight loss in obese populations have used intervention durations similar to those used in our study.[1] Up to this point, most of the studies that have evaluated relationships between genetic variants and weight loss achieved through interventions have focused on genes related to energy expenditure, appetite control, adipogenesis or genes related to insulin resistance and lipid metabolism.[1] Our study expands the realm of current knowledge by showing a potential role for circadian clock genes on weight loss.

In conclusion, in this intervention trial performed in a Mediterranean population, we have replicated previous studies that reported relationships between *CLOCK* gene polymorphisms and obesity. Our results showed that rs1801260 may predict the outcome of weight loss strategies that are based on low-energy diets. Carriers of the G allele may exhibit a greater degree of obesity and experience greater difficulty in losing weight in response to a low-energy diet. The difficulty was consistently observed throughout the treatment period, although it was most evident since the third month of treatment. These data suggest that *CLOCK* gene polymorphisms may predict weight loss magnitude in response to a low-energy diet.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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