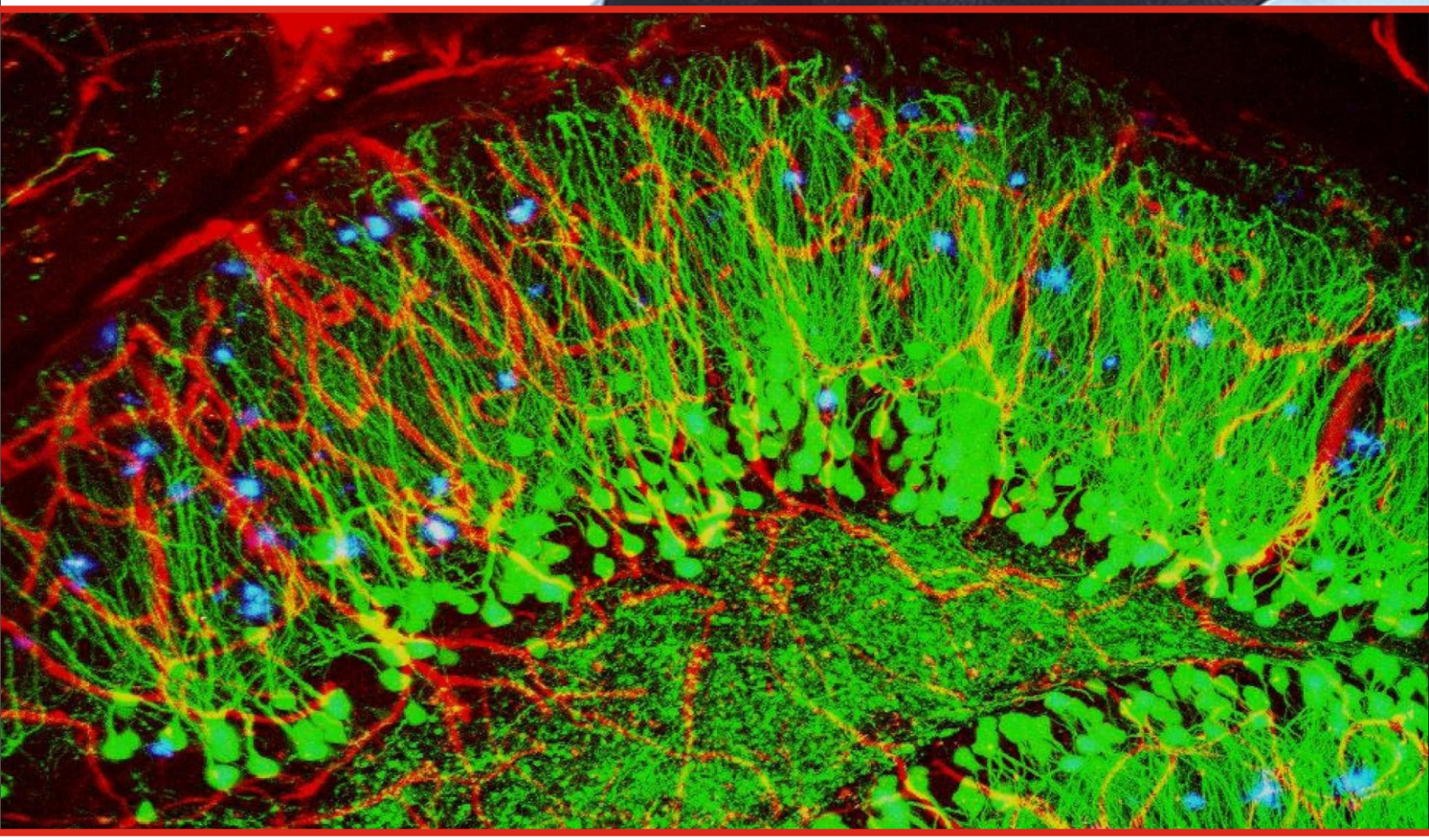
NEWS LETTER

BioGenesis
HEALTH CLUSTER
INDIA

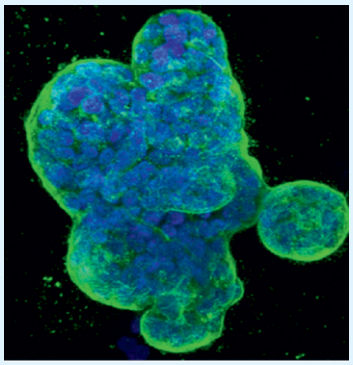
"Together we grow.
Together we prosper. Together we
will build a strong and inclusive India.
India wins yet again!"

Narendra Modi, Hon'ble Prime Minister



HIGH-QUALITY AND TIMELY DATA ARE ESSENTIAL FOR COMPREHENSIVE CERVICAL CANCER CONTROL AND UNDER-PIN EFFECTIVE POLICY-MAKING.

Why Research on Cancer Biology Is Critical to Progress against the Disease



Research on the biology of cancer starts with the simplest of questions: What is—and isn't—normal?

To understand how cancer develops and progresses, researchers first need to investigate the biological differences between normal cells and cancer cells. This work focuses on the mechanisms that underlie fundamental processes such as cell growth, the transformation of normal cells to cancer cells, and the spread, or metastasis, of cancer cells.

Knowledge gained from such studies deepens our understanding of cancer and produces insights that could lead to the development of new clinical interventions. For example, studies of cell signaling pathways in normal cells and cancer cells have contributed greatly to our knowledge about the disease, revealing molecular alterations that are shared among different types of cancer and pointing to possible strategies for treatment.

The last few decades of basic research in cancer biology have created a broad base of knowledge that has been critical to progress against the disease.

In fact, many advances in the prevention, diagnosis, and treatment of cancer would not have occurred without the knowledge that has come from investigating basic questions about the biology of cancer.

Opportunities in Cancer Biology Research

Scientists today have a growing understanding of the biology of a vast array of cancers driven by various mutations and across many body sites. New data and research approaches have created opportunities for researchers to study in detail many aspects of cancer biology, including how the normal biological programs of cell proliferation and death are altered during cancer and how the immune system responds to tumors.

The discovery of tumor stem cells in a range of cancers has created opportunities for researchers to identify these rare cells in both solid tumors and hematologic cancers, as well as to investigate the role of these cells at different stages of disease.

The recognition that the cancer cell is in a symbiotic relationship with the tumor microenvironment has created opportunities to study the interactions of cancer cells within the tumor or the host microenvironment. Researchers are now studying the molecular mechanisms and signaling pathways of cancer cell development, proliferation, and metastasis.

Researchers are also investigating the role of the human microbiome—the community of microorganisms that inhabit the human body—in the initiation and progression of tumors.

New genetic technologies developed over the past decade have helped researchers examine the functional effects of genetic alterations that underlie the development of cancer. These tools have also been used to study epigenetic changes associated with cancer, mechanisms of DNA damage and repair, and gene regulation in cancer cells.

The introduction of increasingly powerful structural biology approaches has allowed researchers to characterize the structures of mutant proteins involved in cancer, such as RAS, and other molecules in greater detail than had been possible previously. And through approaches that allow the characterization of the entire proteome, researchers are integrating genomic analysis with the analysis of the proteins in tumor cells to learn, in detail, how cancer-associated mutant proteins affect other proteins.

Proteogenomics Research: On the Frontier of Precision Medicine

Separately, genomics and proteomics provide a partial picture of cancer biology. Studying them together via proteogenomics research produces a more unified picture of cancer. Researchers are hopeful that proteogenomics, the integrated study of proteomics and genomics, may improve our ability to prevent, diagnose, and treat cancer at the molecular level using precision medicine.

There are also opportunities to explore cancer biology through systems biology approaches. Researchers use a variety of information and tools, such as mathematical modeling, to describe the complex interactions among components of a biological system and make predictions that help guide and further refine experimental science.

Challenges in Cancer Biology Research

Basic research in cancer biology is often viewed as "high risk," in part because the clinical applications of a given research project might not be clear at the outset. However, knowledge gained from studying cancer cell biology not only improves our understanding of the disease but is essential for the development of clinical advances that benefit patients, as recent progress in the areas of immunotherapy and cancer vaccines illustrates.

Nonetheless, because of the uncertainty about the outcomes of basic research in cancer biology, this area of research receives relatively little funding from sources that are driven by profit. For this reason, federal funding for cancer biology research is critical.

Collaboration across disciplines is increasingly necessary to better understand key mechanisms in cancer. Therefore, some investigators may need to develop tools and strategies for sharing and communicating research results.

NCI's Role in Cancer Biology Research

NCI supports and directs research on the biological differences between normal cells and cancer cells through a variety of programs and approaches. For example, the Division of Cancer Biology (DCB) supports extramural researchers who are using a variety of methods to study cancer biology.

In addition to many of the topics mentioned above, DCB supports research on:

Shining a Spotlight on Tumor Exosomes

Pediatric neuro-oncologist David Lyden, M.D., Ph.D., studies exosomes to uncover the steps of cancer cell metastasis.

- the metabolism of cancer cells, the responses of cancer cells to stress, and mechanisms involved in control of the cell cycle
- biological agents (such as viruses and bacteria), host factors (such as obesity, co-morbid conditions, and age), and behaviors (such as dietary intake) that may cause or contribute to the development of cancer
- immune regulation of the development and spread of tumors and approaches to improve immune targeting and destruction of cancer cells
- genomic instability and related molecular, cytogenetic, and chromosomal effects during induction and progression to malignancy
- the role of the microenvironment created by inflammation and the inflammatory signaling molecules in the formation and progression of tumors
- processes and molecular targets where there is potential for therapeutic or preventive intervention
- the effects of hypoxia on tumor cell invasion and

metastasis

- the role of somatic stem cells in determining tumor progression and metastatic behavior, and control of the stem cell niche by tumor microenvironment

NCI-supported research programs in cancer biology include the:

- Physical Sciences in Oncology Network (PS-ON)

  The goal of this initiative is to promote and foster the convergence of physical science and cancer research. Small transdisciplinary teams of physical scientists (engineers, physicists, mathematicians, chemists, and computer scientists) and cancer researchers (cancer biologists, oncologists, and pathologists) collaborate on solving problems such as determining which cell is the cell of origin for brain and hematopoietic tumors and exploring the use of three-dimensional images of single cells as cancer signatures.

- Cancer Systems Biology Consortium (CSBC)

  The CSBC focuses on combining advanced experimental approaches with mathematical and computational methodologies to build and test predictive models of cancer. The initiative takes an integrative approach to cancer research to complement and expand our current understanding of tumor development and progression across many physical and time scales, with the ultimate goal of improving the lives of cancer patients.

- Barrett's Esophagus Translational Research Network (BETRNet)

  This multidisciplinary, multi-institutional collaboration was established to better understand Barrett esophagus and to prevent esophageal adenocarcinoma. BETRNet aims to better understand esophageal adenocarcinoma (EA) biology; examine research opportunities associated with its precursor lesion, Barrett Esophagus; improve EA risk stratification and prediction; and provide strategies for EA prevention. The overriding goal is to decrease the incidence, morbidity, and mortality of this cancer.

- Alliance of Glycobiologists for Detection of Cancer

  This consortium of tumor glycomics laboratories and their research partners study the cancer-related dynamics of complex carbohydrates. This program, which NCI sponsors with the National Institute of General Medical Sciences and the National Heart, Lung and Blood Institute, aims to study the structure and function of glycans in relation to cancer.

- Molecular and Cellular Characterization of Screen-Detected Lesions Initiative

  The goal of this program is to undertake a comprehensive molecular and cellular characterization of tumor tissue, cell, and microenvironment components to distinguish screen-detected early lesions from interval and symptom-detected cancers. Researchers use various technologies and approaches to determine both the cellular and molecular phenotypes of early lesions, with the goal of better predicting the fate of early lesions.

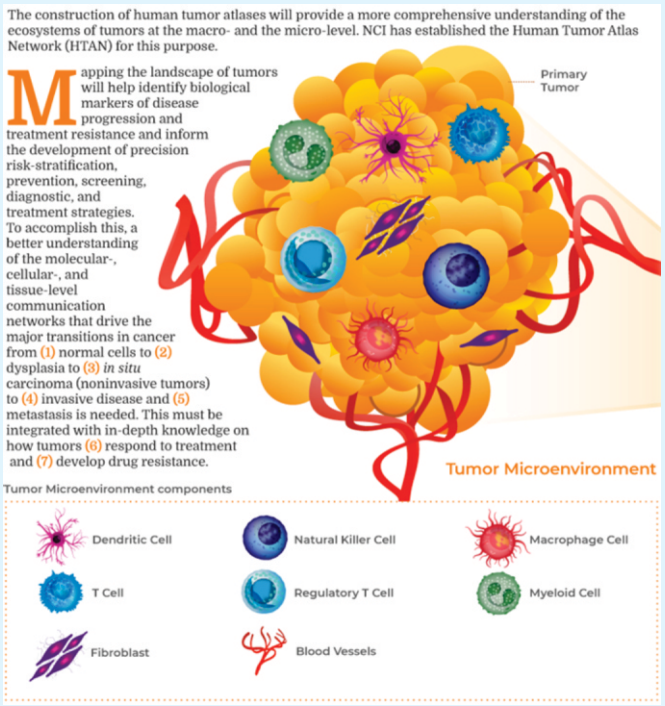
- Clinical Proteomic Tumor Analysis Consortium (CPTAC)

  CPTAC was launched by NCI's Office of Cancer Clinical Proteomics Research (OCCPR) to systematically identify proteins that result from genetic alterations in cancer cells, study how they affect biological processes, and provide this data with accompanying assays and protocols to the public.

- Applied Proteogenomics OrganizationaL Learning and Outcomes Network (APOLLO)

A collaboration between the Department of Defense (DoD), Department of Veterans Affairs (VA), and NCI using the latest genomic and proteomic research methods to more rapidly and accurately identify effective drugs to treat cancer based on the proteogenomic profile of a patient's tumor. Initial collaborative efforts will focus on a cohort of 8,000 patients with lung cancer and will make data broadly available to the research community. Eventually, the effort will be expanded to additional cancer types.

Cancer and the Human Tumor Atlas Network



- Human Tumor Atlas Network (HTAN)

A collaborative network that is constructing multidimensional tumor atlases to document the molecular and cellular alterations and interactions within tumors as they develop and evolve.

The atlases will represent a diverse patient population and describe the dynamics of cancer, focusing on the transition from precancer to malignancy, from local invasion to distant metastasis, and how tumors respond to treatment and develop resistance to drugs.

NCI's Centers of Excellence bring together intramural researchers from NCI's Center for Cancer Research andDivision of Cancer Epidemiology and Genetics to develop new projects and initiatives in various areas of cancer biology, including:

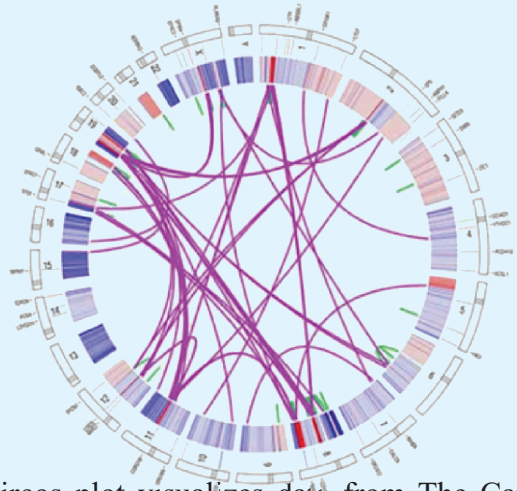
- Chromosome Biology

The experts affiliated with this center study the mechanisms involved in chromosome function through diverse research that includes mapping the dynamic changes of the genome and transcriptome during the development of cancer and translational research for the early diagnosis of cancer.

- Integrative Cancer Biology and Genomics

This center's goal is to use advanced analytic technologies to define homogenous clusters of patients, who can then be treated with appropriate therapies. The researchers in this center build upon the immense amount of basic research data available in an effort to shorten the time between discovery and patient benefit by bringing together expertise in five areas: biomarkers and molecular targets, genomic approaches, human genomics and genetics, cancer inflammation, and integrative/systems biology and bioinformatics.

Cancer Genomics Research



This Circos plot visualizes data from The Cancer Genome Atlas (TCGA) and allows scientists to explore the interrelationships among different data points.

Credit: National Cancer Institute

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- Challenges in Cancer Genomics Research
- NCI's Role in Cancer Genomics Research

Why Genomics Research Is Critical to Progress against Cancer

The study of cancer genomes has revealed abnormalities in genes that drive the development and growth of many types of cancer. This knowledge has improved our understanding of the biology of cancer and led to new methods of diagnosing and treating the disease.

For example, the discovery of cancer-causing genetic andepigenetic changes in tumors has enabled the development of therapies that target these changes as well as diagnostic tests that identify patients who may benefit from these therapies. One such targeted drug is vemurafenib (Zelboraf), which was approved by the Food and Drug Administration (FDA) in 2011 for the treatment of some patients with melanoma who have a specific mutation in the BRAF gene as detected by an FDA-approved test.

Over the past decade, large-scale research projects have begun to survey and catalog the genomic changes associated with a number of types of cancer. These efforts have revealed unexpected genetic similarities across different types of tumors. For instance, mutations in the HER2 gene (distinct from amplifications of this gene, for which therapies have been developed for breast, esophageal, and gastric cancers) have been found in a number of cancers, including breast, bladder, pancreatic, and ovarian.

Researchers have also shown that a given type of cancer, such as breast, lung, and stomach, may have several molecular subtypes. For some types of cancer, the existence of certain subtypes had not been known until researchers began to profile the genomes of tumor cells.

The results of these projects illustrate the diverse landscape of genetic alterations in cancer and provide a foundation for understanding the molecular basis of this group of diseases.

Opportunities in Cancer Genomics Research

Although a large number of genetic alterations that

drive the development and progression of many types of cancer have been identified through large-scale research studies, some tumor types have not been deeply characterized. New technologies and the knowledge gained from previous genomic studies could be used to define the full set of driver mutations and other alterations to DNA and RNA in many cancers. Studies that compare genomic information from tumors and normal tissue from the same patient allow researchers to discover genomic changes that may drive cancer.

Another opportunity is to expand the current use of genomic methods to investigate the molecular basis of clinical phenotypes. This approach could help researchers identify genetic changes that may distinguish aggressive cancers from indolent ones, for example. Similar approaches could be used to study the molecular basis of response to a given therapy, as well as mechanisms of resistance to treatment.

The wealth of data emerging from cancer genome studies increasingly will be integrated with patients' medical histories and clinical data. These integrated results could be used to develop more tailored approaches to cancer diagnosis and treatment, as well as to improve methods of predicting cancer risk, prognosis, and response to treatment.

Genomic tools will also be essential for analyzing results from precision medicine clinical trials, such as those being conducted by NCI's National Clinical Trials Network.

Challenges in Cancer Genomics Research

Comprehensive analysis of cancer genomes has revealed a great deal of diversity in the genetic abnormalities found within cancers of a single type. Moreover, recurrent genetic alterations within these cancers are often involved in only a small percentage of cases. Identifying which genetic changes initiate cancer development and discovering rare genetic alterations that drive cancers are therefore challenges for the field.

Another challenge is acquiring high-quality biological samples needed for genomic studies, particularly for tumor types that are uncommon or rare, or those not treated primarily by surgery.

Developing cell lines and animal models that capture the diversity of human cancer is also an unmet need. Models of rare cancer subtypes may be nonexistent or underrepresented, and there are no models for many recurrent genetic lesions in human cancer.

Managing and analyzing the vast amounts of data involved in genomic studies are additional challenges for the field. This area of research requires an efficient bioinformatics infrastructure and increasingly involves contributions of data and expertise from cross-disciplinary teams.

NCI's Role in Cancer Genomics Research

Pursuing the genetic foundations of cancer is a vital part of NCI's research efforts. NCI's Center for Cancer Genomics (CCG) focuses on the study of how altered genes promote cancer. CCG uses high-throughput techniques to identify and study mutations, large rearrangements of the genome, increases and decreases in DNA copy number, chemical modifications to DNA, and changes in the expression of RNA and proteins. NCI supports diverse cancer genomics research and related efforts to translate these findings into clinical advances for patients.

The institute also encourages collaborations to advance cancer genomics research and discussion about opportunities and research priorities that could lead to new insights into etiology, outcomes, and risk factors for cancer. In October 2018, for example, NCI convened a meeting of experts to discuss future directions in the characterization of mutational signatures in cancer research.

Characterizing Cancer Genomes

NCI investigators analyze the DNA and RNA of cancer cells using advanced technologies such as next-generation DNA sequencing to map the landscape of the cancer genome and discover new changes linked to disease. NCI studies commonly use multiple genomic techniques. Integrating the results from several analyses helps scientists gain a better understanding of cancer, much like combining magenta, cyan, and yellow inks can generate vibrant color prints.

- The Cancer Genome Atlas (TCGA), a collaboration between NCI and the National Human Genome Research Institute (NHGRI), and Therapeutically Applicable Research to Generate Effective Treatments (TARGET) have characterized thousands of genomes and matched normal samples. This large number is important for discovering DNA, RNA, and protein abnormalities that are responsible for cancers in small numbers of patients.

- The Cancer Genome Characterization Initiative (CGCI) also studies cancer genomes, including cancers associated with HIV infection.
- CCG characterizes cancer genomes through its Genome Characterization Pipeline, which converts tissue samples donated by patients into high quality, publicly available genomic data.
- TCGA and TARGET taught the research community the importance of combining patients' medical data with cancer genomics data, leading to NCI programs that integrate rich genomic and clinical datasets.

Analyzing Standard of Care and Novel Treatments at a Molecular Level

Collaborative programs within and outside of NCI are collecting genomic data from patients receiving standard cancer treatments and patients receiving investigational treatments in clinical trials. These research collaborations have the power to answer questions critical to improving cancer outcomes, such as how tumors develop drug resistance, and what treatments are most effective against particular genomic traits.

- The Clinical Trials Sequencing Project (CTSP), a collaboration between CCG and NCI's Division of Cancer Treatment and Diagnosis, and the Cancer Driver Discovery Program (CDDP), characterize tissue samples from patients who have undergone standard-of-care or investigational treatments. These programs seek to understand the genomic basis of cancer development, metastasis, and drug resistance.
- The Exceptional Responders (ER) Initiative analyzes the genetic basis of exceptional responses to therapy. In some cases, a trial of a new drug fails to help most patients, but one or two people treated with the drug benefit. ER investigates the reason for exceptional responses to help assign the right treatments to the right patients in the future.
- ALCHEMIST, a set of precision medicine lung cancer trials, screens participants' genomes for molecular targets of currently available targeted therapies. By analyzing patients' tumors over the course of their treatment, ALCHEMIST aims to uncover how different cancers respond to targeted drugs and how tumors evolve during treatment.

Modelling the Activity of Cancer Genes

To translate genomic insights to the clinic, the activity of potential cancer genes that have been identified must be tested in models of cancer. These models can be cancer cell lines, organoid tissues, mice, or other model organisms. NCI supports research that helps bridge the gap between initial genomic discoveries and translation.

- The Cancer Treatment Discovery and Development Program (CTD2) is a network of scientific laboratories devoted to translating genomics into clinical benefit. CTD2 researchers study how genes linked to cancer work in cells and explore opportunities to target vulnerabilities with new therapies.
- The Human Models Cancer Initiative (HCMI) is generating new cancer models using cutting-edge technologies. These models will provide researchers with more accurate representations of a wide variety of cancers, and genomic characterization of the models may reveal links between genomic traits and how cells behave.

Relating Inherited Risk Factors to Cancer Genomics

Researchers in NCI's Division of Cancer Epidemiology and Genetics (DCEG) integrate tissue profiling into studies examining the causes of cancer to better understand the process by which normal cells are transformed into cancer cells (carcinogenesis) and to pinpoint factors associated with risk for developing specific molecular or genomic subtypes. DCEG investigators are also working to identify novel molecular and genomic signatures in tumors that are linked to germline genetic variants and environmental exposures, such as cigarette smoking and ionizing radiation. This approach will help identify new risk factors and yield novel insights into biological mechanisms of carcinogenesis.

- The DCEG Laboratory of Translational Genomics investigates the biology underlying the association between common and inherited genetic variants and cancer susceptibility with the goal of understanding how genetic variation contributes to cancer etiology and outcomes.
- The DCEG Cancer Genomics Research Laboratory supports epidemiologic research by processing, characterizing, and analyzing tissue collections and other samples using genome-wide association studies, DNA sequencing, and candidate gene studies.

Data Sharing

NCI has spearheaded genomic data sharing practices since the inception of TCGA. NCI's support for cancer genomic data sharing continues by making all the data as open and accessible as possible while protecting patient privacy.

BRCA Exchange: A Global Resource on Gene Variants

The large data sharing project will inform understanding of cancer risk.

- The NCI Genomic Data Commons is a data sharing platform that harmonizes diverse datasets and provides streamlined access for the research community. The GDC contains NCI-generated genomic datasets such as TCGA and TARGET, and continually expands its catalog by incorporating data submissions from research organizations, advocacy foundations, and industry. By standardizing patient medical information and raw genomic data using the latest bioinformatics pipelines, the GDC provides high quality processed data.
- The Cancer Genomics Cloud Pilots facilitate large-scale computing on NCI genomic data by making them accessible through commercial cloud providers. The Cloud Pilots can reduce costs and increase efficiency for big data analyses.

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Childhood Cancers Research

Phineas Sandi, shown here on his first day of kindergarten, participated in an NCI clinical trial that tested genetically engineered T cells to treat acute lymphoblastic leukemia. He was in remission within 11 days of starting the trial and remains free of cancer.

Credit: Kristina Sandi

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Why Research Is Critical to Progress against Childhood Cancer

Cancer is the leading cause of death from disease among children and adolescents in the United States. Although substantial progress has been made in the treatment of several types of childhood cancer over the past five decades, progress against other types has been limited. Even when long-term survival is achieved, many survivors of childhood cancer may experience long-term adverse effects from the disease or its treatment.

Clearly, more research is needed to develop new, more-effective, and safer treatments for childhood cancer. And infrastructure and practices that allow researchers to learn from every child with cancer need to be put in place.

NCI has a number of programs that address childhood cancers specifically, and many of the institute's other research programs are applicable to children with cancer even if they aren't focused specifically on pediatric cancers. The institute supports a broad range of biomedical research that is relevant to this population, including:

- Basic research to enhance our understanding of the fundamental mechanisms of cancer
- Clinical research to test new treatments for safety and effectiveness
- Survivorship research to reduce the long-term adverse effects of cancer and its treatment

Challenges in Childhood Cancer Research

NCI's Rare Cancer Clinics Fostering Collaboration

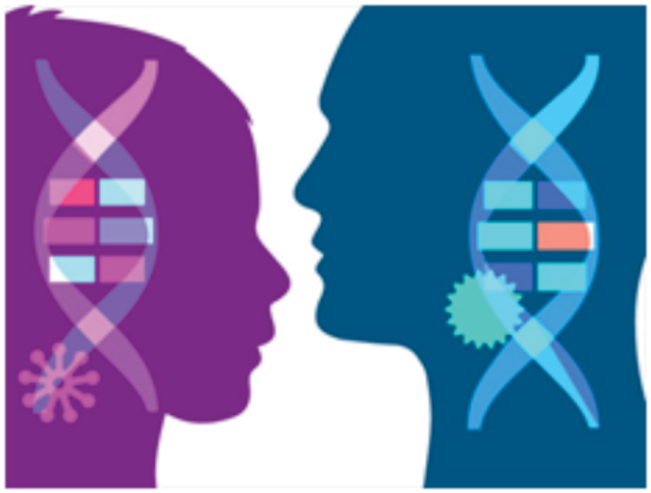
Clinics bring together clinicians, patients, and advocates.

One challenge in conducting research on childhood cancer is that cancers in children and adolescents are relatively uncommon. Childhood cancers represent less than 1% of all new cases of cancer diagnosed in the United States each year. Because the number of children with cancer is small and patients are treated at many different institutions, answering complex biological questions about childhood cancer requires collaboration.

As clinical trials are increasingly restricted to smaller numbers of patients who are defined by the molecular characteristics of their tumors, rather than where the tumors originated in the body, collaboration among children's cancer centers and a strong national clinical research program will continue to be essential to ensure that trials enroll sufficient numbers of participants to produce meaningful results.

In addition, more efficient ways to curate and share research knowledge—from genomic data to clinical outcomes—need to be developed to speed progress against childhood cancers.

Another challenge is that the causes of most childhood cancers are unknown. A small percentage of cancers in children and adolescents can be linked to inherited genetic abnormalities or exposures to diagnostic or therapeutic radiation. Environmental exposures, including infectious agents and toxic chemicals, that may play a role in childhood cancers have been difficult to identify, partly because cancer in children is rare and partly because it is difficult to determine what children might have been exposed to early in their development. As a result, identifying opportunities to prevent childhood cancer may be difficult.



Childhood and Adult Leukemia Differ Genetically

Findings have influenced clinical trials testing therapies for kids with AML.

In addition, the types of cancers children develop, and the biology of those cancers, generally differ from those of cancers diagnosed in adults. For example, tumors of developing organs and tissues (such asretinoblastomas in the eye and osteosarcomas in bone) are more common in children.

Moreover, most childhood cancers have relatively few genetic alterations, and they often lack the genetic targets for treatments that have been developed and approved for cancers occurring in adults. And drugs that target signaling pathways that are active in some adult cancers might be difficult to use in children, given that many of these signaling pathways are essential for normal development.

In fact, childhood cancers are often driven by genetic alterations that are distinct from those that occur in adult cancers. As an example, some childhood cancers are initiated by fusion genes that result from chromosomal translocations that produce "fusion oncoproteins." Few treatments have been developed to date that target these types of cancer-causing genetic alterations. Another contributing factor to the small number of targeted therapies for childhood cancers is that the rarity of these diseases has been an impediment to commercial drug development.

Searching for Less-Toxic Cancer Treatments for Kids

Gregory Aune, M.D., Ph.D., focuses on identifying biomarkers of toxicity caused by powerful cancer drugs.

Additional challenges in childhood cancer research are developing new treatments that are less toxic and cause fewer adverse effects (both acute and late) than current treatments and developing interventions to mitigate the adverse effects of both current and future treatments. The late effects of childhood cancer therapy can have profound physical, emotional, and other consequences for survivors, including a shortened life expectancy. How to minimize and address these late effects to improve both the quality and the length of life of survivors is a research priority.

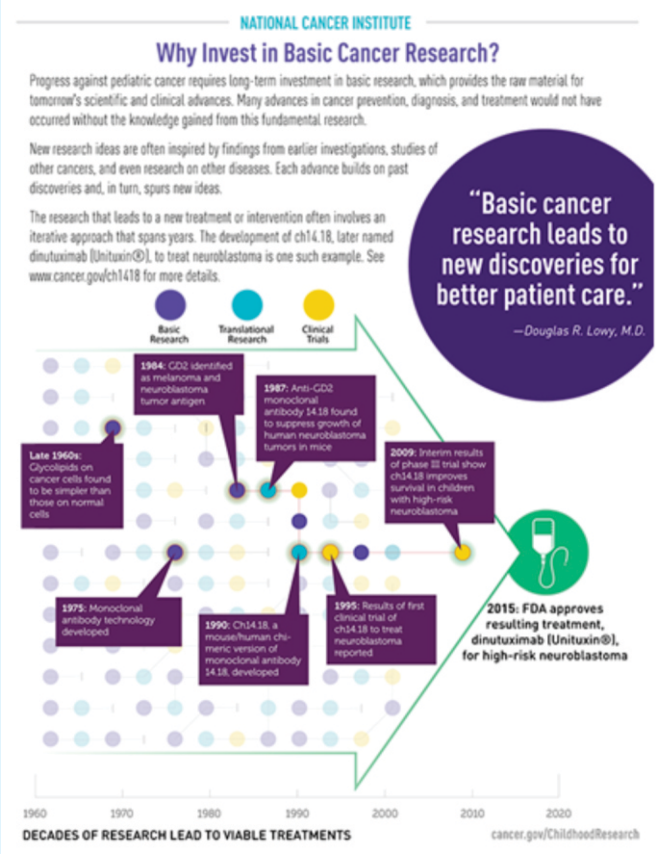
NCI has issued a funding opportunity announcement to improve outcomes for pediatric, adolescent, and young adult cancer survivors. The funding will stimulate the development and testing of interventions to prevent, lessen, and manage the adverse physical and psychosocial effects of cancer and its treatment in these patients.

More information about drug metabolism in children, which varies with developmental age, is also needed, as are better laboratory andanimal models for screening and testing drugs for potential use in children and adolescents. The optimal use of radiation therapy in treating childhood cancers also needs to be defined so that efficacy is maintained or increased while long-term side effects are reduced.

Basic Research Drives Progress against Childhood Cancer.

Virtually all progress against cancer in both children and adults has its origins in basic research, often in areas that are not directly related to the disease.

As an example, the discovery of CRISPR-Cas9 for gene editing has revolutionized the study of genes that control cancer cell growth and survival in both childhood and adult cancers. This discovery came from basic research in microbiology on the mechanisms by which bacteria resist infections by viruses.



Another example had its origins in basic research on proteins called histones, which are DNA-binding proteins that provide structural support for chromosomes and help control the activity of genes. Scientists spent years investigating how these proteins are modified in the cell nucleus and the role of histone modifications in controlling when and to what extent genes are expressed.

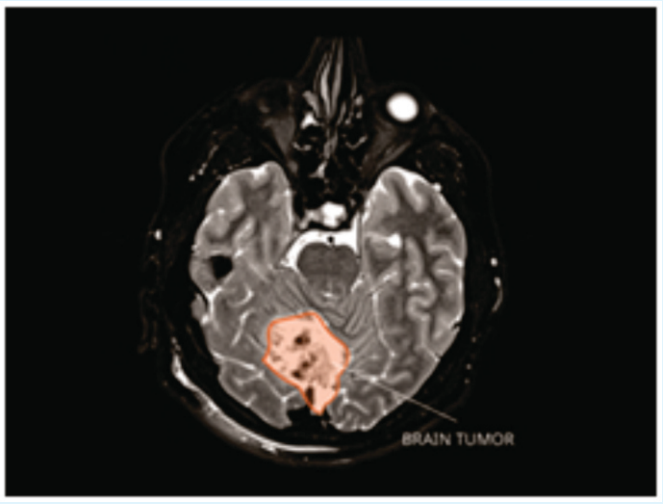
The findings of this research became immediately relevant to a type of pediatric brain tumor called diffuse intrinsic pontine glioma (DIPG) when it was discovered that most DIPG tumors have a mutation in the gene for the histone protein H3.3 that prevents a specific modification of the protein. This mutation in H3.3 is thought to be a driver mutation for DIPG and is associated with aggressive disease and shorter survival.

Promising Areas of Research on Childhood Cancers

Although our understanding of the biology underlying cancers that occur in children and adolescents has increased tremendously in the past decade, there are still critical gaps in our knowledge. NCI has identified several areas in which more research is needed and has identified opportunities to use new approaches to gain additional insights into childhood cancers.

Immunotherapies for Childhood Cancers

Immunotherapies are treatments that restore or enhance the immune system's natural ability to fight cancer. In the past few years, the field of cancer immunotherapy research has produced several new methods for treating cancer.



CAR T-Cell Study Suggests Promise for Childhood Cancers

In mouse models of common pediatric cancers, treatment shrank or eradicated tumors.

One example is chimeric antigen receptor (CAR) T-cell therapy, which has been shown to induce sustained remissions in pediatric patients with acute lymphoblastic leukemia (e.g., as seen in clinical trials ofCTLO19 and CD19-CAR T) and B-cell leukemias and lymphomas (e.g., in trials of CD19/CD22-CAR T). This therapeutic approach arose from decades of research on how the immune system works and how to manipulate it for clinical benefit.

Early investigations by NCI scientists Lawrence Samelson, M.D., and Richard Klausner, M.D., on the structure of the T-cell receptor and the role this receptor plays in T cell activation, as well as the pioneering work of NCI's Steven Rosenberg, M.D., Ph.D., and his colleagues on an immunotherapy

technique called adoptive cell transfer (ACT), helped pave the way for this major treatment advance.

The NCI Center for Cancer Research's Pediatric Oncology Branch (POB) conducts clinical trials of immunotherapy in pediatric and young adult patients, and the Children's Oncology Group (COG) and the Pediatric Brain Tumor Consortium are evaluating immunotherapy treatments for selected childhood cancers. The Cancer Immunotherapy Trials Network (CITN) has a pediatric component that is developing clinical trials to test immunotherapies for children with cancer.

As part of the Cancer Moonshot, NCI has established the Fusion Oncoproteins in Childhood Cancers (FusOnC2) Consortium and Pediatric Immunotherapy Discovery and Development Network (PI-DDN). Learn about FusOnC2 and PI-DDN below, in How NCI Programs Are Making a Difference in Childhood Cancer.

Molecularly Targeted Therapies for Childhood Cancers

Molecularly targeted therapies are drugs or other substances that kill cancer cells by targeting specific molecules that are necessary for cancer cells to grow and survive. These therapies can be small-molecule inhibitors, monoclonal antibodies, or antibody–drug conjugates.

POB conducts clinical trials of targeted therapy in pediatric and young adult patients, and COG and the Pediatric Brain Tumor Consortium are evaluating targeted therapies for selected childhood cancers.

For example, results from an NCI-sponsored clinical trial, conducted by COG and led by Alice Yu, M.D., Ph.D., of the University of California, San Diego, led to the approval of the monoclonal antibody dinutuximab (Unituxin) to treat high-risk neuroblastoma.

Additionally, the Pediatric Brain Tumor Consortium (PBTC) studied the targeted agent selumetinib in children with relapsed or refractory low-grade gliomas. Reductions in tumor size were observed in most patients. Based on these results, COG will be studying selumetinib in phase 3 clinical trials for children with newly diagnosed low-grade glioma.

In 2017, NCI and COG launched the NCI–COG Pediatric Molecular Analysis for Therapy Choice (Pediatric MATCH) trial, which is testing molecularly targeted therapies in children with advanced solid tumors that are not responding to treatment. Tumor DNA sequencing is being used to identify those children whose cancers have a genetic abnormality that is targeted by a drug being studied in the trial.

And, more recently, NCI's Small Business Innovation Research (SBIR) program issued an award to expand and accelerate clinical trials testing the experimental small-molecule inhibitor ONC201 in patients with a specific type of lethal brain cancer called H3 K27M-mutant glioma (2R44CA192427-04).

How NCI Programs Are Making a Difference in Childhood Cancer

NCI recognizes that children and adolescents are not just small adults and that specialized treatments tailored to childhood cancers are needed. Therefore, NCI supports an array of programs specifically to advance childhood cancer care and has renewed these initiatives and programs over numerous funding periods. Some of these programs include:

Improving Outcomes for Patients with Nf1

Philip Moss had run out of treatment options for his neurofibroma tumors when he joined a clinical trial at NIH.

o The Pediatric Oncology Branch (POB) in NCI's Center for Cancer Research conducts high-risk, high-impact basic, translational, and clinical research on childhood cancers. For example, POB Chief Brigitte Widemann, M.D., recently led the only treatment trial in the nation for children with tumors caused by neurofibromatosis type 1 (NF1), a genetic disorder in which painful and often disfiguring tumors of the nerves can grow on or under the skin. The phase I trial showed that children tolerated the drug selumetinib, and nearly all experienced tumor shrinkage.

o The Human Genetics Program (HGP) in NCI's Division of Cancer Epidemiology and Genetics (DCEG) conducts clinical, genetic, molecular pathology, and epidemiological studies of children at high risk of cancer. For example, researchers from the program are leading a genome-wide association

study of Ewing sarcoma to better understand the genetic architecture of the disease and to identify regions of the genome that may increase risk. HGP researchers are also studying osteosarcoma to better understand the role that genetic variation plays in risk and patient outcomes and identify genes or genomic regions that may be important in osteosarcoma. The program also studies familial cancer syndromes, including Li-Fraumeni Syndrome, DICER1 syndrome,NF1, and inherited bone marrow failure syndromes (IBMFS), to better understand these disorders and investigate possible genotype/phenotype relationships that will improve clinical management and aid in genetic counseling.

Fueling Progress against Childhood Leukemia

TARGET initiative leads to clinical trials of targeted therapies.

- The Therapeutically Applicable Research to Generate Effective Treatments (TARGET) program uses genomic approaches to catalog the molecular changes in several types of childhood cancer to increase our understanding of their pathogenesis, improve their diagnosis and classification, and identify new candidate molecular targets for better treatments. For example, TARGET researchers performed a pan-cancer study of somatic alterations in nearly 1,700 pediatric leukemias and solid tumors and found major genomic differences when compared with adult cancers. The related Cancer Genome Characterization Initiative (CGCI) includes genomic studies of various pediatric cancers that often do not respond well to treatment.
- The Children's Oncology Group (COG)Exit Disclaimer, which is part of NCI's National Clinical Trials Network (NCTN), develops and coordinates pediatric cancer clinical trials that are available at more than 200 member institutions, including cancer centers throughout the United States and Canada. In addition to conducting traditional late-phase clinical trials, COG has established the Pediatric Early Phase-Clinical Trial Network (PEP-CTN)Exit Disclaimer to conduct early-phase trials and pilot studies so new anticancer agents can be rapidly and efficiently introduced into pediatric cancer care.
- The NCI–COG Molecular Analysis for Therapy Choice (Pediatric MATCH) precision medicine trial is a nationwide trial to explore whether targeted therapies can be effective for children and adolescents with solid tumors that harbor specific genetic mutations and have progressed during or after standard therapy. The trial, which is funded by NCI and conducted by COG, opened to patient enrollment in 2017. Germline testing is performed on all enrolled patients to assess whether the genetic aberrations identified in their tumors are inherited. The genomic data captured in the trial will serve as an invaluable resource for researchers seeking to understand the genetic basis of pediatric cancer.

Gabriella Miller Kids First PEDIATRIC RESEARCH PROGRAM

- The Pediatric Brain Tumor Consortium (PBTC)Exit Disclaimer is a multidisciplinary cooperative research organization devoted to identifying better treatment strategies for children with primary brain tumors.

Kids First Pediatric Research Program Moves Forward

Researchers developing central resource for data sharing.

o NCI participates in the Gabriella Miller Kids First Pediatric Research Program, which is building a rich data resource (sponsored by the National Institutes of Health) to increase knowledge about the genetic changes associated with childhood cancers and structural birth defects. The program allows investigators from

different research communities to share data and collaborate, and it encourages new ways of thinking about childhood diseases.

- The Childhood Cancer Survivor Study (CCSS) is examining the long-term adverse effects of cancer and cancer therapy on approximately 35,000 survivors of childhood cancer who were diagnosed between 1970 and 1999. The study was created to gain new knowledge about the long-term effects of cancer and its treatment, and educate survivors and the medical community about the potential impacts of a cancer diagnosis and treatment. The results obtained from CCSS are used to help design treatment protocols and interventions that will result in an increase in survival, while minimizing harmful late effects. This research is also used to develop and expand programs for early detection and prevention of late effects in children and adolescent cancer survivors. For example, to better understand the genetic risk of second cancers, DCEG and CCSS researchers are collaborating on studies that aim to identify both common and rare genetic variants that may be associated with second cancers or other late adverse effects among survivors of childhood cancer. In a related study, DCEG scientists also are studying the long-term health of survivors of retinoblastoma, following a cohort of individuals with hereditary or nonhereditary disease to understand how retinoblastoma treatments impact risk for second cancers and long-term mortality.

Using Tandem Transplants to Treat Neuroblastoma

Julie Park, M.D., led a trial that changed the way neuroblastoma is treated in the US.

- The New Approaches to Neuroblastoma Therapy (NANT)Exit DisclaimerConsortium consists of a multidisciplinary team of laboratory and clinical scientists focused on improving outcomes for patients with high-risk neuroblastoma by discovering mechanisms of resistance to therapies, discovering targetable vulnerabilities driving resistance, and translating these insights into clinical trials. NANT works closely with COG to translate their experimental therapy findings into COG phase 3 clinical trials. Their findings regarding the tumor microenvironment, tumor response to therapy, and the application of cellular therapies to solid tumors have implications beyond neuroblastoma.
- The Pediatric Preclinical Testing Consortium (PPTC) systematically evaluates new agents in genomically characterized models of childhood cancer. The primary goal of the PPTC is to develop high-quality preclinical data to help pediatric oncology researchers identify agents that are most likely to show significant anticancer activity when tested in the clinic against selected childhood cancers.
- The Pediatric Genomic Data Inventory (PGDI) is an open-access resource to help researchers access data from genomic sequencing projects for pediatric cancer. The inventory lists ongoing and completed sequencing projects from the United States and other countries, the type of cancer studied, molecular characterization data available, and points of contact for each project.
- The Hyperactive RAS Specialized Programs of Research Excellence (SPOREs) focus on developing better treatments for neurofibromatosis type 1 and related cancers in children, adolescents, and young adults.
- The Fusion Oncoproteins in Childhood Cancers (FusOnC2) Consortium is a multidisciplinary, collaborative network of investigators studying select fusion oncoproteins implicated in childhood cancers that have a high risk of treatment failure and for which there has been little progress in identifying targeted agents.
- The Pediatric Immunotherapy Discovery and Development Network (PI-DDN) is a collaborative research network identifying and advancing research opportunities for translating immunotherapy concepts for children and adolescents with cancer toward clinical applications. Primary goals of the PI-DDN include the discovery and characterization of immunotherapy targets for childhood and adolescent cancers, the development of new immunotherapy treatment approaches, and an improved understanding of theimmunosuppressive tumor microenvironment in order to advance new, more

effective immune-based treatment regimens for high-risk pediatric cancers.

- The Human Tumor Atlas Network (HTAN) is a collaborative network that is constructing multidimensional tumor atlases to document the molecular and cellular alterations and interactions within tumors as they develop and evolve. The atlases will represent a diverse patient population—including children—and describe the dynamics of cancer, focusing on the transition from precancer to malignancy, from local invasion to distant metastasis, and how tumors respond to treatment and develop resistance to drugs.
- The Pediatric Cancer Immunotherapy Trials Network (CITN) is using the clinical trials infrastructure of theCITN to conduct clinical trials of immunotherapy agents of specific relevance to children and adolescents with cancer. Examples of the types of novel treatments to be investigated by the Pediatric CITN include cellular therapies (e.g., CAR T cells targeting pediatric cancer antigens) and antibody-based therapies, including antibody-drug conjugates, that target surface antigens preferentially expressed on childhood cancers.
- The My Pediatric and Adult Rare Tumor (MyPART) network of scientists, patients, family members, advocates, and health care providers is working together to help find new treatments for rare childhood, teen, and young adult solid tumors that have no cures. Working as a team, researchers share data and help design experiments and clinical trials, advocates discuss issues important to patients, and clinicians share their experiences treating rare cancers. MyPART is part of the larger NCI Rare Tumor Patient Engagement Network.
- DCEG researchers collaborate with the International Childhood Cancer Cohort Consortium (I4C) and theChildhood Leukemia International Consortium (CLIC)Exit Disclaimer, collaborations that pool information from cohort studies from around the world to answer questions about childhood cancers. I4C brings together multidisciplinary teams of epidemiologists, basic scientists, and clinicians, to collaborate on investigations into the role of early-life exposures on cancer risk. CLIC includes more than 30 case–control studies and has identified associations between childhood leukemia and environmental risk factors.

Sen. John McCain's death continues to highlight desperate push to cure brain cancer

- The death of Sen. John McCain from glioblastoma on Saturday once again sheds light on this devastating illness and the need to find a cure. It is the same brain cancer that took the life of Joe Biden's son, Beau, back in 2015, and former Sen. Ted Kennedy in 2009.
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"It is very sad about the outcome of Sen. McCain, yet it reminds us how brutal and deadly this disease can be and how much work is still ahead of us to combat it," said Mazen Kamen, president of the Kamen Brain Tumor Foundation in New York City. The nonprofit organization aims to provide new and effective treatment strategies for brain cancer, especially for children. Kamen's own son lost his battle to glioblastoma in 2016.

Daunting statistics

Sen. McCain's brain cancer was determined just a year ago after he underwent surgery to remove a blood clot over his left eye at the Mayo Clinic Hospital in Phoenix. Lab results determined there was a link between the clot and the tumor.

Glioblastoma multiforme is the most common and deadliest of the glial tumors because the cells reproduce so rapidly. They can be found anywhere in the brain or spinal cord. The tumor grows by turning normal brain cells into stem cells, which continuously replicate and regrow. So even if a tumor is surgically removed, it is difficult to extract every cancerous cell; any left behind will result in the growth of a new tumor.

According to the American Brain Tumor Association, there are approximately 700,000 people in the United States living with a primary brain and central nervous system tumor. Of these, 14.9 percent are glioblastomas. Glioblastomas represent the highest number of cases of all malignant tumors, with an estimated 12,760 new cases predicted in 2018.

Since 1985, there have been only four FDA-approved drugs to treat the more than 120 different types of brain tumors , according to

the National Brain Tumor Society. Between 1998 and 2014, claims the NBTS, 78 investigational brain tumor drugs were entered into the clinical trial evaluation process, and 75 of them failed.

More from Modern Medicine:

Immunotherapy drugs slow skin cancer that has spread to the brain

Hope for Lyme disease victims: Race is on to develop new tests — and a vaccine

Heart disease is becoming a big red-state problem

Traditional therapies for brain cancer — surgery, chemotherapy and radiation — haven't been very successful, said Dr. José Baselga, chief medical officer and physician-in-chief at Memorial Sloan Kettering Cancer Center, because the brain is designed so toxins can't infiltrate it. "It is very challenging to have chemotherapy get to the brain with a good dose," he said. Instead, Dr. Baselga says researchers and scientists are making strides in what he calls precision medicine.

Making new inroads in lifesaving research

Kamen and his wife Jill, both founders of the Kamen Brain Tumor Foundation, are committed to the promise of precision medicine.

Their foundation recently provided funding to the Dana-Farber Cancer Institute to continue its research on immunotherapy — testing chemotherapy agents in the lab beforehand to see if they can cross the blood-brain barrier.

"In the past, there was no way of knowing for sure which agents could cross the blood-brain barrier and which ones could not. Now they can see which ones will infiltrate the tumor in the hope of killing it," said Mazen.

The Kamen Brain Tumor Foundation also recently funded Memorial Sloane Kettering Cancer Center's research on intrathecal radioimmunotherapy, in which immune drugs are tagged with radiation and are inserted directly into the fluid surrounding the brain and spinal cord to kill the tumors. Injecting the tumor directly with meds, including immune meds, and watching the progress is a way to avoid surgery, said Mazen.

Their latest research involves growing the actual brain tumor tissue extracted from the patient in the laboratory, said Mazen. "Now we have a way to deliver medicines through the blood-brain barrier, something we were not able to do before," he said. "In the lab, scientists can determine which therapy — meds, radiation, immune therapy — works and then apply that to the patient and be comfortable that it will concentrate in the tumor tissue. This avoids giving therapies that do not work and only give serious side effects," said Mazen.

Another trial under way now: tagging the patient's own T-cell immune cells with a specific virus in the lab and injecting them into the patient's bloodstream in the hope that it will directly attack the tumor's blood supply.

Mazen and Jill Kamen are hopeful that time will tell.

"Sen. McCain was not only a hero in his lifetime but also a hero during his illness. He was a fighter and took his illness with dignity and grace. This is a life lesson to all of us who face adversity in life," said Mazen. "He was a great man, and we owe it to future generations to find a cure to this devastating disease."

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Items and personal notes are left outside the office of Sen. John McCain (R-AZ) as people pay their respects to the late Arizona senator on August 26, 2018 in Phoenix, Arizona.

- Ralph Freso | Getty Images
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Cervical cancer research

Research into the symptoms of cervical cancer

A UK study wanted to find out more about the symptoms and experiences that young women had before being diagnosed with cervical cancer.

The study team found that many of the women (aged 18 to 29) didn't know the symptoms of cervical cancer and delayed going to see the doctor about them.

The team suggested that information about symptoms should be improved and this could help cervical cancer to be diagnosed earlier.

Research into cervical screening tests

We know there is a link between the development of abnormal cells in the cervix and infection with the human papilloma virus (HPV). Researchers have developed tests to find HPV in cervical screening samples. This shows which women should be referred for an examination of the cervix called colposcopy.

If a woman's cervical screening test shows borderline or low grade abnormal cell changes (dyskaryosis), laboratories test the sample of cells for HPV. If HPV is present, the woman is referred for a colposcopy test.

Screening for HPV first

In some areas of England, the screening programme is looking at testing for HPV first. So if HPV is found, the woman's sample is then checked for abnormal cells.

Researchers think this might be better because HPV testing picks up more abnormal cells in the cervix. Also, if your sample has no HPV it is unlikely that cervical cancer will develop in the next few years. So these women might not need to go for screening as often. But more studies are needed to know this for sure.

Researchers are continuing to look into other ways of testing for HPV for cervical screening. One trial looked at whether women could collect their own samples for HPV testing. The researchers found no real difference in test results between the HPV sample taken by the women themselves, and those done by a doctor or nurse.

A recent review of studies has shown that a urine test to pick up HPV might be useful for screening but more studies are needed before it can be used.

Research into preventing cervical cancer

HPV vaccines

Vaccines, such as Cervarix and Gardasil, have been developed to prevent HPV infection. There are many different HPV strains. HPV types 16 and 18 are known to increase the risk of cervical cancer.

Several research trials have tested vaccines as a way of preventing infection with HPV. The trials have shown that the vaccines help to prevent abnormal changes in the cervix that may develop into cancer. In the UK, HPV vaccination is offered in school to all girls aged 12 to 13.

Research suggests that this vaccination programme will greatly lower the number of cases of cervical cancer. It will also reduce the need for colposcopy.

Research into scans for cervical cancer

Researchers are looking at a type of magnetic resonance imaging (MRI) scan called diffusion weighted MRI for cervical cancer. The aim of this study is to find out if the diffusion weighted MRI can show whether cervical cancer is likely to have a good or a bad outcome.

Research into treatment for cervical cancer

Surgery

Surgery is the usual treatment for early stage cervical cancer.

Researchers are looking at different ways of doing surgery for early cervical cancer. They are comparing removal of the womb and cervix (a simple hysterectomy) with the usual treatment (a radical hysterectomy).

A radical hysterectomy involves removing:

- the womb (including the cervix)
- all the tissues holding the womb in place
- the top of the vagina
- all the lymph nodes around the womb

The trial wants to find out if a simple hysterectomy is as good as a radical hysterectomy in treating cervical cancer. It also wants to check if the simple hysterectomy gives fewer side effects and a better quality of life after the surgery.

Chemotherapy

Researchers are:

- comparing chemotherapy before surgery with chemotherapy and radiotherapy (chemoradiotherapy) in early cervical cancer
- looking into giving chemotherapy on its own before chemoradiotherapy starts for locally advanced cervical cancer.

- testing chemotherapy for advanced cervical cancer

Radiotherapy

Researchers are looking into ways of improving internal radiotherapy (brachytherapy) for cervical cancer.

They are also looking at increasing the radiation dose when giving external radiotherapy by using Intensity Modulated Radiotherapy (IMRT). The aim is to see if doctors can increase the radiation dose to the cancer, without causing more side effects than standard radiotherapy treatment.

Research is also looking into using different chemotherapy drugs alongside radiotherapy for cervical cancer. Researchers think they might be able to improve results by investigating other combinations of drugs.

Radiotherapy side effects

Researchers for the HOT II trial looked at whether using a high pressure oxygen treatment called hyberbaric oxygen (HBO) therapy could help to relieve the long term side effects of radiotherapy to the area between the hip bones (the pelvis). The results of the trial disagreed with other reports that say HBO is helpful. So the trial team felt larger trials are needed to know for sure.

Another study is looking at using a palm oil supplement and a drug called pentoxifylline to relieve symptoms caused by pelvic radiotherapy. The trial team want to find out if this combination of treatment helps, and to learn more about the side effects.

Researchers are also using a device called an electronic nose to see if they can predict long term changes in bowel function after pelvic radiotherapy.

Targeted cancer drugs

Targeted cancer drugs change the way that cells work. They can boost the body's immune system to fight off or kill cancer cells. Or they can block signals that tell cells to grow.

Research is looking into different types of targeted drugs for cervical cancer. These drugs are being tested in trials. They are being looked at alone or in combination with radiotherapy or chemotherapy to treat cervical cancer.

The drugs being tested include:

- bevacizumab
- nivolumab
- cediranib

A trial is looking at whether a vaccine against the human papilloma virus (HPV) can work as a treatment against some cancers, including cervical cancer.

Treatment for cervical cancer depends on how far the cancer has spread.

As cancer treatments are often complex, hospitals use multidisciplinary teams (MDTs) to treat cervical cancer and tailor the treatment programme to the individual.

MDTs are made up of a number of different specialists who work together to make decisions about the best way to proceed with your treatment.

Your cancer team will recommend what they think the best treatment options are, but the final decision will be yours. In most cases, the recommendations will be:

- for early cervical cancer – surgery to remove the cervix and some or all of the womb, or radiotherapy, or a combination of both
- for advanced cervical cancer – radiotherapy with or withoutchemotherapy, and surgery is also sometimes used

Cervical cancer is often curable if it's diagnosed at an early stage.

When cervical cancer is not curable, it's often possible to slow its progression, prolong lifespan and relieve any associated symptoms, such as pain and vaginal bleeding. This is known as palliative care.

The different treatment options are discussed in more detail in the following sections.

Removing very early cancer

Large loop excision of the transformation zone (LLETZ)

This is where the cancerous cells are removed using a fine wire and an electrical current.

It's usually done under local anaesthetic (while you're awake but the area is numbed) and can be done at the same time as a colposcopy.

Cone biopsy

A cone-shaped area of abnormal tissue is removed during surgery. This is usually done under general anaesthetic (while you're asleep).

Surgery

There are 3 main types of surgery for cervical cancer:

- trachelectomy – the cervix, surrounding tissue and upper part of the vagina are removed, but the womb is left in place
- hysterectomy – the cervix and womb are removed and, depending on the stage of the cancer, it may be necessary to remove the ovaries and fallopian tubes
- pelvic exenteration – a major operation in which the cervix, vagina, womb, ovaries, fallopian tubes, bladder and rectum may all be removed

Pelvic exenteration is only offered when cervical cancer has come back.

Trachelectomy

A trachelectomy is usually only suitable if cervical cancer is diagnosed at a very early stage. It's usually offered to women who want to have children in the future.

During the procedure, the cervix and upper section of the vagina are removed, leaving the womb in place. Your womb will then be reattached to the lower section of your vagina.

It's usually done by keyhole surgery.

Lymph nodes (part of the lymphatic system, the body's waste-removal system) from your pelvis may also be removed.

Compared with a hysterectomy or pelvic exenteration, the advantage of this type of surgery is that your womb remains in place. This means you may still be able to have children.

However, it's important to be aware that the surgeons carrying out this operation cannot guarantee you will still be able to have children.

A stitch will be put in the bottom of your womb during the surgery. This is to help support and keep a baby in your womb in future pregnancies. If you do get pregnant after the operation, your baby will have to be delivered by caesarean section.

It's also usually recommended you wait 6 to 12 months after surgery before trying for a baby so your womb and vagina have time to heal.

Trachelectomy is a highly skilled procedure. It's only available at certain specialist centres in the UK, so it may not be offered in your area and you may need to travel to another city for treatment.

Hysterectomy

A hysterectomy is usually recommended for early cervical cancer. This may be followed by a course of radiotherapy to help prevent the cancer coming back.

Two types of hysterectomies are used to treat cervical cancer:

- simple hysterectomy – the cervix and womb are removed and, in some cases, the ovaries and fallopian tubes are too; only appropriate for very early-stage cervical cancers
- radical hysterectomy – preferred option in advanced stage 1 and some early stage 2 cervical cancers; the cervix, womb, top of the vagina, surrounding tissue, lymph nodes, fallopian tubes and, sometimes, ovaries are all removed

Short-term complications of a hysterectomy include infection, bleeding, blood clots and accidental injury to your ureter, bladder or rectum.

Although the risk of them is small, long-term complications can be troublesome. They include:

- your vagina becoming shorter and drier, which can make sex painful
- urinary incontinence
- swelling of your arms and legs, caused by a build-up of fluid (lymphoedema)
- your bowel becoming blocked by a build-up of scar tissue – this may require further surgery

Because your womb is removed during a hysterectomy, you will not be able to have children.

If your ovaries are removed, it will also trigger the menopause if you have not already experienced it.

See complications of cervical cancer for more information about the menopause.

Pelvic exenteration

A pelvic exenteration is a major operation that's usually only recommended when cervical cancer comes back. It's offered if the cancer returns to the pelvis but has not spread beyond this area.

A pelvic exenteration involves 2 phases:

- the cancer and the vagina are removed – it may also involve removing the bladder, rectum or lower section of the bowel, or all 3
- 1 or 2 holes, called stomas, are created in your tummy – the holes are used to pass pee and poo out of your body into pouches called colostomy bags

Following pelvic exenteration, it may be possible to reconstruct your vagina using skin and tissue taken from other parts of your body. This would mean you could still have sex after the procedure, although it may be several months until you feel well enough to do so.

Radiotherapy

Radiotherapy may be used on its own or in combination with surgery for early-stage cervical cancer. It may be combined with chemotherapy for advanced cervical cancer, where it can be used to control bleeding and pain.

Radiotherapy can be delivered either:

- externally – a machine beams high-energy waves into your pelvis to destroy cancerous cells
- internally (brachytherapy) – a radioactive implant is placed next to the tumour inside your vagina

In most cases, a combination of internal and external radiotherapy will be used. A course of radiotherapy usually lasts about 5 to 8 weeks.

As well as destroying cancerous cells, radiotherapy can sometimes also harm healthy tissue. This means it can cause significant side effects many months, or even years, after treatment.

Brachytherapy aims to reduce harm to surrounding tissue by delivering the radiation as close as possible to the tumour, but it can still cause side effects.

However, the benefits of radiotherapy often tend to outweigh the risks. For some people, radiotherapy offers the only hope of getting rid of the cancer.

Side effects of radiotherapy are common and can include:

- diarrhoea
- pain when peeing
- bleeding from your vagina or rectum
- feeling very tired
- feeling or being sick
- sore skin, like sunburn, in your pelvis region
- narrowing of your vagina, which can make having sex painful
- infertility
- damage to the ovaries, which will usually trigger an early menopause if you have not already gone through it
- bladder and bowel damage, which could lead to incontinence

Most of these side effects will resolve within about 8 weeks of finishing treatment, although in some cases they can be permanent. It's also possible to develop side effects several months, or even years, after treatment has finished.

If infertility is a concern for you, it may be possible to surgically remove eggs from your ovaries before you have radiotherapy so they can be implanted in your womb at a later date. However, you may have to pay for this.

It may also be possible to prevent an early menopause by surgically removing your ovaries and replanting them outside the area of your pelvis that will be affected by radiation. This is called an ovarian transposition.

Your doctors can provide more information about the possible options for treating infertility and whether you're suitable for an ovarian transposition.

Chemotherapy

Chemotherapy can be combined with radiotherapy to try to cure cervical cancer, or it can be used as a sole treatment for advanced cancer to slow its progression and relieve symptoms (palliative chemotherapy).

Chemotherapy for cervical cancer usually involves using either a single chemotherapy drug, called cisplatin, or a combination of different chemotherapy drugs to kill the cancerous cells.

Chemotherapy is usually given straight into your vein using a drip. You will probably be seen as an outpatient so will be able to go home once you've received your dose.

As with radiotherapy, these medications can also damage healthy tissue. Side effects are therefore common and can include:

- feeling and being sick
- diarrhoea
- feeling tired all the time
- reduced production of blood cells, which can make you tired, breathless and vulnerable to infection
- mouth ulcers
- loss of appetite
- hair loss – cisplatin does not usually cause you to lose your hair, but other chemotherapy drugs may

If you do lose your hair, it usually should grow back within 6 months of the completion of your course of chemotherapy.

Some types of chemotherapy medication can damage your kidneys so you may need to have regular blood tests to assess the health of your kidneys.

Follow-up

After you finish your treatment and the cancer has been removed, you'll need to attend regular appointments for testing. This will usually involve a physical examination of your vagina and cervix (if it hasn't been removed).

Because cervical cancer can return, these examinations will be used to look for signs of this happening. If the examination finds anything suspicious, a further biopsy can be done.

Follow-up appointments are usually recommended every 3 to 6 months for the first 2 years, and then every 6 to 12 months for a further 3 years.

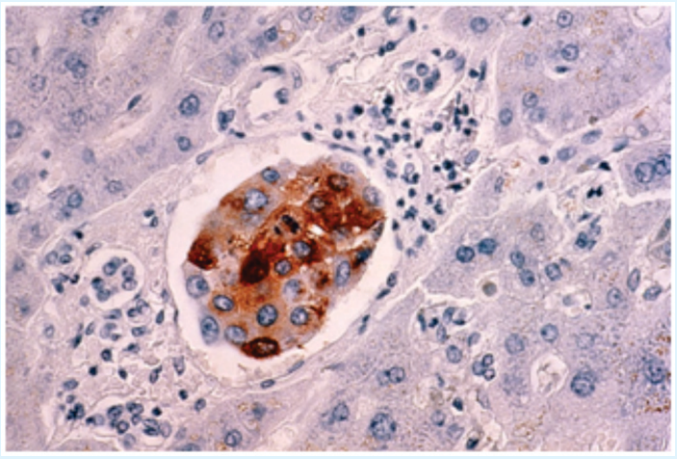
Your multidisciplinary team (MDT)

Members of your MDT may include:

- a surgeon
- a clinical oncologist (a specialist in chemotherapy and radiotherapy)
- a medical oncologist (a specialist in chemotherapy only)
- a pathologist (a specialist in diseased tissue)
- a radiologist (a specialist in imaging scans)
- a gynaecologist (a doctor specialising in treating conditions that affect the female reproductive system)
- a social worker
- a psychologist
- a specialist cancer nurse, who'll usually be your first point of contact with the rest of the team.

New Breakthrough in Breast Cancer Treatment announced in Amsterdam

Breast cancer is one of the most common cancers in women throughout both the developed and less developed world. Estimates from WHO says that over 508, 000 women worldwide died in 2011 due to breast cancer. And although it is thought to be a disease of the developed world, almost 50% of breast cancer cases and 58% of deaths occur in less developed countries.



Metastasizing cancer cells

At times, it seems like conquering all forms of cancer is a Sisyphean task. Made even more pronounced by the fact that since the refinement of modern medicine, we've successfully controlled smallpox, measles, tuberculosis, polio and other potentially fatal diseases. Cancer however, seems to always rear its ugly head like some kind of unassailable whack-a-mole.

The role of breast cancer awareness and treatment of breast cancer in the Netherlands is especially significant, given the fact that in 2012, it ranked fourth from the top out of 20 countries on a chart that recorded the age-standardised rate of breast cancer per 100,000.

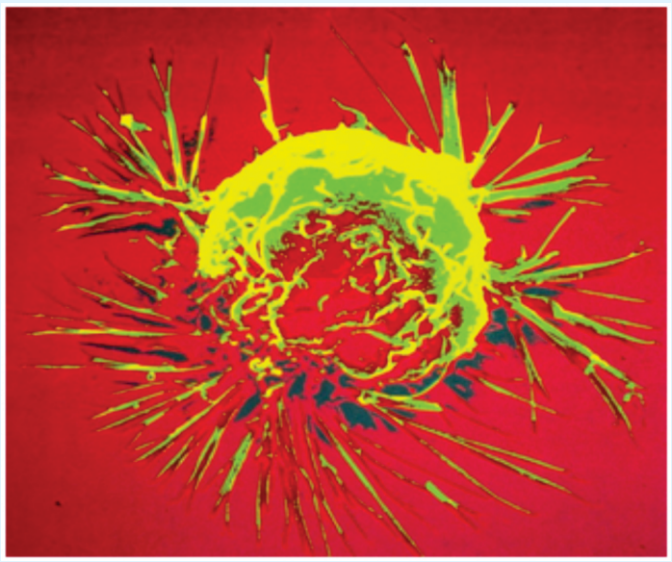
Perhaps, the prevalence of breast cancer in the Netherlands and in fact, the world, is about to take a hit. If you've planned on moving to Amsterdam you now have another great reason to live in this great city. You will get world-class medical attention if you're ever in a battle with breast-cancer.

Research recently presented by Professor Nigel Bundred at the European Breast Cancer Conference in Amsterdam revealed that the effectiveness of two types of drugs known as Herceptin and Lapatinib had been tested, and results looked promising.

Breast cancer awareness. An ongoing battle for the greater good.

This pair of drugs is commonly used in breast cancer treatment at the moment, but this is the first time they had been combined and used both before surgery and chemotherapy. Some types of breast cancer were eliminated in just 11 days.

Cancer Research UK funded the study and testing. They aimed to use these drugs to combat a protein called HER2 (human epidermal growth factor receptor 2), which affects the growth and division of cancer cells. It's also more likely to return than other cancer types.



An artist's depiction of a breast cancer cell.

This treatment is also being deemed a success due to that fact that it may render chemotherapy unnecessary. 257 women with HER2 positive breast cancer were chosen for the study. Half of the 257 women were put on the drug combo while the other half was the control group. What they found was that of those on the drug:

- 11% had no cancer cells remaining within two weeks, and;
- 17% of cases featured dramatically shrunken tumors.

Compared to the control group who were only given Herceptin, they were found to have:

- 0% with no trace of cancer cells, and;
- Only 3% showed a drop in tumor size.

Clearly, the two drugs combined have a major effect on breast cancer cells as opposed to being used on their own.

Following this, they will need to run clinical trials to see if these women really can avoid chemotherapy treatment and if the current standard of care for some HER2 positive breast cancer patients can and should be changed.

It's certainly one to keep an eye on, although there's still a lot of work to be done by the scientific community.

Breast cancer tumours killed in 11 days with 'staggering' new therapy

Experts hail findings as 'astonishing' and suggest results of trial for one of most aggressive forms of breast cancer could revolutionise future treatment.

A "staggering" new breast cancer therapy has destroyed deadly tumours in just 11 days, trials have shown.

Experts hailed the findings as "astonishing" and said it is the first time a drug for the disease has ever shown such a response.

They suggested the results of the trial, in women with one of the most aggressive forms of breast cancer, could revolutionise future treatment of other types of disease.

The combined drug therapy - which would cost around £1,500 for treatment - entirely destroyed tumours as large as 3cm.

"For solid tumours to disappear in 11 days is unheard of. These are mind-boggling results"

Professor Nigel Bundred

The findings mean that in future, thousands of women might be spared gruelling rounds of chemotherapythat normally follow surgery, experts said.

The UK study involved women suffering from one of the most aggressive types of breast cancer - HER2 positive - who were given a combination of two targeted drugs.

Almost nine in 10 women showed some response to the treatment - meaning the number of cancer cells began to fall. In one in four cases, the powerful cocktail saw tumours shrink significantly, and in some cases totally vanish.

The drug was shown to be effective even in cases where disease had spread to the lymph nodes, the study, presented at the European Breast Cancer Conference in Amsterdam, found.

Researchers said those taking part in the trial were left astounded at the "mind-blowing" effect of combining two drugs - Herceptin, the current

standard treatment for such cases, with a second drug called Tyverb.

They said the breakthrough could radically change future treatment of breast cancer, as treatment with high-cost drugs for less than a fortnight was "dirt cheap".

Lead researcher Professor Nigel Bundred, professor of surgical oncology at the University of Manchester, said: "For solid tumours to disappear in 11 days is unheard of. These are mind-boggling results."

Around 10,000 women a year who are diagnosed with breast cancer are HER2 positive, which means the disease ends to grow more quickly.

In recent years, they have been prescribed the drug Herceptin, usually for about 12 months, with costs amounting to around £20,000.

The second drug, Tyverb, has shown some promise, but has been rejected by NHS rationing bodies for not being cost-effective, at £27,000 a year.

The study of 257 cancer patients by the University of Manchester and The University Hospital of South Manchester NHS Foundation trust examined what happened when women were given both drugs together.

Scientists wanted to test the new combination therapy on tumours, to measure how effective it was at killing cancer cells. After 11 days, surgeons operated.

But medics were shocked to learn that in many cases, the cancerous growths had either disappeared, or shrunk significantly. Normally, it takes a matter of months or even years for tumours to respond to treatment.

The vast majority of the patients had shown some response to the treatment by the time their operations were due. In total, 87 per cent underwent biological changes that suggested the number of cancer cells had fallen.

Scientists said they were most excited about a sub-group of 27 per cent of patients, who "responded exquisitely well" to the treatment, Prof Bundred, a cancer surgeon, said.

In 11 per cent of cases, the tumours had disappeared entirely, less than two weeks after they had been diagnosed. In a further 17 per cent of patients, the tumours had become so small they were classed as "minimal residual disease" - less than 5mm.

Prof Bundred said: "We are pretty certain that we are not only getting tumour disappearance – we are getting an immune response as well."

"These results are so staggering that I suspect that we will have to run another trial to prove that they are generalisable".

Fellow researcher David Cameron, an oncologist at Edinburgh university, said: "It was only when the pathologist was scratching around in the lab, saying 'Where is the tumour?' that it became apparent that there was no tumour."

After the first few results came through from the trial, researchers had to surgeons who were due to remove tumours that they might find little or no trace of them, they said.

Scientists said it was not yet clear how the mechanism worked, but said the combined force of the drugs appeared to block cancer cells from multiplying, killing them off and sparking an immune response.

Fellow researcher Professor Judith Bliss, of the Institute of Cancer Research in London, said: "Statisticians tend to be quite sceptical and conservative, but the potential that we can eradicate a tumour in 11 days is remarkable."

At the moment, women with breast cancer usually have their tumour removed during surgery followed by a combination of chemotherapy, radiotherapy, hormonal therapies and targeted drugs such as Herceptin.

The breakthrough could spare thousands of women from gruelling rounds of chemotherapy, charities said.

It also raises the prospect of cost-effective treatment for women who have until now had limited options, and fallen victim to NHS rationing when new drugs come on the market.

Although the drugs are relatively expensive, the length of treatment would be so short that the costs would be small. Tyverb is also close to the end of its patent, meaning the price is likely to drop.

"If you are only using it for 11 days it's dirt cheap," Prof Cameron said. And he said the effects demonstrated by the approach suggested similar treatments could offer hope for other types of disease.

Samia al Qadhi, chief executive at Breast Cancer Care, said: "The astonishing findings in this study show that combining these two drugs has the potential to shrink HER2 positive breast cancer in just 11 days.

"For some HER2 positive breast cancer patients the effect of this drug combination will be amazing, and mean they can avoid chemotherapy and its gruelling side effects completely. For others, their tumours may not shrink, but doctors will know either way very quickly, giving them the ability to rapidly decide on further treatment.

"Although an early study, this has game changing potential," she said.

How the new breast cancer therapy works:

Around 10,000 women a year are diagnosed with HER2 positive breast cancer - one of the most aggressive forms of the disease.

The cancer cells have a high number of receptors for the human epidermal growth factor (HER2) which stimulate the cells to divide and grow.

The new trial involved 257 women who had just received a diagnosis, and were due to have surgery within a fortnight to remove deadly tumours, before starting chemotherapy.

Scientists wanted to test the new combination therapy on tumours, to measure how effective it was at killing cancer cells.

They had no idea it would prove so potent that some of the growths would be destroyed even before surgery went ahead.

The study divided women into three groups:

1. Some received no treatment before their operation - as is normally the case.
2. Others were given the drug Herceptin, which is usually given for a year after surgery.
3. A third group were given Herceptin, combined with a newer drug called Tyverb.

The study, funded by Cancer Research UK, found that among 66 women given the combination therapy, 87 per cent had some kind of immediate biological response.

This was shown by a drop in proteins which indicate cells are growing and dividing, or a rise in cell death of at least 30 per cent. But the findings were yet more remarkable among 28 per cent of women in the group.

In total, 11 per cent of those given the drug combination saw tumours entirely vanish, in less than a fortnight. A further 17 per cent had tumours shrunk to less than 5mm - a growth so small it is classed as "minimal residual disease".

Scientists said they did not know what mechanism had caused the drug cocktail to be so effective.

But they speculated that the treatment had blocked cancer cells from multiplying, killing them off and sparking an immune response in the body.

• NHS breast cancer tests miss 3,500 tumours a year

Enabling the genomic revolution in Africa

H3Africa is developing capacity for health-related genomics research in Africa

Our understanding of genome biology, genomics, and disease, and even human history, has advanced tremendously with the completion of the Human Genome Project. Technological advances coupled with significant cost reductions in genomic research have yielded novel insights into disease etiology, diagnosis, and therapy for some of the world's most intractable and devastating diseases—including malaria, HIV/AIDS, tuberculosis, cancer, and diabetes. Yet, despite the burden of infectious diseases and, more recently, noncommunicable diseases (NCDs) in Africa, Africans have only participated minimally in genomics research. Of the thousands of genome-wide association studies (GWASs) that have been conducted globally, only seven (for HIV susceptibility, malaria, tuberculosis, and podoconiosis) have been conducted exclusively on African participants; four others (for prostate cancer, obsessive compulsive disorder, and anthropometry) included some African participants (www.genome.gov/gwastudies/). As discussed in 2011 (www.h3africa.org), if the dearth of genomics research involving Africans persists, the potential health and economic benefits emanating from genomic science may elude an entire continent. The lack of large-scale genomics studies in Africa is the result of many deep-seated issues, including a shortage of African scientists with genomic research expertise, lack of biomedical research infrastructure, limited computational expertise and resources, lack of adequate support for biomedical research by African governments, and the participation of many African scientists in collaborative research at no more than the level of sample collection. Overcoming these limitations will, in part, depend on African scientists acquiring the expertise and facilities necessary to lead high-quality genomics research aimed at understanding health problems relevant to African populations and to become internationally competitive in genomic science and its applications.

In June 2010, the U.S. National Institutes of Health (NIH) and UK-based Wellcome Trust, in partnership with the African Society of Human Genetics, announced a plan to enhance the ability of African scientists to apply genomic and epidemiological approaches to shed light on the determinants of chronic and infectious diseases in Africa (1). The Human Heredity and Health in Africa (H3Africa) initiative, now funded at $76 million over 5 years, is focused on capacity building, as well as specific scientific goals. H3Africa research grants are awarded directly to African institutions where principal investigators are based (table S1), which allows African scientists to develop and direct their independent research agendas. The program encourages formation of intra-continental collaborations and development of specific infrastructural elements, i.e., African-based biorepositories and a pan-African bioinformatics network (H3ABio-Net). H3Africa also includes training programs aimed at retaining African scientists on the continent to help build a sustainable critical mass of researchers. Open calls for research proposals have emphasized collaborations within Africa, plus accessible biorepositories and a bioinformatics network with nodes across the continent (table S2). The footprint of H3Africa extends across Africa (see the map), comprising 21 grants (table S1). It is anticipated that, together, H3Africa projects will analyze samples from 50,000 to 75,000 participants.

H3Africa is predicated on the belief that diseases and nonmedical issues relevant to Africans can be best explored in partnership with inhabitants of Africa (both researchers and research participants) who can provide a rich context and deep knowledge of the continent's past and present environment. African genomes and the unique genetic structure of African populations harbor many clues to understanding human evolutionary history which, in turn, can help shed light on disease etiology. For example, recent genomic studies showed that African Americans (AA) with chronic kidney disease (CKD) who harbor risk variants of the apolipoprotein L1 gene (APOL1) have a risk for accelerated CKD progression and the development of end-stage renal disease, that is two to five times normal, respectively (2, 3). These variants also confer 29 times the risk of HIV-associated nephropathy (HIVAN) (4). Despite these renal outcomes, the prevalence of the risk genotype is 13% among AA and virtually absent among those of non-African ancestry. The prevailing hypothesis is that APOL1 renal risk variants evolved in sub-Saharan Africa about 10,000 years ago to confer protection against the regionally endemic trypanosome parasite, the cause of African sleeping sickness. Recent studies led by African scientists showed that the frequency of the risk variants, as well as the prevalence of CKD and HIVAN in carriers of the risk variant, are much higher in West Africa (Yoruba, 28%; Igbo, 23%; the major ancestral populations of AA) where the trypanosome parasite is endemic as compared with the non-endemic region of Ethiopia (~1%) (5–7). The association between CKD and APOL1[a component of high-density lipoprotein (HDL) cholesterol] is shedding light on the complicated protective relation between HDL cholesterol and CKD in global populations (8). In another example, African scientists participating in H3Africa have used genomic tools to understand how genes interact with life style (barefoot farming practices) to increase susceptibility to podoconiosis, a neglected tropical disease in Ethiopia and Cameroon (9).

A key challenge to building critical mass for genomic research in Africa is the retention of scientific leadership capable of developing and maintaining sustainable research programs. The dearth of research-intensive institutions on the continent, coupled with a shortage of funded positions, continues to drive Africa's talented scientists to countries where they have better opportunities to develop their potential and pursue their interests.

Furthermore, the African continent lacks a strong history of collaborative scientific endeavor (10), as African researchers have turned to their well-resourced counterparts from Europe, North America, and Asia, rather than to their neighbors, to achieve scientific excellence and strong publication records. Consequently, African scientists have not adequately developed the necessary infrastructure and large-scale biomedical research culture required to promote research in Africa. H3Africa has begun building a strong foundation for genomic research based on collaboration among African scientists. Perhaps more important, H3Africa is facilitating the implementation of the norms and standards for project oversight, goal orientation, and timely dissemination of discoveries and training of the next generation of biomedical researchers across Africa. The consortium is also addressing the use of standardized protocols with detailed attention to community engagement and ethics approval (see below), protocols and policies for sharing biospecimens and data, and publication policies for large collaborative groups.

Approaches to these issues are facilitated by frequent interactions among consortium members to share experiences in developing genomic research programs, to support and promote interactions among the collaborative projects, and to jointly tackle ethical and policy concerns. An important example is data harmonization. By standardizing phenotype measurement and how collected responses are coded to facilitate data merging, statistical power for discovery of genetic variants and for modeling gene-by-environment interactions can be greatly increased.

Implementation of multinational and multiinstitutional genomics research projects in Africa faces additional challenges. Many local ethics review committees have little experience in genomic studies that require broad consent for long-term storage and sharing of biospecimens and data, and some have balked at the concept of global sharing of biospecimens and data with no immediate local benefit, viewing it as another form of exploitation. Several African countries have restrictive legislative policies that hamper sharing across national boundaries. Cultural beliefs and practices regarding donating any body part, including blood, need to be addressed. The growing international debate about return of individual genomic results is also an issue in Africa (11). Finally, there are huge disparities across Africa that span rural communities adhering to long-established cultural beliefs and practices on the one hand to sophisticated "citizens of the world" residing in major cities on the other. These communities share genetic heritage, but require different approaches to engagement and informed consent. Thus, H3Africa includes a grant program that supports empirical research on innovative approaches to informed consent; community engagement; and the ethical, legal, social, and cultural factors unique to the African research environment.

The H3Africa Consortium has developed an approach that attempts to balance (i) protection of the ability of African scientists to be the first to analyze and publish findings about their main research questions, given their limited resources and capacity to deal with data as quickly as scientists in developed countries with (ii) the benefit of global access to H3Africa data and biospecimens. To reach these not completely compatible ends, the H3Africa Consortium has agreed that data will be made initially available to the consortium members via H3ABioNet until submission to the European Genome-phenome Archive, from which they will be publicly accessible (through an independent Data and Biospecimen Access Committee). As is common in genomics, there will be a short lag (12 months) between data submission and publication; this is somewhat longer than the norm (6 to 9 months) to provide resource-challenged African investigators a bit more time to analyze and submit their manuscripts for peer review.

Similar considerations went into development of the policy for the release of biospecimens collected in H3Africa. The biospecimens will be stored in an African biorepository (with backup elsewhere on the continent), and from there shared globally for further research. Data and biospecimen sharing does, however, raise the often contentious issues of ownership and commercialization rights. The H3Africa Consortium is addressing this issue while embracing an ethos that promotes research for the global common good. Resources generated by H3Africa are expected to be useful in future genomic research not only in Africa but also globally.

H3ABioNet has also embarked on a program of training and accreditation of its bio-informatics nodes to carry out specific data analysis techniques, i.e., of GWAS or next-generation sequencing data. Part of the training involves a series of workshops,

often held at the nodes, to prepare for an accreditation exercise. The accreditation involves giving the nodes raw data sets to analyze, with their results being assessed by an international accreditation committee. One of the major challenges in holding training courses or even just joining working-group Skype calls, is poor Internet connectivity. H3ABioNet is using creative approaches to overcome these issues by seeking low latency alternatives and using portable devices that host data and tools and run independently of the network.

There are several criteria for success that have been defined to assess the accomplishments of the H3Africa initiative (see the table). Each of the component grants has a set of specific, yearly milestones, progress toward which is assessed on an annual basis by the funders (with input from an Independent Experts Committee of outside scientists. Both Wellcome Trust and the NIH will also critically evaluate the progress of H3Africa through peer review toward the end of the initial funding period. Accomplishments of both individual grants and the overall program will be considered in each funder decision process to determine whether continued support is justified.

The efforts of the African scientific community and their international colleagues will not in themselves be sufficient. It is essential that national governments and regional political and economic organizations support sustained funding of all research fields, including genomics and research infrastructure development. In fact, H3Africa has been useful in leveraging additional funding from local sources, as demonstrated by support from the South African Department of Science and Technology to enhance data collection in an H3Africa project of cardiometabolic disease genomics, an early promise of potential long-term success.

Interpreting cancer genomes using systematic host network perturbations by tumour virus proteins

Genotypic differences greatly influence susceptibility and resistance to disease. Understanding genotype–phenotype relationships requires that phenotypes be viewed as manifestations of network properties, rather than simply as the result of individual genomic variations1. Genome sequencing efforts have identified numerous germline mutations, and large numbers of somatic genomic alterations, associated with a predisposition to cancer2. However, it remains difficult to distinguish background, or ‘passenger’, cancer mutations from causal, or ‘driver’, mutations in these data sets. Human viruses intrinsically depend on their host cell during the course of infection and can elicit pathological phenotypes similar to those arising from mutations3. Here we test the hypothesis that genomic variations and tumour viruses may cause cancer through related mechanisms, by systematically examining host interactome and transcriptome network perturbations caused by DNA tumour virus proteins. The resulting integrated viral perturbation data reflects rewiring of the host cell networks, and highlights pathways, such as Notch signalling and apoptosis, that go awry in cancer. We show that systematic analyses of host targets of viral proteins can identify cancer genes with a success rate on a par with their identification through functional genomics and large-scale cataloguing of tumour mutations. Together, these complementary approaches increase the specificity of cancer gene identification. Combining systems-level studies of pathogen-encoded gene products with genomic approaches will facilitate the prioritization of cancer-causing driver genes to advance the understanding of the genetic basis of human cancer.

Microbe-Microbe and Microbe-Host Interactions
The oral metagenome in health and disease

Abstract

The oral cavity of humans is inhabited by hundreds of bacterial species and some of them have a key role in the development of oral diseases, mainly dental caries and periodontitis. We describe for the first time the metagenome of the human oral cavity under health and diseased conditions, with a focus on supragingival dental plaque and cavities. Direct pyrosequencing of eight samples with different oral-health status produced 1 Gbp of sequence without the biases imposed by PCR or cloning. These data show that cavities are not dominated byStreptococcus mutans (the species originally identified as the ethiological agent of dental caries) but are in fact a complex community formed by tens of bacterial species, in agreement with the view that caries is a polymicrobial disease. The analysis of the reads indicated that the oral cavity is functionally a different environment from the gut, with many functional categories enriched in one of the two environments and depleted in the other. Individuals who had never suffered from dental caries showed an over-representation of several functional categories, like genes for antimicrobial peptides and quorum sensing. In addition, they did not have mutans streptococci but displayed high recruitment of other species. Several isolates belonging to these dominant bacteria in healthy individuals were cultured and shown to inhibit the growth of cariogenic bacteria, suggesting the use of these commensal bacterial strains as probiotics to promote oral health and prevent dental caries.

Introduction

The oral cavity of humans is inhabited by hundreds of bacterial species, most of which are commensal and required to keep equilibrium in the mouth ecosystem. However, some of them have a key role in the development of oral diseases, mainly dental caries and periodontal disease (Marsh, 2010). Oral diseases initiate with the growth of the dental plaque, a biofilm formed by the accumulation of bacteria in a timely manner together with the human salivary glycoproteins and polysaccharides secreted by the microbes (Marsh, 2006). The subgingival plaque, located within the neutral or alkaline subgingival sulcus, is typically inhabited by anaerobic Gram negatives and is responsible for the development of gingivitis and periodontitis.

The supragingival dental plaque is formed on the teeth surfaces by acidogenic and acidophilic bacteria, which are responsible for dental caries. This is considered the most extended infectious disease in the world, affecting over 80% of the human population (Petersen, 2004). A poor oral health has also been related to the stomach ulcers, gastric cancer or cardiovascular disease, among others (Watabe et al., 1998; Wu et al., 2000). It is therefore surprising that no efficient strategies to combat oral diseases have been developed, despite their dramatic impact on human health. Some of the main reasons that oral pathogens have not been eradicated are related to the difficulty of studying the microbial communities inhabiting the oral cavity: First, the complexity of the ecosystem (several hundreds of species have been reported with multiple interaction levels) makes the potential pathogenical species difficult to target (Socransky et al., 1998); second, not a single ethiological agent can be identified as in classical, Koch's postulates diseases. This has been clearly shown in periodontal disease, where at least three bacterial species that belong to very different taxonomic groups (the so-called ‘red complex’ of periodontal pathogens) are known to be involved in the illness (Darveau, 2010); and third, a large proportion of oral bacteria cannot be cultured (Paster et al., 2001), and therefore traditional microbiological approaches give an incomplete picture of the natural communities inhabiting the dental plaque. However, the development of metagenomic techniques and next-generation sequencing technology now allows the study of whole bacterial communities by analysing the total DNA pool from complex microbial samples.

Pioneering metagenomic studies in the human microbiome centred in the gut ecosystem, initially through a shot-gun approach, in which DNA was cloned in small-size plasmids followed by traditional Sanger sequencing method (Gill et al., 2006; Kurokawa et al., 2007), obtaining reads of about 800–1000-bp long. Recent approaches include the end sequencing of large-size fosmids (Vaishampayan et al., 2010) and the use of Illumina sequencing technology to deliver vast amounts of small-size reads that could be later assembled (Qin et al., 2010).

Studies of the oral cavity microbiota, as well as other body habitats within the human microbiome such as the skin, the vagina or the respiratory tract, have mainly focused on the sequencing of PCR-amplified rRNA genes (Aas et al., 2005; Grice et al., 2008). These PCR-based studies have provided a substantial improvement of our knowledge of oral bacterial communities compared with past culture-based research, but the estimates of microbial diversity are hampered by biases in PCR amplification (de Lillo et al., 2006), cloning bias (Ghai et al., 2010) and when short pyrosequencing reads of the 16S rRNA gene were used, uncertainties in taxonomic assignment (Keijser et al., 2008; Lazarevic et al., 2009) and inflated diversity due to pyrosequencing errors (Quince et al., 2009). Recently, the first study of the oral metagenome has been carried out by directly applying next-generation sequencing to a single sample from a healthy individual (Xie et al., 2010), thus removing potential biases imposed by cloning and PCR. We have applied a similar approach to several samples varying in health status, directly sequencing the metagenomic DNA by 454 pyrosequencing, which has allowed us to compare the total genetic repertoire of the bacterial community under different health conditions.

Materials and methods

Sample collection

Supragingival dental plaque was obtained from 25 volunteers after signing an informed consent. The sampling procedure was approved by the Ethical Committee for Clinical Research from the DGSP-CSISP (Valencian Health Authority, Spain). The oral health status of each individual was evaluated by a dentist following recommendations and nomenclature from the Oral Health Surveys from the WHO, taking samples with sterile curettes. Plaque material from all teeth surfaces from each individual was pooled. In volunteers with active caries, the dental plaque samples were taken without touching cavities. In those cases, material from individual cavities was also extracted and kept separately. The volunteers were asked not to brush their teeth 24 h before the sampling. Information was obtained regarding oral hygiene, diet and signs of periodontal disease. DNA was extracted using the MasterPure Complete DNA and RNA Purification Kit (Epicentre Biotechnologies, Madison, WI, USA), following the manufacturer's instructions, adding a lysozyme treatment (5 mg ml−1, at 37 °C for 30 min). For this study, eight samples were used for subsequent pyrosequencing, selected on the basis of homogeneity in their clinical features, including similar age, periodontal status, smoking habits and mucosal health. Supragingival dental plaque samples were taken from six individuals that were divided in three groups according to the number of caries they had suffered and that represented different degrees of oral health: two individuals had never developed caries in their lives (healthy controls), another two individuals had been regularly treated for caries in the past and had a low number of active caries at the moment of sampling (one and four cavities, respectively); and the last two individuals had a high number of active caries (8 and 15) and poor oral hygiene. In addition, samples from individual cavities were collected, and for two of them enough DNA for pyrosequencing was obtained: one at an intermediate stage and the other one at an advanced stage of caries development (dentin lesion), corresponding to teeth 1.6 and 4.6 following WHO nomenclature. The sequencing was performed at Macrogen Inc. (Seoul, South Korea) using the GS-FLX sequencer (Roche, Basel, Switzerland) with Titanium chemistry. After quality checking, average read length was 425±117 bp. Sequences were deposited, and are publicly available in the MG-RAST server with the following accesions: 4447192.3, 4447102.3, 4447103.3, 4447101.3, 4447943.3, 4447903.3, 4447971.3 and 4447970.3.

Sequence analysis

Artificially replicated sequences (accounting for 1.2–4.54% of the raw reads) were removed from the data set using the '454 replicate filter' (Gomez-Alvarez et al., 2009). The human sequences were identified by MegaBlast (Altschul et al., 1990) against the human genome (e-value cutoff 1e−10) and were removed from the final data set. They accounted for 2.23–74.99% of the replicate-filtered reads (Supplementary Table 1). The metagenomic reads were mapped against 1117 sequenced reference genomes using the Nucmer and Promer v3.06 alignment algorithms, with the default parameters (Kurtz et al., 2004). The nucleotide identity values of each read against its hit in the genome were used to generate frequency histograms. If the mode was 94% or higher the plot was considered to represent sequence identity against the same species (Konstantinidis and Tiedje, 2005).

Stand-alone RPSBlast was used to align reads (translated into all six possible reading frames) to protein profiles (represented by position-specific scoring matrices). Queries were performed against the complete conserved domains database (Marchler-Bauer et al., 2009) and against the COGs (Tatusov et al., 2003) and Tigrfams (Selengut et al., 2007) databases. Fractions of sequences assigned in each case are shown in Supplementary Table 2. TFams classification assignments were integrated into higher hierarchical levels, according to the Tigrfam classification scheme, in subroles and main roles. COGs assignments were also integrated into the higher level of COG's functional categories. In addition, samples were uploaded to the MGRAST server (Meyer et al., 2008) and the functional assignment based on SEED subsystems was retrieved for the three hierarchical levels used: Subsystem, subsystem hierarchy 2 and subsystem hierarchy 1 (bottom up). In all cases, a table containing the counts of functional categories per sample was generated and used for subsequent analysis. All statistical analyses were conducted on R (2.6.2). Heat maps of taxonomic composition were generated using the gplots library of R (Warnes et al., 2009) with relative frequencies per sample, as well as Euclidean distance, or normal medians. The relative rates of over-represented features present in the people without caries were estimated using a control of the false discovery rate, for testing the amount of false positive predictions (q-values) for a given P-value of significance, with the algorithm described by White et al. (2009).

Taxonomic assignment

16S rRNA sequences were extracted from the reads of each metagenome by similarity search using BLASTn (Altschul et al., 1990) against the RDP database, with an e-value cutoff of 1e–10. Sequences <200 bp were removed. Phylogenetic assignment of the sequences was made using the RDP Classifier (Wang et al., 2007), using an 80% confidence threshold. New operational taxonomic units were proposed if the reads were over 400 bp in length and had a nucleotide identity between 80–95% to known 16S sequences. Taxonomic assignments of all open reading frames were carried out based on a lowest common ancestor (LCA) algorithm (Alstrup et al., 2004) with the characteristics described in the MEGAN software (Huson et al., 2007). We implemented the algorithm in a multi-threaded command-line oriented in-house software in order to obtain faster analysis and simplify its integration in pipelines and downstream analysis. To obtain the LCA of each sequence, we carried out BLASTx homology searches against a custom database comprising the non-eukaryotic sequences of the NCBI's non-redundant database. For each query sequence (read), only hits with a bit score at least 90% of the best matches were considered in the LCA computation. We also made use of the script phymmBL (Brady and Salzberg, 2009) that combines the assignment of sequences both by homology and by nucleotide composition using hidden Markov Models. All the available complete and WGS genomes were retrieved from the human oral microbiome database (Chen et al., 2010), as well as the RefSeq of NCBI containing all bacterial and archaea genomes (june 2010), and were used to build a local database to perform taxonomic model constructions and homology searches, using sequences larger than 200 bp to predict taxonomic affiliation. At this read length, phymmBL's performance at the class level has been estimated to be over 75%. All the taxonomic and functional results were parsed into a MySQL database for further analysis.

Results and discussion

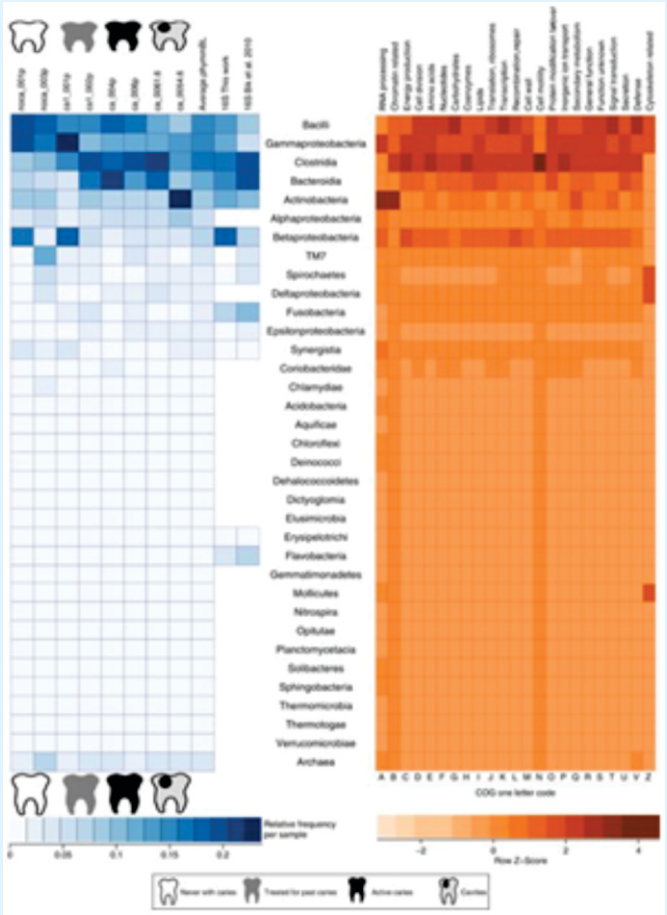
The oral microbiome by pyrosequencing

Supragingival dental plaque samples were taken from six individuals that were divided in three groups according to the number of caries they had suffered and that represented different degrees of oral health: two individuals had never developed caries in their lives (healthy controls), another two individuals had been regularly treated for caries in the past and had a low number of active caries at the moment of sampling; and the last two individuals had a high number of active caries and poor oral hygiene. In addition, samples from individual cavities were collected, and for two of them enough DNA for pyrosequencing was obtained. A total of 1 Gbp of DNA sequence was obtained from the eight samples selected. The amount of human DNA in the metagenomes varied from 0.5–40% in supragingival dental plaque samples (Supplementary Table 1), thus the total size of the studied metagenome was reduced to 842 Mbp of sequence. We obtained an average read length of 425±117 bp, which allowed a functional assignment in a significant fraction of the

metagenome (Supplementary Table 2). In addition, assembly of those reads produced 1103 contigs larger than 5 Kb and 354 longer than 10 Kb. Success in the assembly of large contigs was dependent on sequencing effort. We obtained an average of 129.5 Mbp of filtered, high-quality sequences for each of the six oral samples. In the two cavity samples, around 70% of the reads corresponded to human DNA, and an average of 32.5 Mbp of filtered, high-quality reads were obtained.

Estimating diversity in the oral metagenome

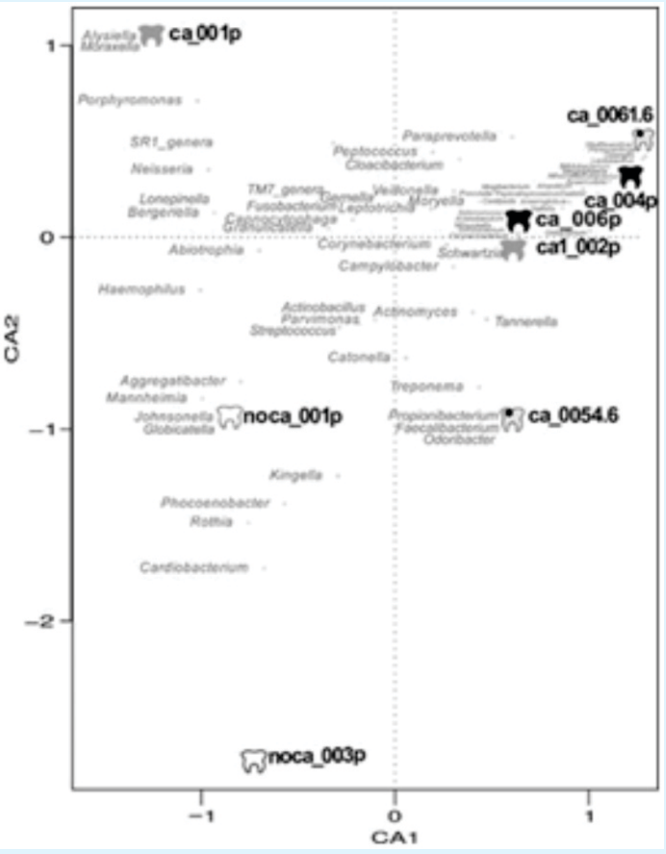
We estimated microbial diversity in all samples by three different methods. First, we selected the reads matching 16S rRNA genes, assigning them to different taxonomic levels. A total of 4254 16S rRNA sequences were obtained (Supplementary Table 1), giving a similar picture of diversity to that obtained through 16S rRNA PCR-dependent procedures (Bik et al., 2010), although the relative proportions of each taxonomic group were different (Figure 1). These 16S rRNA reads identified 186 sequences representing novel operational taxonomic units previously undetected by PCR amplification (Supplementary Table 3). Rarefaction curves and different diversity indexes based on the rRNA sequences obtained from the metagenomic reads indicate an estimate of 73–120 genera for dental plaque samples (Supplementary Table 1 andSupplementary Figure 2). A second approach to estimate diversity was the use of a LCA algorithm to classify all reads giving a hit in public databases at the taxonomic level for which the assignment was unambiguous (Huson et al., 2007). Over 1.5 million reads were assigned by this procedure, confirming the presence of bacterial groups detected by 16S rRNA genes, but suggesting that a wider range of taxonomic groups was present (Supplementary Figure 1). Finally, the recently developed phymmBL binning procedure (Brady and Salzberg, 2009) was used to taxonomically assign 1.94 million reads from our data set. The results agreed again with the taxonomic distribution described by the 16S rRNA and the LCA approaches, but with further implication of other bacterial taxa. The results from these three methods show that the relatively small numbers of 16S genes in directly sequenced metagenomes are enough to describe the main taxonomic groups present without cloning or PCR-based biases, although at the expense of lower sequence depth. Some of the taxa found at low proportions in our data set were also detected by large-scale 16S rRNA cloning studies (Paster et al., 2001; Bik et al., 2010) but others were not (Figure 1). This could be not only due to lower amplification efficiency of these bacteria by universal primers, but also due to the detection of false positive hits by the LCA and phymmBL approaches.



Bacterial diversity in the oral cavity. The graph on the left shows the relative frequency of different bacterial taxa, based on the assignment of the DNA reads by the PhymmBL software and by 16S rRNA reads extracted from the metagenome, and compared with the PCR results obtained by Bik et al. (2010). The graph on the right indicates the relative contribution of each taxonomic group to the coding potential of the ecosystem, based on the COGs functional classification system. It can be observed that the functional contribution is not equal among taxa.

Despite the low number of samples examined, interesting differences in diversity can be seen between healthy and diseased individuals. All three methods showed a tendency for Bacilli and Gamma-Proteobacteria to be more common in healthy individuals, whereas typically anaerobic taxa like Clostridiales and Bacteroidetes are more frequent in diseased samples

(Figure 1, Supplementary Figure 1). Bacilli are particularly depleted in the two samples from within cavities, and one of them showed a high proportion of Actinobacteria. Reads assigned to beta-Proteobacteria (mainly Neisseriales) and TM7 were at very low proportions in diseased samples, and studies based on a larger number of individuals should test whether their presence could be associated to healthy conditions. Correspondence analysis between the metagenomes based on the taxonomic assignation by 16S rRNA reads showed that samples with poor oral health tended to cluster together, whereas different consortia of bacteria can be found in healthy individuals (Figure 2). Some genera, like Rothia or Aggregatibacter appear to be specifically associated to healthy samples, in agreement with PCR-based studies that compared bacterial diversity in healthy controls and diseased volunteers (Aas et al., 2005, 2008; Corby et al., 2005). The metagenomic recruitments also showed Aggregatibacter as one of the prevalent species in individuals without caries (see below).



Correspondence analysis (CoA) of the bacterial diversity in oral samples based on 16S rRNA reads extracted from the metagenomes. The first axis successfully separates healthy from diseased individuals. The graph suggests bacterial genera which are potentially associated with absence of caries.

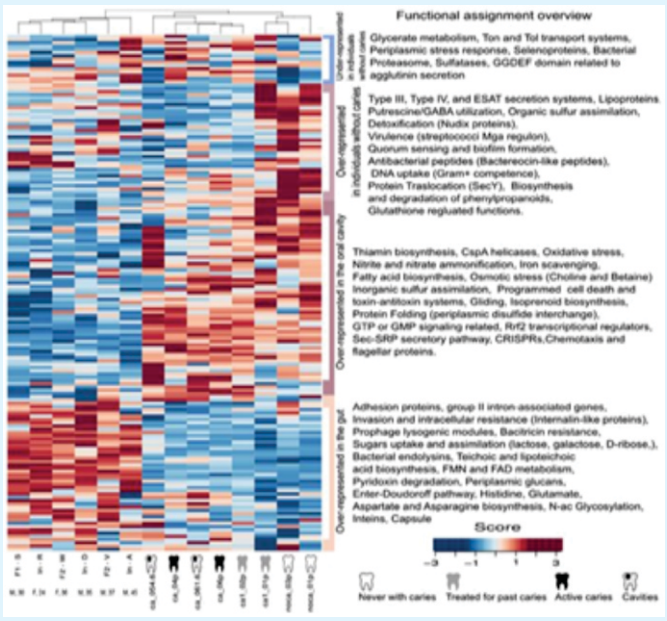
Sequence similarity searches against 18S rRNA databases revealed very few significant hits against eukaryotic species. No rRNA reads were identified from Candida or other fungi that are regular inhabitants of the oral cavity, indicating that although these organisms are frequently detected by PCR amplification (Ghannoum et al., 2010), they are probably present at low proportions. In sample CA-04, significant hits to the rRNA ITS region of the protozoan Trichomonas tenax were found. Trichomonas tenax is found particularly in the oral cavity of patients with poor oral hygiene and advanced periodontal disease (Kleinberg, 2002), and it has been shown to be involved in bronchopulmonary infections.

An effective tool to quantify the presence of selected species in metagenomes is provided by sequence recruitments (Rodriguez-Valera et al., 2009). Individual metagenomic reads that give a hit over a certain identity threshold against a reference bacterial genome are 'recruited' to plot a graph, which will vary in density depending on the abundance of that organism in the sample. If the average nucleotide identity displayed is above 94%, the recruitment is very likely made against reads of the same species (Konstantinidis and Tiedje, 2005). By comparing our metagenomes against the genomes of 1117 fully sequenced genomes available in databases, we were able to estimate the abundance of close relatives of these reference species in our samples (Supplementary Figure 3A). Interestingly, bacteria closely related to Aggregatibacter andStreptococcus sanguis were among the three with the highest level of recruitment in individuals without caries, in agreement with these species being more frequently amplified from the oral cavity of healthy individuals (Aas et al., 2005; Corby et al., 2005). On the other hand,Streptococcus gordonii and Leptotrichia buccalis were abundant in individuals with caries. Strains of Veillonella parvula were the most abundant in all individuals with caries and appeared to be common to all samples, but interestingly the recruitment plots show differences between strains (Supplementary Figure 4). For instance, the Veillonellapresent in the two healthy individuals shows a genomic island without recruitment, even at the protein level, between positions 2066–2094 Kb of the reference genome. Individuals with caries CA-04 and CA1-01 do contain this region, which includes CRISPR-associated genes, hypothetical proteins, a protein involved in DNA uptake and an

amidophosphoribosyltransferase. This way, differences between strains of the same species can be identified which would pass unnoticed by 16S rRNA studies, and future work should identify whether those differential genes might be involved in pathogenesis. In addition, recruitment plots indicate that few taxa are normally dominant in each metagenome (Supplementary Figure 3B). This suggests that although bacterial diversity is indeed very large in the oral cavity, very few taxa account for most of the bacterial cells, and a big portion of the identified species are present at very low densities.

Functional diversity in the oral ecosystem

One of the powerful applications of LCA and phymmBL approaches is that each read with a significant hit can be assigned a taxonomic origin, and at the same time can also be related in many cases to a putative function. By relating taxonomy to function we have been able to predict what ecological or metabolic role each bacterial group can have. An example of this 'who can do what' approach can be seen in Figure 1 by using the COGs function classification system. It shows that categories are not equally distributed, and that some taxonomic groups are especially endowed for performing concrete functions. For example, a large portion of genes involved in defence mechanisms (that is, restriction endonucleases and drug efflux pumps) appear to be encoded by Bacilli. Other functions unequally distributed were cell motility genes in Clostridiales (mainly flagellar proteins) or signal transduction and carbohydrate metabolism in Bacilli (Figure 1, right). A more detailed functional analysis of the metagenome was performed using several systems for gene classification at different hierarchical levels. All pyrosequencing reads were compared against the conserved domains database, the Subsystems annotation environment (SEED) and the Tigrfams profiles (see Materials and methods section). Correspondence analysis (CoA) of the eight samples according to the functional assignment of the reads gave similar clustering patterns for the three function classification systems (Supplementary Figure 5). Samples from diseased individuals tended to cluster together, indicating that a similar set of functions were encoded in their metagenomes, and the two samples from individuals that had never suffered from caries, together with sample CA1-01 (with only one cavity at the moment of sampling), could be separated from the rest by the principal component. When the functional assignment of the oral microbiome was compared with that of the adult gut microbiome (Kurokawa et al., 2007) a $\chi 2$-test of independence revealed that the overall gut and oral functional roles depicted in the RAST subsystems are significantly different ($\chi 2(df=158)=17\ 057.42$, $P<2.2e{-}16$, $\varphi=0.123$), and this was supported also by clustering analysis where the oral samples clustered together (Figure 3), indicating that the gut and the mouth are two different ecosystems in terms of the relative frequencies of functions encoded in their metagenomes. It had previously been shown that the taxonomic diversity of the gut and oral ecosystems is clearly distinct (Bik et al., 2010), despite the fact that clear examples of horizontal gene transfer have been shown between these two interconnected niches (Mira, 2007). Our data show large blocks of over-represented functions in the gut microbiome, while others appear over-represented in the oral samples (a detailed list of these functional categories is represented in Supplementary Figure 6). It is interesting to note that metabolic genes, like those involved in sugar uptake and assimilation, are enriched in gut bacteria together with adhesion proteins and prophage genes, whereas gene families related to oxidative and osmotic stress or iron scavenging are more frequent in the oral microbiome (Figure 3). Thus, the relative proportion of these functional categories provides important insights into the ecology of each ecosystem and the potential role of the corresponding microbiotas for human health.



Functional profiles from oral and adult-gut metagenomic samples. Classification was based on Subsystem hierarchy 2 of MG-RAST.

Counts were normalized to the total number of reads per sample and then normalized by function. Blue to red gradient indicates levels of under/over-representation. Large blocks of gene categories are over-represented in each of the two microbiotas, indicating that the gut and the oral cavity are two functionally distinct ecosystems. Within the oral microbiome, some functional roles are over-represented in individuals without caries. A full version of this figure indicating all 101 functional categories is included in Supplementary Figure 6. Sequences from the healthy adult-gut metagenomes were taken from Kurokawa et al. (2007). The age and sex of each individual are indicated below each label.

Within the oral samples, individuals are clustered according to their health status (Figure 3). From an applied viewpoint, it is interesting that several functional categories are over-represented in samples from individuals without caries. Remarkable uprepresented genes in healthy individuals are involved in antibacterial peptides like bacteriocins (P-value=2.95 e−7; q-value=4.63 e−8), periplasmic stress response genes likedegS, degQ (P=2.46 e−46; q=3.22 e−46), capsular and extracellular polysaccharides (P=7.04 e−5; q=8.5 e−6) and bacitracin stress response genes (P=3.4 e−3; q=3.24 e−4). Other functional categories were also over-represented but the difference was not statistically significant, like genes involved in quorum sensing and phospholipid metabolism. The higher presence of bacteriocin-related genes points at these bioactive compounds as promising potential anti-caries agents. Some gene features over-represented in individuals with active caries are involved in mixed-acid fermentation (P=2.85 e−260; q=2.65 e−259) and DNA uptake and competence (P=6.29 e−8; q=1.13 e−8). Finally, it must be underlined that some over-represented genes in healthy individuals have an unknown function, and future studies should elucidate whether they are involved in the protection of the teeth against cariogenic conditions.

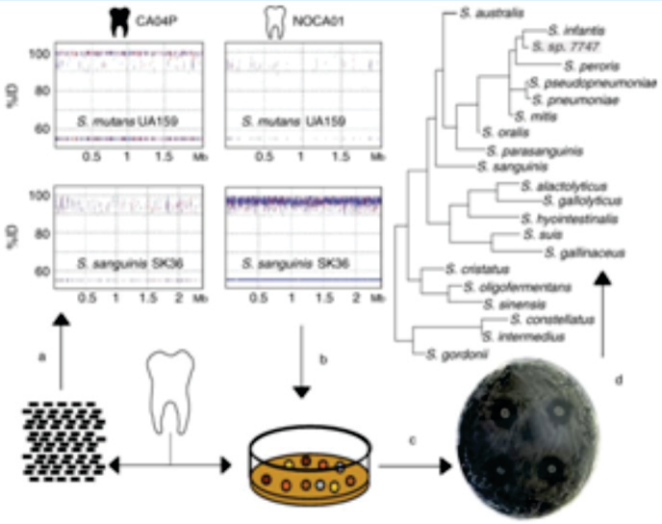
Cavities are complex ecosystems

We were able to extract sufficient DNA for 454 pyrosequencig in two samples from individual teeth, one at an intermediate stage and the other one at an advanced stage of caries development (dentin lesion). Given that mutans streptococci initially were considered to be the main ethiological agents of dental caries (Loesche, 1986), it is not surprising that most strategies against this disease have aimed at targeting Streptococcus mutans. These include the development of a vaccine using known surface antigens, passive immunization strategies that could neutralize the bacterium, the co-aggregation of S. mutans to probiotic strains or the use of specific inhibitors of S. mutans proteins, among others (Russell et al., 2004). In addition, the presence of mutans streptococci in children is typically associated to caries risk in oral-health evaluation protocols (Ge et al., 2008). However, pioneering molecular-based studies of cavities have failed to amplify mutans streptococci by PCR or hybridization in a significant proportion of cavities, suggesting that other bacterial genera like Lactobacillus, Actinomyces or Bifidobacterium could be involved in the disease (Aas et al., 2008; Becker et al., 2002). Recent molecular work has confirmed this finding and expanded the list of potential cariogenic bacteria to other species like Veillonella, Propionibacterium andAtopobium (Aas et al., 2008), most of them are poorly studied bacteria. The metagenomes of cavities studied here showed an almost complete absence of S. mutans. However, they displayed a large taxonomic diversity, which are included among the most common genera, Veillonella, Corynebacterium or Leptotrichia (Supplementary Table 4). Some of these bacteria, particularly Veillonella, have been shown to be predominant at all stages of caries progression (Aas et al., 2008) and under high-glucose conditions, and appear to be implied in acid production (Bradshaw and Marsh, 1998). Interestingly, consortia between Veillonella alcalescens andS. mutans were shown to produce more acid than any one of these species separately (Noorda et al., 1988), suggesting that synergistic effects probably take place, as it has been demonstrated in other complex microbial communities. Thus, although these data are based on the metagenomes from only two cavities, they favour a nonspecific plaque hypothesis for the development of dental caries (Marsh, 1994; Kleinberg, 2002). Further work should elucidate the potential role these bacteria had other than mutans streptococci in the progression of caries, as well as their synergistic and antagonistic interactions. The forecoming improvements in the amount of DNA required for next-generation sequencing techniques will allow a metagenomic study of cavities at different stages of development, including initial, white-spot lessions.

This is important because mutans streptococci could be instrumental at initial stages of caries, after which other species could colonize the niche. If caries is confirmed to be a polymicrobial disease, this should be taken into account for future therapeutic strategies. For instance, a potential solution for immunization strategies could pass through the selection of vaccine targets shared by different pathogens involved in the process of tooth decay (Mira et al., 2004; Mira, 2007).

Search for potential probiotics through metagenomics

The existence of a small proportion of the human adult population that has never suffered from dental caries has led some authors to suggest the presence of some bacterial species with a potential antagonistic effect against cariogenic bacteria (Corby et al., 2005). Bacterial replacement of pathogenic strains by innocuous isolates obtained from healthy individuals has been successfully shown to prevent pharynx infections and is the basis for probioticts preventing infectious disease in the gut and other human niches (Tagg and Dierksen, 2003). Metagenomic recruitment of cariogenic bacteria against the oral microbiome of healthy individuals shows a complete absence of S. mutans and S. sobrinus. Interestingly, the lack of detection of the cariogenic bacteria is accompanied by an intense recruitment of other streptococci (mainly those related to S. sanguis) and Neisseria, which comprise the most abundant genera in these individuals (Supplementary Figure 3B). Given the possibility that isolates of these dominant genera could be involved in antagonistic interactions with cariogenic bacteria, fresh dental plaque samples from 10 healthy individuals (including those from which the metagenomic sequences were obtained) were collected and used for culturing under conditions optimal for the growth of neisserial and streptococcal species. After microscopic examination, diplococci and streptococci were selected, providing a collection of 249 isolates. Those that could be grown on the same culture medium as S. mutans and S. sobrinus were transferred to a loan culture of these cariogenic bacteria. This simple screening identified 16 strains that displayed inhibition rings (Figure 4). PCR amplification of the 16S rRNA gene identified most of them as streptococci, with a 96–99% sequence identity to S. oralis, S. mitis andS. sanguis. Thus, this metagenomic approach allowed us to quantify the most abundant bacteria and confirms the previously hypothesized presence of bacteria with a protective effect against cariogenic species. This effect appears to be direct (that is, inhibitory), but other indirect effects such as stimulation of the immune response or direct competition for the same substrate or niche cannot be ruled out. Future research on these isolates should aim at identifying the secreted compounds responsible for the inhibition of caries-producing bacteria, and metagenomic libraries of dental plaque DNA may prove useful in this respect (Seville et al., 2009). Our own inhibition screenings performed on metagenomic fosmid libraries from dental plaque of healthy individuals against cariogenic bacteria suggest that antimicrobial peptides are among the products causing the inhibition. We propose the probiotic use of these anti-cariogenic bacteria or the utilization of the antibiotics they encode as promising new therapies against dental caries and other oral diseases (Devine and Marsh, 2009).



Searching of bacterial strains with a potential antagonistic effect against cariogenic bacteria. Metagenomic recruitment plots are used to detect the species (a), which are at low frequencies in individuals with caries but are among the most common in caries-free subjects. These species are then selected based on culture conditions and microscopic examination (b). The isolates are grown in solid media to provide an inhibition screening against caries-producing bacteria (c), selecting the strains that display inhibition rings (d), such as the Streptococcus strain 7747. Sequencing the genome of these inhibitory strains and comparing it against the metagenome of caried individuals must confirm that these strains are absent under diseased conditions.

Conclusion

We have shown that the direct pyrosequencing of human samples is a feasible approach to study the human microbiome, which would obviate the biases imposed by cloning and PCR and that would provide a more complete view of human-related bacterial communities beyond their composition inferred from the 16S rRNA gene (Ghai et al., 2010; Xie et al., 2010). Even in samples with a large proportion of human DNA such as cavities, the large throughput of next-generation sequencing has provided enough sequences to gain insights into the microbiology of caries, suggesting that it is the outcome of a complex bacterial community.

Despite the limited number of samples analyzed in this first study, important differences between healthy and diseased sites and individuals can be observed at the taxonomic and functional level, suggesting that the dental plaque of individuals that have never suffered from caries can be a genetic reservoir of new anticaries compounds and probiotics. Future population-based studies must evaluate whether the trends described in this study hold when higher sample sizes are used. We hope that these results stimulate further sequencing of the oral metagenome and metatranscriptome in the future as a tool to understand and combat the development of oral diseases.

Cardiovascular Imaging: A Glimpse Into The Future

W.A. Zoghbi, M.D.

A Half-Century of Change and More to Come

It was not that long ago that the only tools available to physicians to assist in the diagnosis of cardiovascular conditions were a stethoscope and a simple electrocardiogram. In a relatively short span of about 50 years, technological developments in cardiovascular imaging have infiltrated every aspect of practice, with noticeable improvements in diagnosis and outcome for patients. The ability of current imaging modalities to reveal details of various cardiac structures and physiology has made them an essential component of training and practice of cardiovascular professionals (Figure 1).

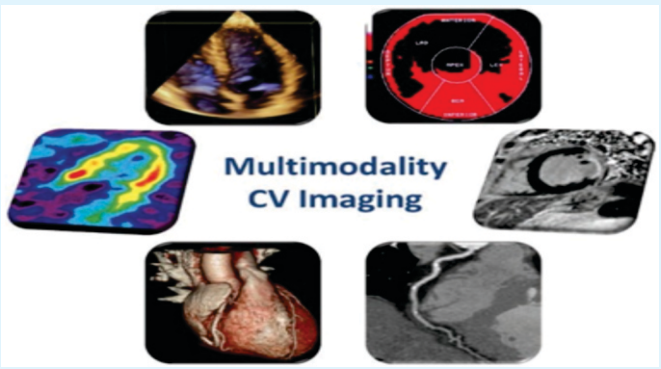


Figure 1.

Various imaging modalities currently used for diagnosis and management of cardiovascular disease.

As other articles in this issue detail, all imaging technologies have undergone continual improvements since their inception. Transformations in technical capabilities, spatial and temporal resolution, and processing speed have led to new applications for echocardiography, nuclear imaging, computerized tomography (CT), and cardiac magnetic resonance (CMR) imaging in research and clinical practice. In nuclear imaging, the newer cameras with CZT detectors have improved both sensitivity and efficiency; newer positron emission tomography (PET) agents enable better measurements of blood flow. CT imaging has increased both the number of slices that can be obtained simultaneously to cover a larger area of the heart as well as temporal resolution that is crucial in cardiac imaging, thus allowing imaging with much lower radiation and better accuracy. In CMR, newer sequences have allowed quantitation of collagen, scar burden, and its distribution, which can be fused with perfusion imaging and anatomy. Echocardiography has evolved from the early days of M-mode and 2-dimensional (2D) imaging to encompass Doppler, transesophageal studies, 3-dimensional (3D) real-time acquisition, and tissue Doppler and speckle tracking technologies.

With such rapid progress, we might be forgiven for taking the future of imaging for granted (Table 1). Better temporal and spatial resolution of real-time 3D echocardiography, which would improve efficiency and the reproducibility of measurements,

is already on the horizon. Refinements in technology should foster miniaturization of ultrasound and electrocardiography into small, pocket-size devices along with the latest in computer gadgetry and "apps." We can particularly look forward to the fusion of multiple imaging modalities, particularly in assessing structural heart disease and in the interventional arena (Figure 2).

Table 1.

Current Trends in Imaging

· 3-dimensional proliferation in echocardiography
· Automation: quantitation of chamber size and cardiac function
· Tissue characterization: viability, scar, collagen, and infiltration
· Improvement in protocols and technology aimed at reduction in radiation
· Miniaturization, hand-held devices
· Multimodality imaging integration and fusion

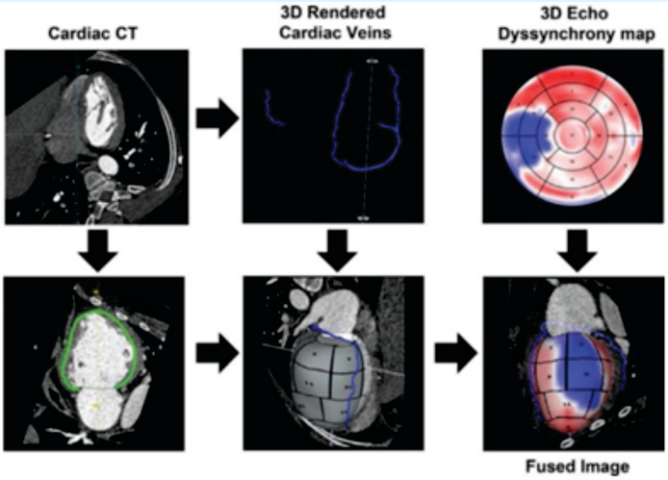


Figure 2.

Fusion of cardiac computed tomography, imaging of cardiac veins, and echocardiographic imaging with a dyssynchrony map (courtesy of Dr. Roberto Lang). This could optimize the localization of biventricular pacing leads in patients with heart failure and ventricular dyssynchrony.

The world of health care is changing, even as the population of patients at cardiovascular risk keeps expanding. In this article, I will highlight areas of imaging that still need improvement with a view to improving health care practice,1 where efficiency and value are becoming ever more dominant criteria throughout the continuum of care—from early detection and prevention of cardiovascular disease to treating advanced illness.

Advances in Evaluation of Cardiac Function

A major determinant of prognosis is cardiac function. The two primary modalities for assessing function are echocardiography and cardiac MRI (CMR), which provide structural imaging and good temporal resolution. Cardiac MRI is the current standard for quantitation of cardiac chambers and ejection fraction of both right and left ventricles. Its characterization of myocardial tissue is unique among imaging technologies in that it is the only one that can image scar tissue through the use of delayed gadolinium enhancement. Newer methodologies are aimed at refining quantitation of diffuse scarring or increases in collagen content, which would complement investigations on diastolic function. Software for automation of such quantitative parameters is currently being enhanced.

Progress in two areas of echocardiography should soon greatly aid the assessment of cardiac function. First, high volume rate imaging with 3D echocardiography, with real-time image capture in a single beat, will facilitate quantitation of ventricular volumes and ejection fraction. Second, automated quantitation of flow at valve annular sites, arising from combined 3D annular size without geometric assumptions and from 3D flow maps at the same site, should soon become a clinical reality.2 This information will be particularly useful in the quantitation of valvular regurgitant lesions.

Diastolic function assessment has relied on Doppler echocardiographic techniques, which offer high temporal resolution. Noninvasive assessment of diastolic function and ventricular filling pressure currently requires a combination of Doppler mitral inflow dynamics and tissue Doppler at two mitral annular sites (septal and lateral). The limitation of this approach is its focal, regional nature, which is not representative of total diastolic myocardial function and properties. In the future, improved 3D time resolution will enable an evaluation of global diastolic function that is more representative of cardiac function than currently inferred from limited interrogation of annular sites.

The advent of speckle tracking technology has allowed current measurements of strain and strain rate using 2D approaches to quantitate regional and global function.3,4 With expected improvements in the technology and standardization of the methodologies among industry vendors, application of speckle tracking to 3D echo will further enhance the accuracy of strain and strain rate measurements without assumptions of deformation in the 3D space. This will make quantitation of regional and global function even more robust.

Imaging in Coronary Artery Disease

The gold standard for detecting ischemia with noninvasive imaging techniques has been during stress testing with echocardiography and nuclear perfusion techniques. Cardiac MRI is gradually making its mark in this field as well. In the future and with more competitive pricing going forward, one can foresee an important role for CMR, especially in patients with depressed ventricular function, since it can provide a comprehensive evaluation of cardiac function, assess the extent of previous infarction along with residual viability, and determine the extent of peri-infarct or distant ischemia all in the same setting. In fact, CMR has an edge in ascertaining myocardial viability, as it is currently the only methodology that can image a scar directly with good spatial resolution.

While stress echocardiography and stress nuclear techniques can identify low- and high-risk individuals and help select patients for medical or invasive management in the vast majority of patients, there are some challenges in the evaluation of coronary artery disease that require better approaches. The presence of left bundle branch block presents a difficulty for both stress echo and nuclear imaging. Recent investigations with CMR suggest that it may have an edge in this situation by providing a comprehensive evaluation of stress wall motion, perfusion, and late gadolinium enhancement. Assessment of the inferior base can present difficulties for wall motion techniques or nuclear imaging, and left ventricular hypertrophy presents a challenge for detecting wall motion abnormalities, particularly during dobutamine stress and with perfusion techniques. Lastly, poor detection of balanced ischemia in patients with multivessel disease remains an underappreciated weakness of nuclear techniques, whereas a hypertensive response during exertion poses specificity concerns for echocardiography and other wall-motion-based techniques. In some of these equivocal situations, one usually proceeds to further imaging with either CT or invasive coronary angiography to delineate the presence or absence of coronary artery disease, depending on the clinical scenario and findings.

Going forward, we need to shift the focus from ischemia detection to addressing total cardiovascular risk for a particular individual. Identifying and decreasing total cardiovascular risk has been highlighted in the recent ACC/AHA guidelines on the treatment of hypercholesterolemia.5 Imaging can enhance this risk assessment by identifying the phenotype (e.g., coronary calcifications or atherosclerosis) in addition to detecting ischemia and other cardiovascular changes that portend an adverse outcome, such as left ventricular hypertrophy and diastolic dysfunction (Table 2). This has been substantiated in recent investigations using a coronary calcium score in individuals with normal stress nuclear techniques; patients with higher calcium scores despite a normal stress nuclear test were at incrementally higher risk of cardiovascular events because of a higher burden of coronary atherosclerosis.6 This approach may also be applied to vascular imaging. Thus, we should aim to identify total cardiovascular risk, which encompasses clinical risk factors, the cardiac phenotype as defined by cardiac imaging, exercise tolerance, stress-induced ischemia, and the extent of vascular atherosclerosis with imaging. Studies are needed to substantiate this approach and thus improve our prevention strategies.

Table 2.

Towards Identifying Total Cardiovascular Risk

· Clinical risk factors (age, gender, symptoms, smoking, hypertension, cholesterol, diabetes, etc.)
· Cardiac phenotype
– Coronary calcifications
– Left ventricular hypertrophy
– Left ventricular global/regional dysfunction
– Diastolic properties/left atrial volume
· Exercise tolerance
· Stress-induced ischemia and extent
· Vascular atherosclerosis imaging

Atherosclerosis assessment will drive technological development in two more areas over the coming years: molecular imaging for earlier detection, and methods to characterize and predict plaque instability and acute coronary syndromes.7,8 The evolution of imaging techniques, particularly with PET/CT fusion and more recently CMR/CT fusion, will enhance the diagnostic power of imaging and may unravel dynamic changes that lead to earlier detection and prediction of the unstable patient.

Interventional Imaging

As imaging has evolved, so has the catheterization laboratory with the arrival of interventional cardiology and catheter-based techniques for repair of structural heart disease. These currently include tools or devices for atrial and ventricular septal defects, transcatheter aortic valve replacement

(TAVR), mitral valve clip or other novel devices for mitral repair, occluders for repair of paraprosthetic valvular regurgitation, and, more recently, left atrial appendage occluder devices (Figure 3). Many of these procedures need imaging guidance for accurate deployment and evaluation of immediate results.9 Thus has the hybrid field of "interventional imaging" gradually evolved. Interventional imaging holds the promise of improving patient care and outcome with less invasive procedures, but to fulfill the promise we need to provide this service with appropriate resources, training, and skill. The field is slated for impressive growth with a cohesive "Heart Team" approach, in which the imager is an integral member.10

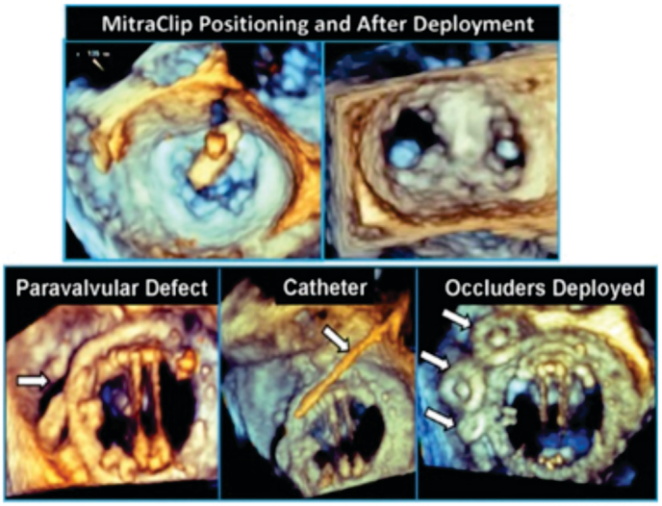


Figure 3.

Interventional imaging in the catheterization laboratory. Upper panels: positioning of a MitraClip for repair of mitral regurgitation with 3-dimensional echo transesophageal guidance and images of the valve after deployment. The lower panels show guidance of occluder devices in a patient with paravalvular prosthetic mitral regurgitation.

Valvular Heart Disease

One of the great strengths of echocardiography has been in the evaluation of valvular heart disease. In fact, echocardiography has evolved over the years to become the first-line diagnostic modality for the assessment of native and prosthetic valves.11,12 Echocardiography is quite robust at assessing the structure and significance of valvular lesions, particularly those with stenosis. More recently, 3D echocardiography, particularly with the transesophageal approach, has allowed us to see valve structure and motion in exquisite detail.

Nevertheless, evaluation of valvular regurgitation, particularly of the mitral valve, remains a challenge and accounts for significant variability among interpreters. Although guidelines have proposed a few parameters,11 they require integration of several findings and thus are amenable to variability. Future developments would aim at more robust, automated quantitation of valvular regurgitation. This could be from comparative volumetric 3D flow through the mitral valve and a systemic valve2 and/or determination of vena contracta using 3D color Doppler or other novel methods.13 While flow convergence using 2D technology has been helpful in this assessment, limitations of this technique are evident in eccentric jets and in crescent-shape regurgitant orifices as seen in functional mitral regurgitation.13

The advent of catheter-based valve implantation or repair has presented new challenges in the assessment of valvular regurgitation. In the case of TAVR, paravalvular regurgitation may occur because of focal asymmetrical valve calcification or inadequate seating of the valve. Paravalvular regurgitation is quite variable (single or multiple sites; circular or crescent shapes) and has proven difficult to evaluate with conventional 2D Doppler. Similarly, the use of the mitral valve clip for repair of mitral regurgitation not infrequently results in residual 1–2 jets from the two created mitral orifices, complicating the assessment and quantitation of regurgitation severity. Future validation of quantitative methods and recommendations on how to approach these lesions will be helpful. Along these lines, a few lingering questions are worth pursuing: How best to evaluate the severity of valvular regurgitation in eccentric or multiple jets? What is the role of cardiac MRI in valvular regurgitation (native or prosthetic) since it is quite accurate in quantitation of flow, and when should it be used?

Another area for future technological development is that of 3D dynamic and automated mapping of valve motion. Software is being developed for better geometric assessment and, importantly, for quantitation of valve strain and stress. Early data show that the distribution of strain is much higher in organic valvular regurgitation and improves significantly after mitral valve repair (Figure 4).14 Further quantitation of stress and strain could provide more insight into the pathophysiology and natural history of valve disease and possibly improve mitral repair techniques that aim at preserving the mitral valve apparatus while at the same time reducing strain and stress for longer durability.

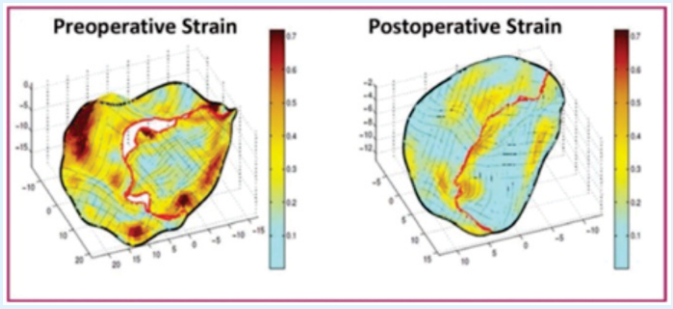


Figure 4.

A depiction of mitral valve strain in a patient with mitral regurgitation prior to mitral valve repair and after surgical repair. With obliteration of the area of noncoaptation (defect) and adequate surgical repair, a significant decrease in the strain pattern is seen postoperatively.

Imaging and the Digital Revolution

At the bedside, we have consistently relied on the stethoscope, essentially a 200-year-old technology. Studies in the late 1990's showing less than 30% accuracy of cardiac diagnoses at the bedside with a stethoscope are sobering15; the rate is likely similar or worse at present. While there are worthwhile discussions to be had about whether we are losing important skills, advances in miniaturization and digital technologies may soon make such discussions moot. Smartphones and tablets now carry apps for auscultation, heart rate and rhythm, and heart rate variability, not to mention health tips for patients. Although "hand-held" ultrasound devices have been produced over the years, miniaturization of echocardiography has only very recently provided us with a portable machine that truly fits in one's pocket. I believe this is the beginning of a true revolution in bedside diagnostics, as one can foresee a combination of ultrasound with other devices that are important to the healthcare professional such as an electronic stethoscope and electrocardiogram, among other tools.16,17 Empowering the physician and healthcare professional at the bedside with simple yet powerful diagnostic tools allows earlier and more accurate diagnosis and management of patients and improved workflow. Even more importantly, bedside diagnostics through portable devices restores the conversation between the physician and patient; instead of test results being reported much later through third parties, the patient receives direct attention from the doctor and a prompt discussion of test results. Physicians in the emergency department and other clinical settings could easily use such devices to determine whether or not patients need further, more costly, imaging.

In order for cardiologists to optimize the use of these technologies in practice, however, we will need studies on the impact of such devices as well as proper education and training (Table 3). Ideally, hand-held devices will be integrated into high-end healthcare systems so that findings are immediately incorporated into the electronic health record (Figure 5). Such devices are not just for wealthy healthcare systems, however; in underdeveloped countries where the equipment is used in rural settings, such devices would afford quick and accurate diagnoses in the field and assist busy healthcare professionals in population risk assessment.

Table 3.

Incorporation of Imaging at the bedside in CV Examination

· Improves CV examination and delivery of care
· Needs further education and training
· Selective Ultrasound use: questionable murmurs, follow-up pericardial effusion, heart failure…
· Better triage for more advanced imaging & faster medical decisions
· Adoption predicated on ease of use, impact & payment models

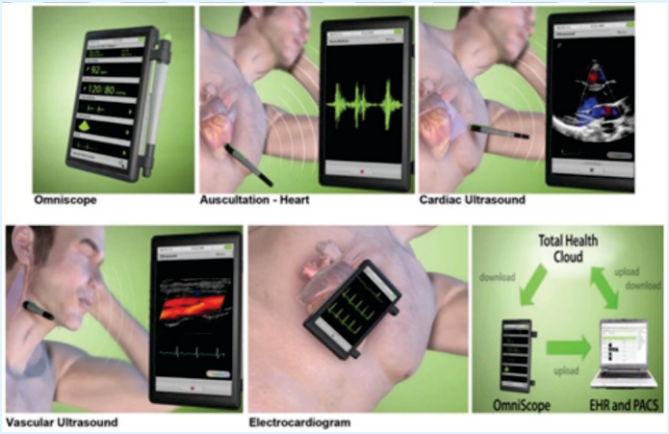


Figure 5.

A rendition of a proposed futuristic small hand-held device (OmniscopeTM) developed by the author, incorporating vital signs, electronic auscultation, ultrasound, electrocardiographic rhythm strip, and other features that would also synchronize with electronic health records.17

The Future of Cardiovascular Imaging: Opportunities and Challenges

The past few decades have witnessed significant improvements in cardiovascular imaging, which is all to the benefit of better diagnosis, management, and early prevention of cardiovascular disease.1 Going forward, I expect the observed growth and refinements in imaging technology and applications to continue unabated, from high-end equipment of

CT, nuclear, CMR, 3D echocardiography, and molecular imaging to miniaturization with hand-held devices. The future promises an unprecedentedly wide spectrum of opportunities (Table 4). Early detection of disease and assessment of the cardiac phenotype at early stages is paramount in preventing cardiovascular disease and particularly relevant in inherited diseases where genetic markers are not yet available and conventional risk factors are absent. There is also the possibility of using imaging for novel drug development and as a surrogate to patient outcome, where appropriate.

· Imaging is crucial in cardiovascular health care delivery
· Multimodality imaging ranges from High-end, molecular imaging to Hand-Held devices
· Allows detection of early disease, identification of the cardiovascular phenotype
· Possible use for novel drug development and as surrogate to patient outcome
· Multimodality imaging: adding complexity, looking for value, appropriate use, comparative effectiveness
· Novel technologies need to demonstrate value: impact on efficiency, patient care & outcome
· Health care providers need to avoid layering of testing with various imaging modalities and use the least radiation
· Research is needed to identify best and cost-effective approaches to disease detection and management in a Digital & Multimodality Imaging world

Table 4.

The Future of Multimodality CV Imaging: Opportunities & Challenges

Imaging will always be an integral part of clinical cardiovascular medicine. With new realities in health care emphasizing quality and cost-effectiveness, future technologies will need to demonstrate value through greater efficiency and efficacy of care and/or patient outcomes. Greater emphasis will be placed on appropriate utilization of technology and resources, including imaging.1,18,19 It is therefore imperative to avoid layering of multiple tests in individual patients; we need to address both cost and safety in the context of patient-centered care. Ultimately, we need to identify, through research, the best approaches to disease detection and management with a focus on providing the best care to the patient.

Respiratory medicine

Caring for patients with chronic obstructive pulmonary disease (COPD) will present a major challenge over the next decade. Due to a combination of factors including past and present smoking habits and an ageing population it is the only major chronic disease that is still associated with rising mortality. Rising rates of smoking in developing countries and the impact of women “catching up” with men's smoking habits will further affect the development of COPD, as well as lung cancer. This review focuses primarily on COPD, asthma, oncology, and lung transplantation, where in each case recent evidence has been, or is likely to be, associated with advances in clinical management.

Methods

After discussion within our large group of pulmonary physicians we selected topics under four broad headings. Three of these—COPD, asthma, and lung cancer—were chosen because of the prevalence of the disease. We added a fourth, lung transplantation, because it is the treatment of last resort for several pulmonary diseases. Articles from 1999 onwards were considered.

Chronic obstructive pulmonary disease

Inhaled corticosteroids

Many patients with COPD are still treated with inhaled corticosteroids despite the lack of evidence on their value. Four recent large scale multicentre trials have now established that inhaled corticosteroids have no effect on the rate of progressive decline of lung function in patients with COPD.1–4 This was the primary outcome in all four studies, and although two found that corticosteroid treatment had a favourable effect on secondary outcomes (the frequency of exacerbations and the use of health care services), these findings need to be confirmed in trials specifically designed to address these questions. Until then, most patients with COPD should not be treated with inhaled corticosteroids because of the significant risk of adverse effects including skin bruising2 and osteoporosis.4

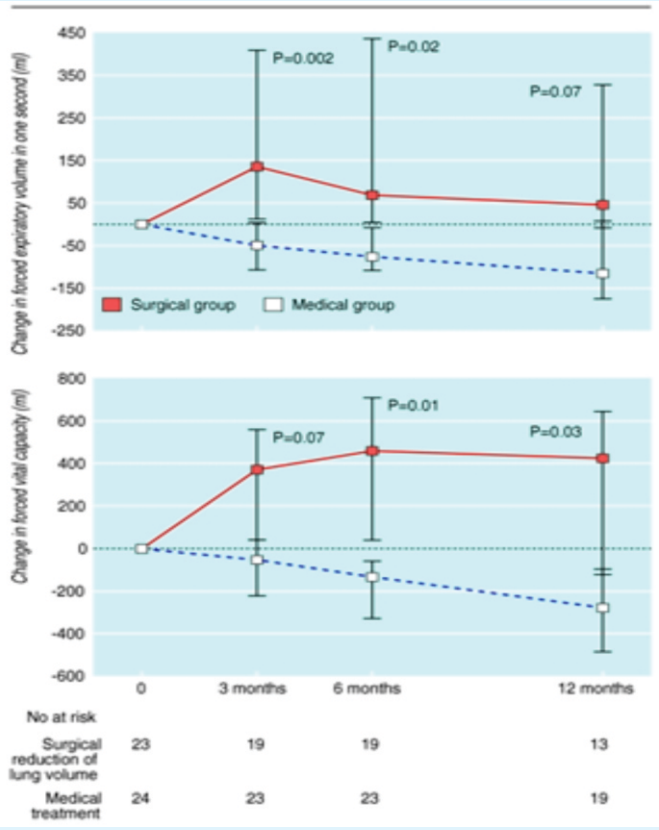
Recent advances

- Recent trials have shown that inhaled corticosteroids do not prevent progressive decline in lung function in chronic obstructive pulmonary disease
- Surgery to reduce lung volume is a promising intervention for emphysema
- Leukotriene blocking agents are a new class of drugs which have bronchodilator and anti-inflammatory properties in asthma
- Positron emission tomography is a new highly sensitive and specific diagnostic tool for staging lung cancer
- Use of non-beating heart donors might help to alleviate the severe shortage of donor lungs for transplantation.

Surgical reduction of lung volume

Surgery to reduce lung volume has attracted considerable attention from both doctors and patients since it was rediscovered in 1995.5 It involves serial non-segmental wedge resections of the most severely diseased portions of the lung with the intent of reducing overall lung volume by 20-30%.

Data on the effects of reducing lung volume, nearly all from case series, show considerable short term physiological, functional, and subjective benefits.6 There have also now been two small randomised controlled trials (n=37 and n=48, maximum follow up two years).7,8 Significant increases in forced expiratory volume in one second (FEV1) and in forced vital capacity (fig (fig1)1) were accompanied by significant reductions in total lung capacity and arterial carbon dioxide tension. In one trial assessing quality of life, significant improvements were also observed.7 Unfortunately, it has been suggested that improvements in pulmonary function and gas exchange after surgical reduction in lung volume are transient and also not related to the improvements in quality of life.9 Selection of suitable patients is critical but is not yet scientifically based; most centres exclude patients with appreciable preoperative hypercapnia, and cor pulmonale. In both the above trials, less than 30% of patients originally thought eligible for the study were finally randomised.



Median changes in forced expiratory volume in one second (top) and forced vital capacity (bottom) in patients after surgical reduction of lung volume and after medical treatment for emphysema. The median changes were obtained by comparing the responses of each subject with baseline values. Reproduced with permission from Geddes et al7

The results so far of surgical reduction of lung volume therefore need to be confirmed in large trials with sufficient numbers of patients and duration of follow up. Data on perioperative mortality, long term maintenance of gains from surgery, and cost effectiveness can then be assessed.

The attention and enthusiasm aroused by this operation before data were available from proper randomised controlled trials have led to great difficulty recruiting patients for such trials. Fortunately, at least two large scale randomised controlled trials are in progress.10,11 Recently a preliminary report has been published on the effects of lung volume reduction surgery in severe emphysema (an FEV1 below 20% of predicted value and either a homogeneous distribution of emphysema or a carbon monoxide diffusing capacity no more than 20% of predicted value). This concerned patients with the most severe disease, a subgroup from an ongoing trial. There was a markedly increased risk of death after surgery compared with that with standard medical treatment.12

Asthma

Self management plans

The 1990s were the decade of the development of self management plans for asthma. These programmes involve self monitoring either of peak expiratory flow or of symptoms, coupled with regular medical review and a written action plan; its beneficial effect in terms of improvement of health outcome has recently been reviewed.13 Self management, however, is not yet implemented on the scale it deserves.

For development of self management plans the severity of asthma has been classified into four groups, based largely on the need for treatment. When symptoms persist despite moderate doses of inhaled corticosteroids, newer guidelines offer a choice between doubling the dose of inhaled corticosteroid and adding a long acting β2agonist twice daily (see US National Heart, Lung, and Blood Institute/WHO guidance on management and

prevention of asthma, www.ginasthma.com). The introduction of preparations combining a long acting β2 agonist and a corticosteroid (such as fluticasone with salmeterol or budesonide with formoterol) will simplify the addition of the long acting component to the treatment regimen.

Data published this year from a large group of patients with asthma using "rescue" medication three to eight times daily have added the information that formoterol gives better control than terbutaline in terms of frequency of exacerbations, use of rescue medication, and improvement in peak expiratory flow, without an increase in adverse effects.14 This contradicts current teaching that long acting β2 agonists should be used only as maintenance medication.

Leukotriene receptor antagonists

Leukotriene blocking agents have proved efficacy in asthma and have recently been introduced in many countries. These agents act by interfering in the 5-lipoxygenase pathway of the metabolism of arachidonic acid, exerting bronchodilator and anti-inflammatory actions.15,16 Taken by mouth once or twice daily they offer the (as yet unproved) prospect of better concordance compared with conventional use of an inhaler several times a day. Montelukast and zafirlukast are the most widely available preparations at present. Although monotherapy with antileukotrienes in asthma is more effective than placebo, it is still less effective in improving lung function and symptoms than monotherapy with low doses of inhaled corticosteroids.17,18 Antileukotrienes are effective when added to low doses of inhaled corticosteroids (beclomethasone 400 μg daily)19 and also in patients who still have symptoms despite taking high doses (1000-4000 μg) of inhaled corticosteroids.20 Whether the ease of use of the antileukotrienes outweighs the clinically superior efficacy of inhaled corticosteroids still needs to be established. Additionally, antileukotrienes are the most effective drugs available for asthma induced by aspirin.21

Adverse effects from these agents are generally mild, but a few cases of Churg-Strauss syndrome have been reported. Whether these cases were due to the antileukotriene itself or to the reduction in corticosteroid dose made possible by the addition of the antileukotriene is not clear.

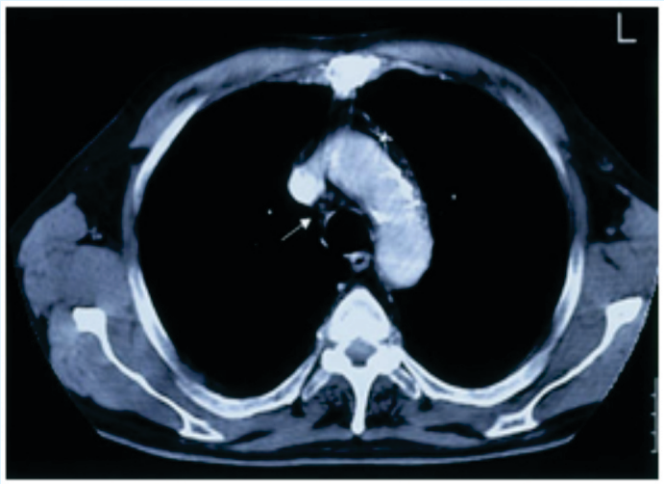
New drugs in development

Many new drugs are being tested for the treatment of asthma, most of them for their putative immunomodulatory properties. A monoclonal antibody against immunoglobulin E, which plays a pivotal role in atopic disease, seems to be closest to being marketed. The clinical development programme has involved mainly studies in patients already taking inhaled corticosteroids. Reductions in doses of inhaled steroids were accompanied by improvements in symptoms.22 Promising first results have also been published for an interleukin 4 receptor antagonist that inactivates naturally occurring interleukin 4, an important proinflammatory mediator in asthma.

New diagnostic techniques in pulmonary oncology

Positron emission tomography

Conventional diagnostic staging of suspected lung malignancies involves not only history, physical examination, chest radiography, and bronchoscopy but also the selective use of different imaging tests such as computed tomography of the chest, ultrasound, bone scans, radiography for suspected metastases, mediastinoscopy, or explorative thoracotomy. Recent advances have been made in the diagnostic evaluation and staging of lung malignancies with metabolic imaging techniques using positron emitting drugs such as 2-deoxyglucose labelled with18F (FDG), which is preferentially taken up in metabolically active tissues such as malignancies. The spatial resolution possible with currently available equipment is about 5 mm. Positron emission tomography with FDG has recently been shown to be superior to conventional staging in determining local, regional, and haematogenous spread of a tumour (fig (fig22).24 The sensitivity of positron emission tomography for detecting metastases almost anywhere in the body was 95% and the specificity was 83%. In 10-20% of patients tumours were reclassified.



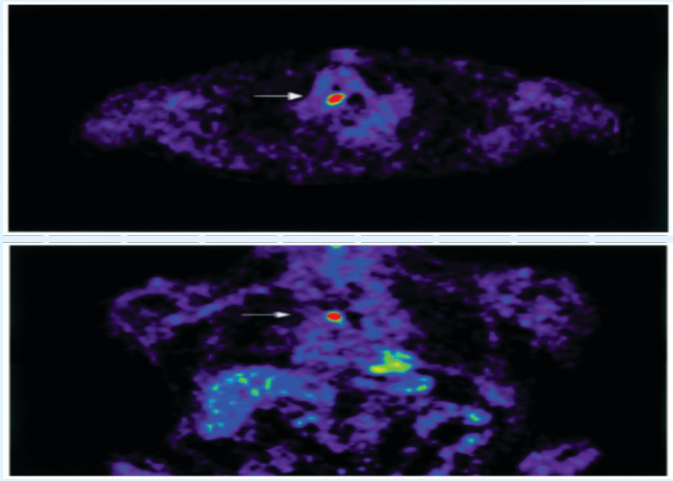


Figure 2

Computed tomography (CT) and positron emission tomography (PET) findings in a patient with squamous cell carcinoma of the right lung. Top: Level 2 CT. Centre and bottom: Axial and coronal PET respectively of area shown by arrow in CT. No abnormal mediastinal lymph nodes were seen on CT, but uptake of 2-deoxyglucose labelled with 18F was increased on PET (arrows). Reproduced with permission from Pieterman et al24

The most common sites of metastases are the local, regional, and mediastinal lymph nodes. Positron emission tomography is superior to computed tomography for mediastinal staging to the extent that no subsequent mediastinoscopy is needed in the case of a negative mediastinal positron emission scan,26 though "hotspots" in the mediastinum still require invasive procedures to confirm malignancy. To avoid invasive procedures, a promising new technique for exploring especially the left side of the mediastinum is endoscopic oesophageal ultrasonography with needle aspiration.27 Whether this technique can replace mediastinoscopy needs to be investigated.

Lung transplantation

Between 1200 and 1500 lung transplantations are carried out annually, in over 150 centres worldwide, the majority in the United States and United Kingdom. Common indications for lung transplantation are pulmonary emphysema (accounting for over 45% of transplantations), cystic fibrosis (15-20%), pulmonary fibrosis (10-15%), and pulmonary hypertension (5%). Survival at one, five, and 10 years after lung transplantation is currently 75%, 50%, and probably 25%, respectively (fig (fig3).3). Lung transplantation results in appreciable survival benefit, especially for patients with cystic fibrosis and pulmonary fibrosis, though not for those with chronic obstructive pulmonary disease.28 Successful bilateral lung transplantation usually leads to return to normal of pulmonary function, as measured by spirometry. Exercise performance after lung transplantation usually approximates 50% of the standard age specific prediction. Furthermore, a successful transplantation results in improvement in virtually all aspects of quality of life.

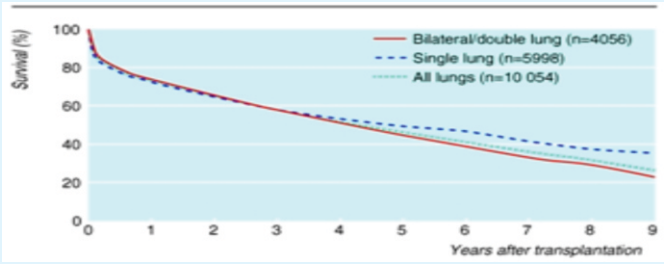


Figure 3

Actuarial survival after lung transplantation. Modified data from the International Society for Heart and Lung Transplantation, 2001 (www.eshlt.org)

Transplantation from non-beating heart donors

Up to a third of patients awaiting lung transplanation die before a suitable donor is found. Several approaches have been tried to increase the supply of donor lungs. It has been suggested that lowering the threshold to allow use of organs from so called "marginal donors" does not compromise results unacceptably.30 Additionally, separate lobes from two related living donors have been simultaneously transplanted successfully to single recipients with cystic fibrosis.

This year, the first successful lung transplantation was performed with lungs from a donor who had had sustained cardiac arrest.31 Xenotransplantation of lungs is currently still at the stage of preclinical experiment.

Rejection versus infection

Delayed complications after lung transplanation are common. They are mainly related either to rejection or to infections during immunosuppression.

The sensitivity to infections is probably due to the open communication of the lung with the environment. Moreover, in the immunosuppressed patient the (transplanted) lung is very sensitive to opportunistic micro-organisms.

Distinguishing between rejection and infection is often difficult. Accurate techniques have been developed for the early diagnosis and monitoring of infections related to the Epstein-Barr virus and of post-transplant lymphoproliferative disease by quantitative polymerase chain reaction; these are an important advance.32 Additionally, the advances in multiplex (combined) polymerase chain reaction techniques for the diagnosis of viral infections offer clear prospects of earlier detection of infection and its distinction from rejection.

Long term prognosis after lung transplantation is determined by whether chronic transplant dysfunction (bronchiolitis obliterans) develops. This condition is characterised by progressive bronchiolar obstruction. This has a variable but often unsatisfactory response to medical treatment, which usually takes the form of increased immunosuppression. It seems that transplanted lungs are more prone than other solid organ transplants are to chronic transplant failure. More potent immunosuppressive drugs, the adverse toxic effects of which are acceptable but which do not increase susceptibility to infection, are being sought. The proportion of patients experiencing at least one episode of acute rejection after renal transplantation has been reduced from 40% to approximately 20% or even lower with the new immunosuppressive drugs mycophenolate mofetil, CD25-blocking monoclonal antibodies, and rapamycin. These findings are of promise for lung transplantation as well, and they are all at some phase of development.

Lack of selective resistance of influenza A virus in presence of host-targeted antiviral, UV-4B

Abstract

Development of antiviral drug resistance is a continuous concern for viruses with high mutation rates such as influenza. The use of antiviral drugs targeting host proteins required for viral replication is less likely to result in the selection of resistant viruses than treating with direct-acting antivirals. The iminosugar UV-4B is a host-targeted glucomimetic that inhibits endoplasmic reticulum α-glucosidase I and II enzymes resulting in improper glycosylation and misfolding of viral glycoproteins. UV-4B has broad-spectrum antiviral activity against diverse viruses including dengue and influenza. To examine the ability of influenza virus to develop resistance against UV-4B, mouse-adapted influenza virus was passaged in mice in the presence or absence of UV-4B and virus isolated from lungs was used to infect the next cohort of mice, for five successive passages. Deep sequencing was performed to identify changes in the viral genome during passaging in the presence or absence of UV-4B. Relatively few minor variants were identified within each virus and the ratio of nonsynonymous to synonymous (dN/dS) substitutions of minor variants confirmed no apparent positive selection following sustained exposure to UV-4B. Three substitutions (one synonymous in PB2, one nonsynonymous in M and PA each) were specifically enriched (>3%) in UV-4B-treated groups at passage five. Recombinant viruses containing each individual or combinations of these nonsynonymous mutations remained sensitive to UV-4B treatment in mice. Overall, these data provide evidence that there is a high genetic barrier to the generation and selection of escape mutants following exposure to host-targeted iminosugar antivirals.

Introduction

Rapidly acquired genetic variability due to error-prone polymerases has made rational drug development against RNA viruses, such as influenza virus, arduous. The fundamental problem underlying this difficulty is the inevitable development of drug resistance, where changes in a very small number of amino acid residues in the targeted viral protein is sufficient to reduce or completely block efficacy of a drug. For example, a single amino acid substitution (H274Y) in influenza A virus (IAV) isolates confers resistance to the neuraminidase inhibitor, oseltamivir1. Rapid development of resistance necessitates new approaches to the development of antiviral drugs. Since viruses are obligate intracellular parasites, they are critically dependent upon host factors for infection, replication, and spread. Identification and targeting of host factors that are critical for the viral replication cycle provides an opportunity for the development of novel classes of antiviral drugs2,3. Due to the improbability of changes to the host genome during an acute viral infection cycle, evasion of host-directed antiviral drugs is much less likely to occur.

IAV is a good target for the development of a host-directed antiviral therapy as the host molecular pathways that interact with the immunodominant viral proteins, hemagglutinin (HA) and neuraminidase (NA), have been well described. HA and NA are the two major surface glycoproteins of IAV that play essential roles in virion attachment and budding4,5. HA attaches the incoming virions to target cells by binding terminal sialic acid residues on cell-surface glycans5, whereas NA is a sialidase that assists with the budding process by cleaving sialic acid on the cell surface6. As a prototypical homotrimeric type I integral membrane protein, HA is synthesized in the endoplasmic reticulum (ER) of infected cells and transported through the Golgi complex to the plasma membrane, where it is incorporated into budding virions7,8,9. A variable number (dependent on the strain) of N-linked oligosaccharides are added co-translationally to HA as it is extruded into the ER through the translocon, and are subsequently trimmed and modified extensively during transport to the cell surface8,10,11,12. HA folding begins co-translationally, as demonstrated by the acquisition of intrachain disulfide bonds and the binding of monoclonal antibodies (mAbs) specific for discontinuous epitopes within HA to nascent chains8,11,12,13,14,15. N-terminal glycosylation at the globular head of HA helps the virus escape the immune response, while glycosylation of some sites at the stem of HA are critical for protein folding and stability11,13,14,15. Similarly, NA is a transmembrane tetrameric protein that uses the ER-Golgi route to the cell surface and is glycosylated in the process16. As both HA and NA rely heavily on glycosylation and other processing in the ER, a host-directed drug that inhibits this process has high potential for antiviral activity.

Key targets for inhibiting the host ER glycosylation pathway include the α-glucosidase I and II enzymes. These enzymes are responsible for making modifications to the co-translationally attached N-linked oligosaccharides, which are necessary for the proper folding of many glycoproteins17,18. Enveloped viruses that express surface glycoproteins, such as HA and NA of IAV, are dependent on the host cell α-glucosidase I and II enzymes for their replication. If viral proteins are not properly glycosylated, protein folding, stability, functionality, and immune evasion are impaired, and may result in reduced viral secretion or the production of defective virions8,9,10,11.

Iminosugars are glucomimetics with structural similarity to sugar molecules that can competitively inhibit glucosidase enzymes. Some iminosugars specifically target the α-glucosidases in the ER19. As a result, their therapeutic potential has been investigated against a range of viruses both in vitro and in vivo20,21,22,23. The iminosugar UV-4B has demonstrated in vitro and in vivo activity against a phylogenetically diverse set of glycosylated, enveloped viruses, including dengue (DENV) and influenza viruses24,25,26,27,28. It was previously demonstrated that DENV has a high genetic barrier for development of resistance against UV-4B27.

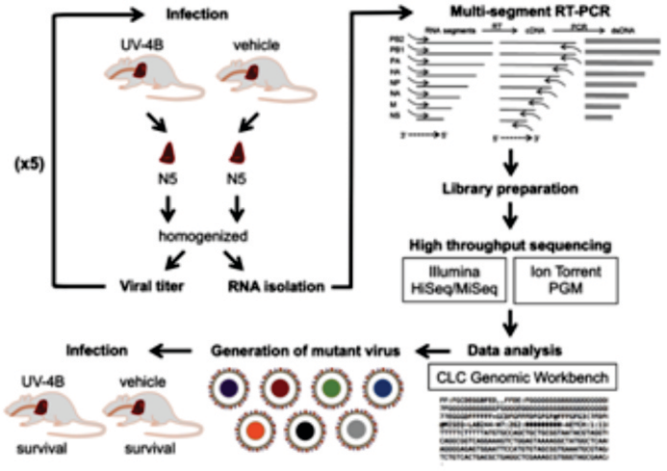
Here, we assessed the development of viral resistance to UV-4B treatment in vivo using a murine model of IAV infection. Mouse-adapted influenza A/Texas/36/91 (H1N1) was passaged in mice treated with UV-4B or vehicle for five successive passages. The sensitivity of the 5-times passaged viruses (P5) to treatment with UV-4B or the unrelated antiviral oseltamivir, which is currently approved for use to treat IAV infections, was confirmed in mice. The passaged viruses were deep-sequenced and relatively few minor variants were identified within each virus. Three substitutions (one synonymous in PB2 and two nonsynonymous in M and PA) were specifically enriched in P5 viruses passaged in the presence of UV-4B. However, these substitutions did not impact the efficacy of UV-4B or oseltamivir against the P5 viruses as evident from in vivo efficacy studies. Recombinant viruses containing each individual or combinations of these mutations remained susceptible to UV-4B treatment, showing no increased replication in vitro or enhanced disease severity in vivo.

Results

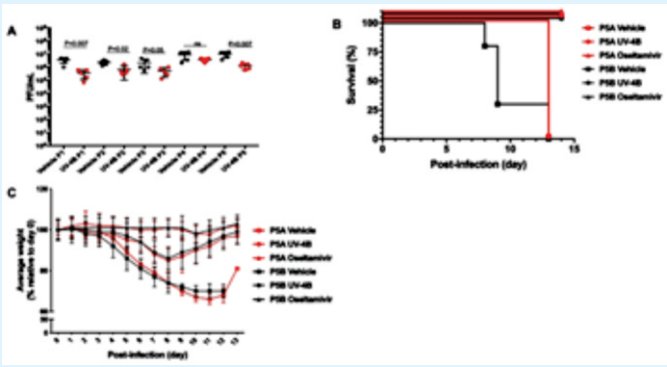
Passaging IAV in vivo in the presence of the host-targeted antiviral UV-4B does not decrease susceptibility to the drug

A murine model of IAV infection was used to test for the development of viral resistance to the iminosugar UV-4B. The dosing route and regimen were selected based on available data from tolerability and pharmacokinetic studies in uninfected mice25,28 and previous efficacy studies using IAV murine models of disease24,25.

Groups of mice were challenged intranasally (i.n.) with influenza A/Texas/36/91 (H1N1) (passage 0; P0) and treated by intragastric administration of UV-4B or vehicle three times a day (TID) for seven days (Fig. 1). A portion of mice (ten) in each group were observed for morbidity and mortality for 14 days. The remaining five mice in each group were sacrificed on Day 4 post-infection (p.i.) and their lungs were harvested and homogenized for virus titration and deep sequencing. Following virus titration of the lung homogenates, a portion of the homogenates were pooled by group and used as the challenge virus for the next passage (P1) in mice. Virus passaging continued for a total of five successive passages. As expected, and similar to our previously published work24,25, viral titers in the lungs of UV-4B-treated mice were significantly lower ($P \leq 0.05$) than that of vehicle-treated mice after each passage except for passage 4 ($P = 0.052$) (Fig. 2A).



Schematic diagram of the study design. Two groups of 15 female BALB/c mice were challenged i.n. with ~1 LD90 (~52 PFU) of mouse adapted A/Texas/36/91 (H1N1) and treated by intragastric administration with 100 mg/kg UV-4B or vehicle (water) TID for 7 days, starting 1 h after infection. Mice from each group ($n = 5$) were sacrificed on day 4 post-infection and their lungs were isolated and homogenized. A portion of the lung homogenates were pooled by group and used as the challenge virus (~1 LD90 or ~52 PFU/mouse) for the next passage in mice, successively for a total of 5 passages. The remaining portion of the lung homogenates were used to measure viral titer and isolate RNA for amplification by multi-segment RT-PCR. Sequencing libraries were prepared and sequenced on either the Illumina HiSeq 2000 or Illumina MiSeq v2 instruments (with repeat sequencing on the Ion Torrent PMG). Virus sequence assembly and identification of SNPs were performed using the CLC Genomics Workbench. Mutant viruses recapitulating the corresponding nucleotide changes of 3 SNPs identified (individually and in combination) were generated using site directed mutagenesis. Lethality and susceptibility to UV-4B of the mutant viruses was measured in vivo using similar experimental conditions.



Properties of IAV after in vivo passaging in the presence or absence of UV-4B. (A) Mouse-adapted INFV A/Texas/36/91 (H1N1) was passaged five times in the presence or absence of UV-4B. BALB/c mice were infected i.n. with the P0 parent virus or virus isolated from lungs of infected mice after 1 (P1) to 4 (P4) passages and treated by intragastric administration with 100 mg/kg UV-4B or vehicle (water) TID beginning 1 h after infection. Lungs were isolated from mice ($n = 5$/group) on day 4 post-infection and viral titers were measured by plaque assay after each passage in mice. UV-4B-treated groups in passages (P)0–4 were significantly lower ($p < 0.05$) than vehicle-treated groups at each passage, except for P4 ($p = 0.052$). (B,C) Survival outcome and relative weight of mice infected with IAV passaged 5 times in vivo in the presence or absence of UV-4B. BALB/c mice ($n = 10$/group) were exposed i.n. to virus (~1 LD90) that was passaged 5 times in mice in the presence of UV-4B (P5A) or water (P5B), and treated via intragastric administration with UV-4B (100 mg/kg, TID), water (TID), or oseltamivir (20 mg/kg, twice daily) beginning 1 h after challenge. Survival (B) and relative average weight compared to day 0 (C) were measured daily through the end of the study (Day 14).

An efficacy study was performed to determine whether IAV isolated after 5 passages (P5) from animals treated with UV-4B (P5A) or vehicle (P5B) were still sensitive to treatment with UV-4B or the influenza virus (INFV) antiviral oseltamivir. Mice were challenged i.n. with P5A or P5B IAV and then treated with UV-4B (100 mg/kg, TID), vehicle (water), or oseltamivir (20 mg/kg, twice daily). The UV-4B treatment dose selected was based on previous mouse efficacy studies25,26. Mice were observed daily for changes in health, weight and mortality for 14 days. All mice treated with UV-4B or oseltamivir survived infection with P5A and P5B viruses (Fig. 2B). All mice infected with P5A or P5B IAV and treated with vehicle succumbed to disease with a median survival time of 13 or 9 days, respectively (Supplemental Table 1). There were no differences in the average decrease in body mass of similarly treated groups infected with either P5A or P5B viruses (Fig. 2C). The onset and severity of clinical signs of disease (health score) were comparable between groups receiving similar treatments whether they were infected with the P5A or P5B viruses. Overall, this suggests that IAV passaged in mice five times in the presence of UV-4B did not acquire mutations that could potentially cause resistance to UV-4B or oseltamivir treatment or increase in vivo pathogenicity.

Sequencing of IAV identified limited selective mutations acquired during in vivo passaging in the presence of UV-4B

IAVs from the five individual mice at each passage that were treated with UV-4B (n = 25 samples, 5 per passage) or vehicle (n = 25 samples, 5 per passage), along with the input challenge virus, were deep-sequenced to achieve at least 200-fold coverage at each base of the coding sequence. Variant analysis of deep-sequence data was performed to identify selection of variants in UV-4B-treated animals versus vehicle-treated animals. The consensus sequence for the parental input mouse-adapted influenza A/Texas/36/91 (H1N1) challenge virus used for this study was previously unpublished. Therefore, we compared it against 2 deposited consensus sequences in the National Center for Biotechnology Information (NCBI) database for the A/Texas/36/91 (H1N1) strain. Five nonsynonymous nucleotide differences were found in the HA and NA sequences for the input parental virus compared with both NCBI sequences, only one of which (HA residue N104D) removes a potential N-linked glycosylation sequon (Supplemental Table 2).

Deep sequencing analysis of the in vivo-passaged IAV was performed to determine the consensus sequence and analyze consensus differences and low frequency nucleotide variations (quasi species/ minor variants) observed in the IAV samples to identify potential genomic population variation in UV-4B-treated and vehicle-treated mice. The sequence analysis identified that the genomic population in the virus samples is fairly uniform for an RNA virus (relatively few minor variants) and most were not specifically associated with UV-4B treatment. There were 31 selectively enriched coding sequence variants with >3% Single Nucleotide Polymorphisms (SNPs) identified at P5 which were unequally divided among 7 of the 8 RNA segments (Table 1). A cut off of >3% was considered true polymorphism (with minimum of 200X coverage at each base) to account for errors we observed from systematic sequencing (as high as 1%) with the Illumina platform, in addition to errors due to reverse transcription and library preparation29,30.

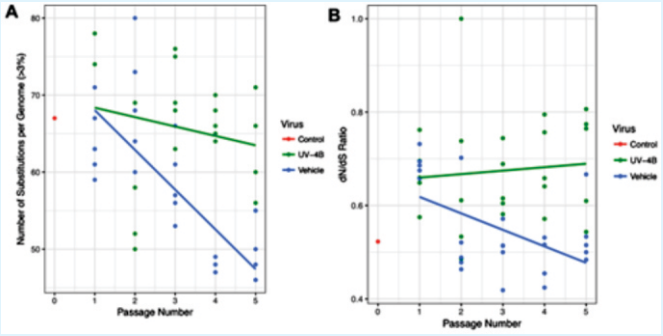
Table 1 Summary of selectively enriched coding sequence variants at passage five.

The summary of results for P5 sequence changes are shown in Table 2. Fourteen SNPs did not result in an amino acid substitution (i.e., synonymous). Of the 17 nonsynonymous substitutions, 6 were enriched in samples from UV-4B-treated mice. The six nonsynonymous substitutions associated with UV-4B treatment were limited to the polymerase acidic (PA), nucleoprotein (NP), and matrix (M) proteins. Other investigators have observed mutations in PA, polymerase basic 2 (PB2), and NP, which were attributed to mouse adaptation; however, not specifically at these amino acid positions identified here31. Only two of the six nonsynonymous substitutions (one each in M and PA) and one synonymous substitution in PB2 were specific to UV-4B treatment and were not also present in viruses from the vehicle-treated samples.

Table 2 Selectively enriched coding sequence variants of influenza A/Texas/36/91(H1N1) virus in untreated vs. UV-4B-treated P5 mice.

Plotting of the substitution rate (minor variant >3%) for each passage shows no significant change in the rate of substitution over passaging in UV-4B-treated mice (linear regression analysis of non-zero slope p-value = 0.2795).

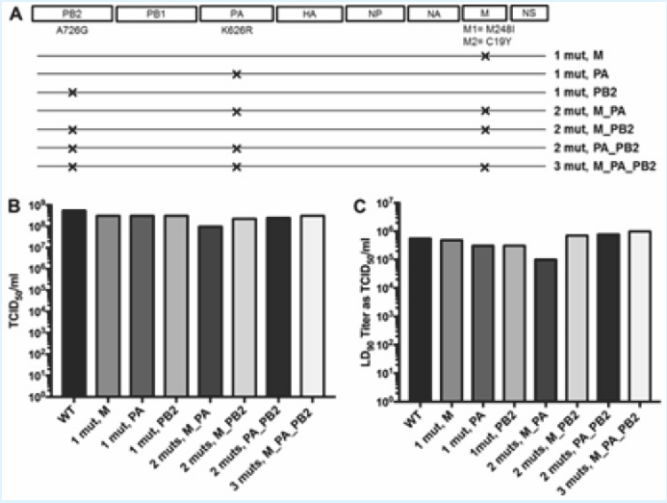
In contrast, linear regression analysis shows a significant decrease in the number of substitutions in vehicle-treated mice (p-value < 0.0001) (Fig. 3A). Unlike what has been observed with direct antivirals (e.g., oseltamivir)32, we found neutral selection in viruses from UV-4B treated mice based on the ratio of nonsynonymous to synonymous substitutions (dN/dS), despite the constant number of substitutions over time (linear regression of non-zero slope p-value = 0.65) (Fig. 3B), and negative selection was observed in vehicle-treated mice over successive passages (linear regression of non-zero slope p-value = 0.0063). Regardless of being under pressure, as evident from neutral (UV-4B group)vs. negative (vehicle group) selection, the lack of any positive selection in the presence of UV-4B after 5-pasages further confirms the high genetic barrier for the emergence of drug resistance in the presence of host-targeted antivirals (e.g. UV-4B)



Regression lines plotting the relationship between passage number and either (A) number of substitutions per genome (>3%) or (B) the dN/dS ratio (number of nonsynonymous substitutions divided by the number of synonymous substitutions). Analysis was performed using the R package ggplot2 with method "lm" (linear model).Recombinant IAV with substitutions enriched in P5 show no increased replication in MDCK cells

All 8 gene segments of A/Texas/36/91 (H1N1) virus were synthesized and cloned to develop reverse genetics systems for further evaluation33. M, PA, and PB2 gene segments were mutagenized to generate the three specifically enriched substitutions found in P5A viruses to determine whether these acquired substitutions have any significant selective advantage for the virus to escape UV-4B treatment. The nonsynonymous substitution in the M gene segment results in an amino acid substitution in both M1 (M248I) and M2 (C19Y), whereas the nonsynonymous substitution in PA results in the amino acid change K626R. We also generated a synonymous nucleotide substitution in PB2 (A726G). A total of 8 recombinant (r) mouse-adapted influenza A/Texas/36/91 (H1N1) viruses were rescued (Fig. 4A) using an established reverse genetics rescue system34,35.

Figure 4



In vitro and in vivo replication efficiency of recombinant wild-type and mutant viruses. (A) Schematic of recombinant viruses generated. (B) Viral titers for wild-type and recombinant mutant viruses were determined by TCID50 assay in MDCK cells at Day 4. (C) Mice were infected i.n. with decreasing challenge doses of wild-type or mutant viruses. Survival was monitored to the end of the study (Day 14) and the LD90 was determined for each virus.

No selective advantage of the substitutions enriched in P5 over the input mouse-adapted wild-type virus

Titration of viruses in Madin-Darby Canine Kidney (MDCK) cells after rescue showed very similar titers for each of the recombinant mutant viruses, demonstrating that these substitutions were not detrimental to viral replication/fitness in vitro (Fig. 4B). Next, we compared the in vivolethality of recombinant IAVs containing substitutions that were enriched in the P5A virus to that of recombinant wild-type mouse-adapted influenza A/Texas/36/91 (H1N1) virus in BALB/c mice. Mice were infected i.n. with decreasing doses of recombinant viruses, and changes in weight loss and survival were monitored. Less pronounced weight loss and a higher survival rate were observed with decreasing challenge doses in a largely dose-dependent manner (Supplemental Figs 1 and 2).

Overall, the LD90 titers (converted as TCID50 titer) were similar among wild-type and mutant viruses in BALB/c mice (Fig. 4C and Table 3), suggesting that no increase in lethality was acquired by viruses with these specific mutations.

Table 3 Estimated 90% lethal dose (LD90) of various influenza A (H1N1) viruses in BALB/c mice treated with water three times daily for 7 days.

Substitutions enriched in P5A are sensitive to both UV-4B and oseltamivir

UV-4B treatment (100 mg/kg TID for 5 days beginning 8 h after infection) protected mice against lethal infection with all the recombinant wild-type and mutant IAV ($p < 0.01$ in all cases) (Fig. 5). Increased survival (60–100%) was observed with UV-4B treatment when mice were infected either with the wild-type or recombinant mutant viruses as compared to those treated with water alone (0–10%). Protection from weight loss was also apparent during the period of UV-4B treatment (day 4 p.i.) (Supplemental Fig. 3). Oseltamivir was included as a positive control compound and performed as expected against both recombinant wild-type virus and the recombinant virus with all three mutations (3 muts, M_PA_PB2) (Fig. 5A,H).

Figure 5

Survival outcome of mice infected with recombinant influenza A (H1N1) mutants and treated with UV-4B, oseltamivir, or vehicle. (A–H) BALB/c mice ($n = 10$/group) were infected i.n. (~1 LD90) with a wild-type recombinant virus or mutant recombinant viruses containing substitutions specific to UV-B treatment. Mice were treated via intragastric administration with UV-4B (100 mg/kg) or vehicle (water) three times daily or oseltamivir (20 mg/kg) twice daily for 5 days beginning 8 h post-challenge. $^{**}P < 0.01$, $^{***}P < 0.001$ when compared to the vehicle group.

Discussion

In this study, we evaluated the ability of mouse-adapted IAV (A/Texas/36/91 (H1N1)) to mutate in vivo in the presence of the host-targeted iminosugar UV-4B. Viruses passaged 5 times in mice in the presence or absence of UV-4B remained sensitive to UV-4B treatment inin vivo efficacy studies, providing evidence that UV-4B treatment does not promote the generation of viral escape mutants. Amongst the passaged viruses, relatively few minor variants were identified, and only three substitutions (one synonymous in PB2 and two nonsynonymous in M and PA) were specifically enriched in UV-4B-treated viruses at passage five. Recombinant viruses containing these mutations showed no detectable selective advantage and maintained their sensitivity to UV-4B treatment in vivo, corroborating a predicted high genetic barrier to escape mutations with host-targeted iminosugar antivirals36.

We identified minimal mutations in IAV after multiple passages in mice, and most of those mutations were not specifically associated with UV-4B treatment, indicating nonspecific selective pressure. Six of the 17 nonsynonymous substitutions identified were enriched in UV-4B-treated samples, which were limited to proteins that are not glycosylated by the host-cell machinery in the ER. Mice infected with recombinant viruses containing mutations in M, PA and/or PB2 were still susceptible to treatment with UV-4B; however, a slightly lower survival benefit (60–90%) was observed for some groups compared to the control group infected with the wild-type recombinant virus (100% survival). These differences could be due, in part, to challenges during the treatment procedure, possibly resulting in early deaths, or because UV-4B doesn't consistently protect every animal from lethal infection, which would be more evident in a larger population. However, it is also plausible that these mutations could alter the HA/NA balance on the virions themselves or through some unknown mechanisms in the context of recombinant viruses. Although, it is important to note that all three of these unique substitutions were at sub-consensus levels in the passaged viruses, even after 5 rounds of passaging, suggesting limited to no selective advantage of these substitutions.

Based on the mechanism of action of UV-4B, it was expected that mutations unique to UV-4B treatment may be identified in viral glycoproteins, especially since this has been shown for selection of IAV in presence of mAbs10,37.

The lack of variants in these proteins indicates that the virus did not acquire mutations necessary to overcome the selection pressure elicited by UV-4B, confirming a high genetic barrier for development of resistance. Unlike previous studies that showed concentration dependent positive selection of the IAV in the presence of oseltamivir and rapid emergence of oseltamivir resistant mutation after only one or two passages in mice32, our dN/dS

analysis showed neutral selection during five successive passages in mice for four days in the presence of UV-4B, which accounts for a total of ~25–50 replication cycles of the virus (calculated based on ~5 to 10 replication cycles over the course of 4 days in mice/passaging38), further confirming the genetic barriers to escape UV-4B treatment. At the same time, as expected, passaging without drug showed negative selection (purifying selection) over time as this virus is already well adapted to the host (mice) and only purged changes that are deleterious on viral fitness in mice.

The generation of resistant mutants following UV-4B treatment was previously evaluated in mice following infection with DENV, a mosquito-borne pathogen that can cause severe and potentially life-threatening illnesses27. Like IAV, DENV requires the host's ER α-glucosidase I and II enzymes for replication. Plummer et al., evaluated the evolution of two populations of passaged virus in the presence or absence of UV-4B in mice: DENV passaged in individual mice after a single passage, and pooled virus over the course of 4 serial passages. Similar to the results reported here for IAV, passaging of DENV in mice in the presence of UV-4B did not provide evidence for generation of viral escape mutants. Only 13 nonsynonymous SNPs in DENV were identified in pooled samples from at least one time point, and 12 of those 13 mutations were found in virus isolated from both the vehicle-treated and UV-4B-treated animals. However, the authors noted that by pooling samples for sequencing, some of the individual mouse-specific responses may have been diluted below detection. For this reason, combined with consideration of the normal passaging of DENV from invertebrate (mosquito) to vertebrate hosts rather than from vertebrate to vertebrate, they evaluated mutations acquired in DENV following a single passage in mice in the presence or absence of UV-4B. Synonymous and nonsynonymous mutations were identified in all DENV proteins after a single passage in mice. Unlike the current study, the dN/dS was higher when passaged in the presence of UV-4B compared to vehicle. Looking specifically at DENV glycosylated proteins (membrane, M; envelope, E; and NS1), nineteen nonsynonymous mutations were present at significant levels only in UV-4B-treated mice, possibly related to the mechanism of the drug. This was not observed for IAV in the present study, where no mutations specific to UV-4B treatment were identified in any of the viral glycoproteins.

Prior to this study, the development of drug resistant IAV strains was primarily evaluated for direct-acting antivirals. Unlike host-targeted iminosugars, approved anti-influenza virus drugs that are currently available directly target viral proteins (M2: amantadine and rimantadine; NA: oseltamivir, zanamivir, peramivir; PA: Xofluza39). The potential for influenza viruses to develop resistance to direct-acting antivirals is well established. Amantadine and rimantadine directly block the M2 proton channel to prevent the delivery of the viral genome into the cytoplasm of cells infected with IAV40. The Centers for Disease Control and Prevention (CDC) no longer recommends the use of amantadine or rimantadine to treat IAV infections in the United States because of the prevalence of resistant IAV strains that are no longer sensitive to treatment with these drugs41. Since the early 1970s, multiple groups have reported instances of resistance to amantadine and rimantadine42,43,44,45. As a result, neuraminidase inhibitors (NAI) are currently recommended by the CDC for treatment of influenza virus infections. NAIs, which inhibit the enzymatic activity of the viral NA protein, prevent virus release and spread and are effective against both IAV and IBV viruses. The active site of NA is highly conserved among all influenza viruses, making NA an ideal target for antiviral therapeutics directed against influenza virus32,46. However, mutations in any of the viruses that alter the shape/charge of the NA catalytic site can reduce binding and result in decreased sensitivity or resistance to treatment47 In addition, evidence shows that in the absence of NA-specific drugs, antibody escape in HA results in epistatic compensatory mutations in NA that could also result in drug resistance1,48.

Of the approved NAIs, oseltamivir is the only drug with sufficient bioavailability to be administered orally49,50,51,52,53,54,55. Oseltamivir-resistant strains can arise from a single mutation that results in a decrease in the binding affinity of oseltamivir to NA56,57. Additionally, combinations of multiple mutations can result in a synergistic or enhanced effect on oseltamivir antiviral resistance32,58,59,60,61 and can potentially restore mild reduced viral fitness that is observed with some single mutations1,59. As the potential risk for the transmission of drug resistant strains is a continuous concern, the impact on the efficacy of other antivirals against such resistant strains must be considered.

Stavaleet al., demonstrated that UV-4B maintains efficacy against an oseltamivir-resistant strain in a murine model of disease25. Here, we report that IAV passaged in mice five times in the presence of UV-4B did not acquire mutations that cause resistance to the commonly used anti-influenza drug oseltamivir. While the proposed mechanism for the antiviral activity of UV-4B is through perturbation of N-linked glycan processing, further studies need to be performed to determine which, if any, glycosylation sites on viral glycoproteins are affected and whether other mechanisms contribute to the anti-IAV activity of UV-4B. Using influenza virus reassortants, Hussain et al. demonstrated that changes in glycosylation of HA are the likely antiviral mechanism for iminosugars but more detailed analysis of which glycosylation sites are most sensitive has not yet been determined62.

It is interesting to note that UV-4B protects against fatal influenza infection in vivo (Fig. 2B,C); however, treatment with UV-4B does not robustly reduce viral replication in the lung (Fig. 2A) to a level that would be biologically significant. Based on these data, it appears that UV-4B may prevent influenza-induced morbidity and mortality through multiple biologic effects that may include mechanisms other than inhibition of viral replication. One such mechanism could be reduction in localized inflammation due to blunted cytokine responses. The freebase form of UV-4B (UV-4) itself does not induce a cytokine response in the blood of naïve, UV-4-treated mice28 or in vitro using mouse splenocytes, or human PBMC (Warfield et al., unpublished data) nor does it alter cytokine responses in vitro following mitogen stimulation (ex. PHA or LPS) (Warfield, et al., unpublished data). However, UV-4B treatment does result in reduced systemic cytokine responses in dengue virus infection mice28and could also result in a reduced local (lung) cytokine responses following influenza infection. Alternately, other novel mechanisms that limit influenza pathogenesis by host-targeted antiviral iminosugars in addition to established mechanisms (i.e., direct impact on the hosts glycosylation pathway or indirectly by protein mis-folding due to ER stress) may be discovered22. The limited reduction in virus titer observed in the mouse lung following UV-4B treatment supports the potential use of UV-4B in a combination therapy. The effect of UV-4B treatment when co-administered with an NA inhibitor, and the potential for the development of drug resistant strains following combined treatment, should be considered and evaluated in future studies.

Taken together, our data suggests that the iminosugar UV-4B, which is effective against a broad range of influenza A and B viruses in vitro and in vivo, is at a low risk for selecting for mutant, drug-resistant influenza viruses. Iminosugars have positive drug-like properties and a history of safe use in humans, including five iminosugar compounds in clinical use: miglustat (Zavesca®) for the treatment of Gaucher's disease and Niemann-Pick Type C; migalastat (Galafold™) for the treatment Fabry disease; and miglitol (Glyset®), acarbose (Precose®), and voglibose (Basen®) for the treatment or prevention of type II diabetes mellitus. Therefore, iminosugars remain a promising class of compounds for the future development of an antiviral therapeutic targeting a range of diverse viral pathogens, including DENV and influenza viruses.

Materials and Methods

Animal welfare, husbandry and observations

All procedures of the study were performed in accordance with the guidelines and protocols set and approved by the Noble Life Sciences or Utah State University Institutional Animal Care and Use Committee (IACUC). Both institutions are fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC). Laboratory animals were observed and veterinary care was provided for all laboratory animals as required on a 24 hr basis, including weekends and holidays, in accordance with the Public Health Service Policy, U.S. Dept. of Agriculture (USDA) and AAALAC International requirements.

Female BALB/c mice (6–8 weeks of age) maintained under specific pathogen-free conditions were procured from Charles River Laboratories and were quarantined for >48 h prior to study initiation. During in-life study duration, all animals were observed carefully 2–3 times daily for changes in clinical signs, including morbidity and mortality. Survival and health were evaluated daily using a scoring system to assess signs of clinical disease (e.g. appearance of fur coat, posture, mobility, activity level and attitude) and provide a numerical value (1–7) directly related to disease severity as previously described25. Mice were euthanized when they met a 30% weight loss cut-off or scored at ≥6 in the standard scoring system.

Animals were euthanized by administration of inhaled CO2followed immediately by cervical dislocation and in accordance with the 2013 American Veterinary Medical Association (AVMA) Guidelines on Euthanasia.

Passaging description

The original virus stock of the mouse-adapted influenza A/Texas/36/91 (H1N1) was obtained from the Baylor School of Medicine (kind gift of P. Wyde and B. Gilbert). Using 6-8-week-old female BALB/c mice, a new working virus stock (P0) was prepared using infected-lung homogenates and the LD90 was determined to be 52 PFU/mouse. Influenza A/Texas/36/91 (H1N1) was passaged successively five times in mice before determining the efficacy of UV-4B against the P5 virus. One group of 30 mice (P0) was challenged with influenza A/Texas/36/91 (H1N1) (~1 LD90 or ~52 PFU) intranasally and were treated via intragastric administration with either 100 mg/kg UV-4B or its vehicle (water) control starting one hour after infection. This treatment continued TID for seven days. A portion of these mice (n = 10) were observed daily for weight changes, morbidity (assessed using standard health scores) and mortality for 14 days. The remaining mice (n = 5) were sacrificed on Day 4 post-infection and their lungs were bisected. Half of each lung was immersed in RNALater for RNA extraction and subsequent virus sequencing. The other half of each lung was snap-frozen in liquid nitrogen and stored frozen until homogenization in PBS on wet ice. Homogenate samples from individual mice were maintained for titration with 0.2 mL from each mouse combined into a pool homogenate to be used in the next passage. Following virus titration of the lung homogenate pool, these viruses were then used as the challenge agent (P1-4) in the next round of virus challenge at ~52 PFU/mouse (~1 LD90) of input virus. Virus passaging continued for a total of five times.

Lethal dose 90% (LD90) and efficacy determination of passage 5 viruses

The LD90 was determined for influenza viruses isolated from lungs after 5 passages in mice treated with UV-4B (P5A) or vehicle (P5B). Groups of ten BALB/c mice were used to test the lethality of six different challenge doses (1, 5, 10, 25, 50, or 100 PFU) of each virus following i.n. exposure. All mice were orally administered 100 µL of water TID starting 1 h p.i. for a total of ten days to mimic treatment. Mice were monitored daily for changes in health (assessed using standard scoring system), weight, and mortality for 14 days.

Groups of 30 mice were challenged with either P5 virus isolated from animals treated with UV-4B (P5A, ~1 LD90 = 25 PFU) or from animals treated with the vehicle control (P5B, ~1 LD90 = 10 PFU). One hour following infection, the animals in these groups were treated via intragastric administration with either 100 mg/kg of UV-4B (N = 10), vehicle control (N = 10), or 20 mg/kg of the positive control, oseltamivir. The treatments with UV-4B and vehicle continued TID for seven days, while the treatment with oseltamivir continued twice a day for five days. Mice were observed daily for 14 days for changes in health, weight, and mortality.

Influenza virus next-generation sequencing

Viral RNA from the infected lung lysates were individually evaluated using multi-segment reverse transcriptase-polymerase chain reaction (M-RT-PCR)63, followed by library preparation using the Nextera DNA library preparation kit (Illumina) and the Ion Xpress™ Plus Fragment Library Kit (Thermo Fisher Scientific) for sequencing using Illumina MiSeq and Ion Torrent PGM, respectively, to overcome platform specific errors. The IAV genomic RNA segments were simultaneously amplified from 3 µl of purified RNA using M-RT-PCR34. Illumina libraries were prepared from these M-RT-PCR products using the Nextera DNA Sample Preparation Kit (Illumina, Inc., San Diego, CA, USA) with half-reaction volumes. PCR products were quantified using QIAxcel (Qiagen, Hilden, Germany), and 25 ng of DNA amplicons for each sample were tagmented (fragmented and tagged) at 55 °C for 5 min. Tagmented DNA amplicons were cleaned with the ZR-96 DNA Clean & Concentrator Kit (Zymo Research Corporation, Irvine, CA, USA) and eluted in 25 µl resuspension buffer. Illumina sequencing adapters and barcodes were added to tagmented DNA via PCR amplification by combining 20 µl tagmented DNA with 7.5 ul Nextera PCR Master Mix, 2.5 ul Nextera PCR Primer Cocktail, and 2.5 µl of each index primer (Integrated DNA Technologies, Coralville, IA, USA) for a total volume of 35 µl per reaction. Five cycles of PCR were performed as per the Nextera DNA Sample Preparation Kit protocol (3 min at

72 °C, denaturation for 10 sec at 98 °C, annealing for 30 sec at 63 °C, and extension for 3 min at 72 °C) to create a dual-indexed library for each sample. After PCR amplification, 10 µl of each library derived from M-RT-PCR products were pooled into a 1.5-ml tube; separately, 10 µl of each library derived from HA-specific amplicons were pooled into a 1.5-ml tube. Each pool was cleaned two times with Ampure XP Reagent (Beckman Coulter, Inc., Brea, CA, USA) to remove all leftover primers and small DNA fragments. The first and second cleanings used 1.2x and 0.6x volumes of Ampure XP Reagent, respectively. The cleaned pool derived from M-RTPCR products was sequenced on the Illumina HiSeq 2000 instrument (Illumina, Inc.) with 100-bp paired-end reads, while the cleaned pool derived from HA-specific amplicons was sequenced on the Illumina MiSeq v2 instrument with 300-bp paired-end reads.

Separately, influenza M-RT-PCR products were randomly amplified and prepared for NGS using a sequence-independent single-primer amplification (SISPA) method as previously described64. The methodology used 100 ng of amplified viral DNA that was denatured in the presence of DMSO and a chimeric oligonucleotide containing a known 22-nt barcode sequence followed by a 3′ random hexamer. A Klenow reaction was prepared with the denatured DNA template by adding NEB buffer II, 3′-5′ exo- Klenow (New England Biolabs, Ipswich, MA, USA), and dNTPs (Thermo Fisher Scientific, Waltham, MA, USA). The Klenow reaction was incubated at 37 °C for 60 min, followed by incubation at 75 °C for 10 min. The resulting cDNA was randomly amplified by PCR using the Promega GoTaq Hot Start Polymerase (Promega Corporation, Madison, WI, USA) for 35 cycles (denaturation: 30 sec, 94 °C; annealing: 30 sec, 55 °C; extension: 48 sec, 68 °C). PCR reactions contained primers corresponding to the known 22-nt barcode sequence from the oligonucleotide utilized in the previous Klenow step. The resulting cDNA was then treated with Exonuclease I at 37 °C for 60 min, followed by incubation at 72 °C for 15 min. SISPA products were normalized and pooled into a single reaction that was purified using the QIAquick PCR purification kit (Qiagen). Pooled samples were further purified to select for SISPA products 300–500 bp in size for Illumina Miseq paired-end (2 × 300) sequencing.

For additional sequencing coverage, samples were re-sequenced using the Ion Torrent platform. M-RT-PCR products were sheared for 7 min, and Ion-Torrent-compatible barcoded adapters were ligated to the sheared DNA using the Ion Xpress Plus Fragment Library Kit (Thermo Fisher Scientific) to create 400-bp libraries. Libraries were pooled in equal volumes and cleaned with the Ampure XP Reagent. Quantitative PCR was performed on the pooled, barcoded libraries to assess the quality of the pool and to determine the template dilution factor for emulsion PCR. The pool was diluted appropriately and amplified on Ion Sphere Particles (ISPs) during emulsion PCR on the Ion One Touch 2 instrument (Thermo Fisher Scientific). The emulsion was broken, and the pool was cleaned and enriched for template-positive ISPs on the Ion One Touch ES instrument (Thermo Fisher Scientific). Sequencing was performed on the Ion Torrent PGM using a 318v2 chip (Thermo Fisher Scientific).

Virus genome assembly and variant analysis

For virus sequence assembly, all sequence reads were sorted by barcode, trimmed, and de novo assembled using CLC Bio's clc_novo_assembleprogram (Qiagen). The resulting contigs were searched against custom full-length influenza segment nucleotide databases to find the closest reference sequence for each segment. All sequence reads were then mapped to the selected reference influenza A virus segments using CLC Bio's clc_ref_assemble_long program.

Minor allele variants (i.e., nucleotide sequences that differ from the reference sequence and are supported by sequencing reads at or above a pre-defined threshold) were identified usingFindStatisticallySignificantVariants (FSSV) software (http://sourceforge.net/projects/elvira/). The FSSV software applied statistical tests to minimize false-positive Single Nucleotide Polymorphism (SNP) calls generated by Illumina sequence-specific errors (SSEs)65. SSEs usually result in false SNP calls if sequences are read in one sequencing direction. The FSSV analysis tool requires observing the same SNP at a statistically significant level in both sequencing directions. Once a minimum minor allele frequency threshold and significance level are established, the number of minor allele observations and major allele observations in each direction and the minimum minor allele frequency threshold are used to calculate p-values based on the binomial distribution cumulative probability. If the p-values calculated in

both sequencing directions are less than the Bonferroni-corrected significance level, then the SNP calls are accepted. A significance level of 0.05 (Bonferroni-corrected for tests in each direction to 0.025) and a minimum minor allele frequency threshold of 3% were applied for this analysis, and the consensus sequence from the input parental virus was used as the reference sequence for each sample.

Differences in the consensus sequence compared to the reference sequence were identified using CLC Bio's find_variations software. The identified consensus and minor allele variations were analyzed by assessing the functional impact on coding sequences or other regions based on overlap with identified features of the genome. For each sample, the reference sequence was annotated using VIGOR software31, and then the variant data and genome annotation were combined usingVariantClassifier software66 to produce records describing the impacts of the identified variations.

Recombinant viruses

To generate the recombinant A/Texas/36/1991 (H1N1) viruses, the appropriate gene segments were cloned into reverse genetics plasmids as previously described33. Substitutions in PA, PB and M were performed using site-directed mutagenesis. Wild-type and recombinant mutant viruses A/Texas/36/1991 (H1N1) were rescued using reverse genetics systems with 293 T and MDCK co-culture as previously described35,63,67.

Virus titration

The 50% tissue culture infectious dose (TCID50) assay was performed forin vitro titrations of IAV. Briefly, the supernatants from the lung homogenates were evaluated using 10-fold serial dilutions in 96-well plates (six dilutions total, 10−2 to 10−7) to determine the viral titers. MDCK cells were seeded at a density of 1 × 104 cells per well in 100 µL of medium into 96-well plates and incubated overnight (20–24 h). Cells were infected with the dilutions in 9 replicates, three plates per yield dilution, with one column serving as the negative control. Cells were incubated at 35 °C until the cytopathic effect (CPE) in wells containing infected cells remained constant (6–10 days). The cells were then fixed with 5% glutaraldehyde and stained with 0.1% crystal violet to measure CPE. The number of infected wells at each dilution was used to calculate the TCID50 value based on the Reed-Muench method68. The calculation of PFU from TCID50 is based on the ratio PFU/TCID50 of the limiting dilution which would infect 50% of the challenged cell layers69.

LD90 and efficacy determinations of recombinant IAV

To determine the LD90 of each recombinant virus (wild-type or substituted), groups of five mice were exposed intranasally (75 µl) to an individual virus using dilutions of 1:100, 1:320, 1:1000, and 1:3200. A group of 5 mice were not infected and served as weight/survival controls. Mice in all groups were treated with water via intragastric administration 3 times daily for 7 days, beginning 8 hours after infection. Mice were monitored for weight loss, morbidity and mortality as described above. LD90 values were graphically estimated and used to set challenge inoculum dilutions for each individual recombinant virus (ranging from 1:320-1:1000).

Groups of mice were exposed intranasally (75 µl) to the wild-type or recombinant viruses at ~1LD90. Groups of mice were treated with UV-4B (100 mg/kg/dose or vehicle (water) via intragastric administration TID for 5 days beginning 8 hours after infection. For two of the viruses, a group treated with oseltamivir was also included as a positive control, where mice were treated with oseltamivir (10 mg/kg/day) twice daily for 5 days beginning four hours after infection. Mice were monitored for weight loss, morbidity and mortality as described above.

Statistical analyses

Statistical analyses were performed using GraphPad Prism. Virologic titers were log10-transformed and statistical analysis between vehicle- and UV-4B-treated groups at each passage performed using an unpaired parametric t-test. Survival analysis was performed using the Kaplan-Meier graphing method and log-rank test. Linear regression analysis was performed to test if slopes of the number of substitutions or the dN/dS ratio over passage number were significantly different than zero.

Diabetes treatment in 2025: can scientific advances keep pace with prevalence?

Abstract

Introduction

Before the availability of insulin in the 1920s, hailed not only as the cure for diabetes but also as one of the greatest advances in the treatment of any disease, a person diagnosed with diabetes would have faced death within a few years. Today, diabetes is not the devastating diagnosis it would have been 100 years ago; in fact, it is now a common misconception among the public that diabetes is not a serious disease. In reality, the impact of diabetes is so significant that it is affecting overall life expectancy: in the United States (US), life expectancy is falling for the first time since statistics were collected, due to obesity and diabetes [Olshansky et al. 2005], and estimates of diabetes prevalence over the coming years suggest many of us reading this article will develop diabetes during our lives [Whiting et al. 2011]. The predictions of the increased prevalence of diabetes are rarely accompanied by predictions of improvements in the treatment of diabetes; however, given the impact of diabetes, it has been the focus of intensive research, resulting in major advances in our understanding of diabetes as well as in treatment options. As the centenary of the discovery of insulin approaches, it seems timely to consider how treatment options may look in the 2020s, and the likelihood that the elusive cure for diabetes could be found by that time.

Technological solutions

The majority of cases of diabetes are type 2 diabetes mellitus (T2DM), and the predicted rise in diabetes prevalence is expected to be driven by increases in the number of T2DM cases. However, it is likely that significant advances in therapy for T2DM will result from the research in type 1 diabetes mellitus (T1DM), as they are both essentially disorders of glucose management (Box 1).

Box 1.

Pathophysiology of type 1 and type 2 diabetes mellitus.

Type 1 (T1DM) and type 2 diabetes mellitus (T2DM) are both characterized by abnormally high levels of glucose in the bloodstream and until the 1930s, when ‘insulin-sensitive’ and ‘insulin-insensitive’ diabetes were differentiated, all patients with diabetes were thought to have a shortage of insulin production [Saltiel, 2000].

Since then the pathophysiology of the two diseases has been researched extensively, and T1DM is relatively well understood. In short, the patient’s immune system attacks and destroys beta cells in the islets of the pancreas, resulting in insulin deficiency. The factors behind the immune response are still uncertain, but are thought to be both genetic and environmental [van Belle et al. 2011].

Even today, the pathophysiology of T2DM is less well understood. At diagnosis, most patients have insulin resistance: the pancreas is producing insulin, but the body cannot use it effectively. Initially, the pancreas compensates by producing more insulin, and patients have larger beta-cell mass. At some point, typically several years after diagnosis, insulin production will decrease, with a corresponding drop in beta-cell mass, and many people with T2DM eventually need to take insulin. Although the underlying cause is unknown, it is thought that liver, fat, and muscle cells all play a role, in addition to the pancreatic beta cells [Saltiel, 2000].

In T1DM, the complete lack of endogenous insulin has focused research on ever-more sophisticated ways to deliver insulin, with the eventual goal of developing an ‘artificial pancreas’. The elements are already available: a sensor to detect blood glucose readings, a computer to calculate insulin requirements, and a pump to automatically deliver insulin. The feasibility of bringing these elements together has already been demonstrated in clinical trials, with sensor-augmented pump therapy, integrating a sensor and a pump, shown to improve glycemic control compared with a regimen of multiple insulin injections per day [Bergenstal et al. 2010; Hermanides et al. 2011]. A true artificial pancreas would also deliver glucagon to raise blood glucose and prevent severe hypoglycemia, a concept that has already been shown to be feasible [El-Khatib et al. 2010].

Several technological challenges need to be overcome to produce a clinically useful artificial pancreas. First, currently available continuous glucose monitors measure glucose levels in interstitial fluid rather than directly in the blood, resulting in a time lag before changes are measured. As a consequence, accuracy is not sufficiently reliable, with reported error rates of between 12% and 17%. Accuracy is likely to be lowest when blood glucose levels are low; hence, continuous glucose monitoring of interstitial fluid is currently recommended only as an adjunct to standard blood

glucose monitoring [Weinzimer and Tamborlane, 2008]. Insulin pump technology is more advanced; nevertheless, today's pumps deliver insulin subcutaneously, and the delay while insulin is absorbed into the bloodstream limits the ability of software to regulate blood glucose accurately [Renard, 2008]. Catheter complications have prevented intravenous delivery of insulin, and surgically implanted pumps are expensive. It is clear that none of these technological challenges are trivial, but given the pace of developments in technology, we can expect more practical options for patients within the next 10 years. For example, so-called 'smart tattoo' biosensors are capable of detecting glucose levels continuously using a simple infrared detector and providing results in real time. These biosensors, which are based on single-walled carbon nanotubes wrapped in glucose-sensitive polymers that fluoresce in the presence of glucose, are currently being researched in animal models [Barone and Strano, 2009].

Biological solutions

Even as technological solutions advance closer and closer to an artificial pancreas, it is unlikely that technology could ever regulate insulin as precisely as beta cells in a healthy pancreas. Research therefore continues into replacing damaged beta cells with functioning cells, or replacing the entire pancreas [Claiborn and Stoffers, 2008;Sachdeva and Stoffers, 2009]. As with the artificial pancreas, most research to date has been conducted in T1DM, but the results will ultimately translate into therapies for T2DM.

Pancreas transplants have been performed since the late 1980s, with more than 30,000 pancreas transplants recorded in the past 25 years [Gruessner, 2011]. In principle, pancreas transplants offer the promise of excellent outcomes for patients with diabetes. Indeed, stricter donor criteria, as well as improvements in surgical techniques and immunosuppression, have led to improved success rates, with the majority of patients no longer needing insulin therapy after the transplant [Gruessner, 2011]. In practice, the vast majority of pancreas transplants are done in patients who have end-stage renal disease and also need a kidney transplant; this is partly due to the shortage of donor organs, but also because the risks of the necessary post-transplant immunosuppressant therapy usually outweigh the health risks of diabetes itself.

A less invasive option that has already been shown to be viable, at least for some patients, is replacing pancreatic beta cells via islet cell transplants [Truong and Shapiro, 2006]. Isolating these cells from a donor pancreas and infusing them into the patient's portal vein has been researched since the 1960s, and a successful protocol using islets from multiple donors, improved cell culture techniques, and reduced toxicity was optimized during the 1990s at the University of Alberta in Edmonton, Canada. Using the Edmonton protocol, initial studies reported success; however, over time transplanted islets lose function and patients still require immunosuppressive drugs, which are known to increase the risk of infections and the incidence of malignancy, as well as being toxic to the islet cells themselves [Alejandro et al. 2008; Shapiro et al. 2000].

The treatment is still considered experimental and is only available to patients with very poor glycemic control and severe hypoglycemic events but, given the benefits of a successful therapy, there is significant drive to overcome the challenges of limited availability of donor tissue and graft survival after transplant. As well as optimizing the yield of islets from donor pancreata, basic science research into cell differentiation has identified possible alternative sources of beta cells, including differentiating stem cells and reprogramming somatic cells [Baiu et al. 2011; Kellyet al. 2011]. Various strategies are also being researched to improve graft survival after transplantation, by developing immunosuppression regimens that are less toxic to islets and inducing revascularization/reinnervation of the islets [Plesner and Verchere, 2011].

In the longer term, other biological solutions using nonislet cells from the patient themselves are possible options, such as transdifferentiation (mediated by growth factor treatments or gene transfer) of nonislet pancreatic cells or liver cells [Claiborn and Stoffers, 2008; Kojima et al. 2003], and regenerating beta cells and/or expanding beta-cell mass using mediators of beta-cell differentiation and maintenance of adult beta cells [Sachdeva and Stoffers, 2009].

Pharmacological solutions

For patients with T1DM, who make no insulin, the only pharmacologic option is replacement insulin. Astonishing progress has been made since replacement insulin first became available, when insulin batches were of variable quality and large, twice-daily injections were needed. Today, it is hard

to imagine how difficult it must have been to manage T1DM without disposable needles or patients self-testing glucose. The possibilities for improvement in pharmacological care for these patients should not be underestimated, although most likely they will be essentially improvements in the convenience of insulin delivery.

In patients with T2DM, a range of pharmacologic treatments have been developed, and continue to be developed (Figure 1). For many years, treatment was dominated by two drug classes, sulfonylureas and biguanides. These two drug classes demonstrate not only the advances in clinical research, but also the role that luck, both good and bad, plays in the progress of treatment. Sulfonylureas were discovered serendipitously in France during the Second World War after hypoglycemia was induced in soldiers in whom the drug was being tested as an antimicrobial agent for typhoid fever [Vaisrub, 1972]. In the US, sulfonylureas first became available in 1955 and for many years were the first-line option for treating T2DM. These drugs were no miracle cure: as is well known, the first-generation sulfonylureas were associated with a high risk of hypoglycemia; the second-generation sulfonylureas, which are still used today, were introduced in 1984.

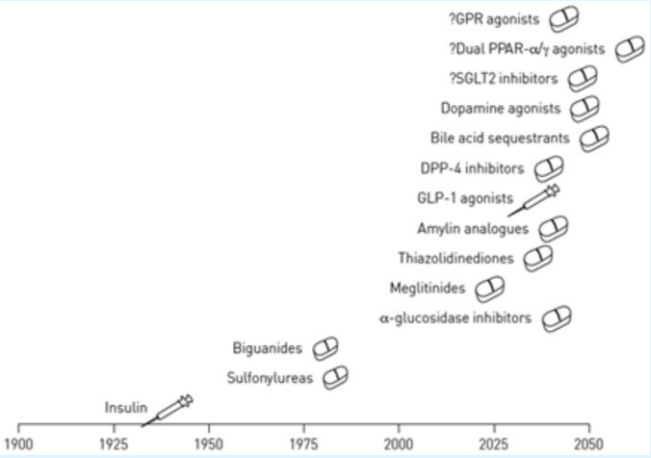


Figure 1.
US Food and Drug Administration approval of pharmacological options for type 2 diabetes mellitus.

Like sulfonylureas, drugs of the biguanide class were observed to have antihyperglycemic properties before their mechanism of action was understood. In fact, the class had been studied in the 1920s but, perhaps because of the excitement over insulin, was largely ignored and it was not until the 1950s that phenformin, buformin, and metformin were researched in clinical trials. Unfortunately, the only biguanide marketed in the US, phenformin, was associated with lactic acidosis, leading not only to withdrawal of phenformin but, understandably, to mistrust of other medications in the same class [Witters, 2001]. It was not until the 1990s, when the large United Kingdom Prospective Diabetes Study (UKPDS) showed the benefits of metformin, especially in terms of weight-neutrality, that it became available and more widely used in the US [United Kingdom Prospective Diabetes Study Group, 1995]. More recently, metformin has been associated with reduced risk of cancer in observational studies, although this potential additional benefit needs to be confirmed in long-term randomized controlled trials [Noto et al. 2012].

The last 20 years have seen an astonishing pace in research into the molecular pathology of diabetes. Our improved understanding of diabetes has facilitated the development of drug classes that target specific metabolic pathways such as the thiazolidinediones, dipeptidyl peptidase-4 (DPP-4) inhibitors, glucagon-like peptide-1 (GLP-1) receptor agonists, and sodium–glucose cotransporter type 2 (SGLT2) inhibitors (Figure 1) [Tahrani et al. 2011]. In the next 10 years, based on current research, we can expect more purposely targeted drugs for T2DM, and better combinations of drugs with simpler treatment regimens [Fakhrudin et al. 2010].

Beyond the drugs already in the pipeline, where might research take us? Can we begin to think about therapies that would cure or even prevent T2DM?

For some time, researchers have known that the brain plays an important role in eating behavior and satiety, and 'gut–brain' connections may be the next therapeutic targets, with newer drugs targeting the central nervous system [Pagotto, 2009]. With the currently available GLP-1 receptor agonists, for example, the brain is likely to mediate at least some of the important effects, such as improved satiety and weight loss, regulation of gastric emptying, and possibly suppression of glucose [De Silva et al. 2011; Knauf et al. 2008]. Indeed, the relatively new use of the centrally acting therapy, quick-release bromocriptine, illustrates the potential of the brain as a treatment target for T2DM. Bromocriptine, a D2 dopamine receptor agonist, has been available for many years as a treatment for acromegaly and hyperprolactinemia, and was approved by the US Food and Drug Administration in May 2009 as a treatment for T2DM [Gaziano et al. 2010]. Similar to when metformin was first used, the mechanism by which bromocriptine improves glycemic control is

as-yet unknown, although it is clear that improvements in glycemic control are seen without increases in insulin concentration [Cincotta et al. 1999; Vero Science, 2010]. Investigators believe the drug acts at a central target in the hypothalamus and may affect circadian rhythms to favor improvements in metabolism. This concept was apparently suggested by the metabolism of migrating birds that develop seasonal insulin resistance before migrating, and then return to a lean state after migration [Scranton and Cincotta, 2010].

Bromocriptine is likely to be used in only a minority of patients due to its fairly modest glucose-lowering effects, although it could spark new avenues of research for diabetes treatments, as is always the case when new drug classes are identified. Intriguingly, the theory of seasonal insulin resistance in migrating birds is consistent with studies of insulin resistance in hibernating animals, suggesting the brain is involved via regulation of dopamine and prolactin, and plays a role in accumulation of fat and development of insulin resistance in winter [Martin, 2008]. Humans have no seasonal variations in dopamine, but this neurotransmitter may still play a role in insulin resistance, with people who develop diabetes essentially trapped in a constant state of 'winter', characterized by chronic insulin resistance and fat accumulation. Because there is no seasonal food shortage, there is no loss of adipocytes, and the body never returns to 'summer' [Martin, 2008]. This pathway, which bromocriptine appears to reset, could open up entirely new approaches to preventing or even curing T2DM.

Could an imposed food shortage help reset metabolism? Studies of bariatric surgery (gastric bypass, sleeve gastrectomy, biliopancreatic diversion or duodenal switch procedure) in obese patients with T2DM seem to suggest that it does, with patients receiving bariatric surgery significantly more likely to eliminate the need for antidiabetic therapies compared with patients receiving only medical therapy for T2DM [Mingrone et al. 2012;Schauer et al. 2012; Sovik et al. 2011]. These surgical procedures improve diabetes not only by causing weight loss, but also by affecting hormones such as GLP-1 and ghrelin, which help signal satiety and hunger, respectively, to the human brain [Peterli et al. 2012]. Increasing satiety signals and reducing hunger signals to the brain can help patients tolerate extreme caloric restrictions.

Dietary restriction is known to increase lifespan in rodents, and can delay or prevent diseases such as cancer, heart disease, and diabetes; however, studies in nonhuman primates have reported conflicting findings, indicating the effects of dietary restriction are likely to be complex [Mattison et al. 2012]. Such studies are hard to replicate in people, but trials showing a severely restricted diet can improve beta-cell function and insulin sensitivity in patients with a relatively short duration of T2DM suggest that humans, like hibernating mammals, have the capacity for recovering from insulin resistance [Lim et al. 2011]. The problem, the study investigators believe, is that few people could maintain such a limited calorie intake, and any successful nonsurgical solution may therefore rely on drugs mimicking the effects of dietary restriction. Given the current and predicted prevalence of obesity, there is already intensive research into agents that decrease appetite or increase satiety. The endocannabinoid system is known to have a role in the regulation of appetite, but cannabinoid receptor antagonists such as rimonabant have been associated with unacceptable side effects [Eckel et al. 2011], although more selective agonists may be an avenue targeted in the future. To date, other therapies developed for the treatment of obesity have also been plagued by safety issues, but there are now several promising molecules in the early stages of development [Eckelet al. 2011].

Among the currently available therapies for T2DM, various classes also promote weight loss. For example, the subcutaneous agent pramlintide is associated with reduced food intake and body weight in obese people with and without diabetes, although it is associated with only modest hemoglobin A1c (HbA1c) reductions and the amount of weight loss induced is also relatively small [Lee et al. 2012]. Pramlintide is a synthetic form of amylin, which is secreted after meals and signals short-term satiety, and may therefore be more useful in combination with other agents. Therapies combining pramlintide with long-term signaling molecules are in the early stages of development, along with other therapies targeting appetite [Powell et al. 2011].

Metformin is also associated with weight loss, although the amount of weight shed is insufficient to meet FDA criteria for a weight loss drug (at least 5% of body weight). Guidelines now recommend physicians consider metformin for preventing or delaying diabetes in individuals with elevated glucose measurements and a body mass index >35

kg/m2 [American Diabetes Association, 2012]. Intriguingly, research has shown that metformin-induced alterations mimic many of the same transcriptional changes in the liver that occur with dietary restriction [Dhahbi et al. 2005]. The effects of metformin are still incompletely understood, but activation of AMP-activated protein kinase (AMPK) appears to play a central role [Zhou et al. 2001]. AMPK is a sensor of energy shortage within cells, acting as a metabolic switch (Box 2). The role of AMPK, and possible activators, are being intensively investigated and, at present, this route appears the most exciting line of enquiry into a possible cure for T2DM.

Box 2.

AMP-activated protein kinase: master switch in metabolism.

We often think of glucose as the fuel for cells, but glucose is just one of the fuels used to produce the actual energy source in every cell: adenosine triphosphate (ATP). The energy balance within individual cells is maintained by the enzyme AMP-activated protein kinase (AMPK), which is activated when the ratio of ATP to adenosine monophosphate (AMP) falls [Viollet et al. 2009]. Because AMPK is the 'master switch' of energy intake and energy expenditure, this enzyme is a theoretical key target for therapeutic intervention in patients with T2DM. If it is possible to activate AMPK, the resulting signaling pathways could be manipulated to restore energy balance, making people more fit and less likely to develop insulin resistance, without the need to decrease energy intake or lose weight [Gruzman et al. 2009].

Investigations of AMPK activators are already confirming some of these theoretical effects. For example, giving an oral AMPK agonist AICAR to sedentary mice was shown to improve treadmill performance [Narkar et al. 2008]. The polyphenol resveratrol, an AMPK modulator present in red wine, appears to protect mice against diet-induced obesity and insulin resistance [Lagouge et al. 2006], and has also been shown to mimic the effects of calorie restriction in people with obesity [Timmers et al. 2011]. These studies are at early stages, although a phase II/III trial of the effect of resveratrol on inflammatory mediators and insulin resistance in patients with T2DM or obesity is underway [ClinicalTrials.gov identifier: NCT01158417].

The current antidiabetes drug metformin reportedly increases AMPK activity; however, the mechanism of action is incompletely understood and metformin may also act via AMPK-independent pathways [Foretz et al.2010; Fryer et al. 2002]. The antihyperglycemic effect of metformin was discovered by chance, but the subsequent discovery that this drug activates AMPK raises the intriguing possibility of developing drugs to extend the benefits of metformin with fewer side effects. This is certainly possible: although AMPK is found in all cells, different complexes localize to the liver, adipocytes, or skeletal muscles, and a drug targeting these complexes with high specificity could selectively restore energy balance without harming other tissues [Gruzman et al. 2009].

Prevention of diabetes-related complications

Using the World Health Organization cut-offs for diagnosing diabetes may seem rather arbitrary to patients and we often need to explain that an HbA1c level of 6.5% was chosen because people with blood glucose over this level are at much higher risk of diabetic retinopathy. In clinical practice today, patients rarely die from the immediate effects of high blood sugar; instead, we screen for and treat diabetes primarily to prevent complications. It is clear that good control of glucose levels is associated with reduced risk of complications [Diabetes Control and Complications Trial Research Group, 1993; United Kingdom Prospective Diabetes Study Group, 1998], but what exactly is it about high glucose that causes complications? Could identifying and targeting those pathways bring the residual rate of complications down to that seen in people without diabetes?

Microvascular complications such as neuropathy, retinopathy, and nephropathy are highly correlated with glucose levels. Various pathways leading to the damage that high glucose levels cause have been proposed, including osmotic stress from sorbitol accumulation [Lorenzi, 2007]; oxidative damage to cells, with free radical production and reactive oxygen species formation [Ceriello and Motz, 2004]; and toxicity from advanced glycation end-products (AGE). As proof of principle, animal studies have shown that inhibition of AGE accumulation might represent an effective strategy to reduce the rate of diabetes progression and/or prevent diabetes-related complications [Schmidt and Stern, 2000; Watson et al. 2011].

Macrovascular complications of T2DM, such as myocardial infarction and ischemic stroke, may not just be related to hyperglycemia; inflammation

mediated by macrophages appears to contribute to, or even be responsible for, insulin resistance [Olefsky and Glass, 2010]. Atherosclerosis is also thought to result from chronic inflammation leading to injury to the arterial wall [Ross, 1999]. Anti-inflammatory drugs could therefore provide a direct method to reduce the risk of macrovascular complications. In fact, aspirin, one of the oldest anti-inflammatory medications, is recommended in certain groups of patients with diabetes, but use is limited by the high risk of bleeding [American Diabetes Association, 2012]. Development of compounds to inhibit mediators of inflammation, such as tumor necrosis factor (TNF)-α and interleukin (IL)-6, as well as other cytokines from fat and immune cells, could reduce inflammation-associated insulin resistance if the proteins could be selected to act in a highly tissue-specific manner without affecting other functions of the immune system [Olefsky and Glass, 2010]. Research in this field is still in the early stages. A trial of etanercept (a TNF-α blocker) failed to improve insulin sensitivity in patients with the metabolic syndrome [Bernstein et al. 2006]. However, the IL-1 receptor antagonist drug used to treat rheumatoid arthritis, anakinra, improved beta-cell function in patients with T2DM [Larsen et al. 2007, 2009], suggesting anti-inflammatory compounds may be part of the future treatment of diabetes.

How might treatment evolve?

The current standard of care for T2DM consists of screening for elevated HbA1c levels or, in some cases, fasting plasma glucose, with diagnosis followed by management with lifestyle modifications and metformin except where contraindicated [American Diabetes Association, 2012]. For patients who do not achieve HbA1c targets, antidiabetes medications are added to metformin; subsequently, patients are monitored and further oral antidiabetes drugs or insulin are added if needed.

Clearly, the care of patients with T2DM is currently suboptimal, largely because our healthcare system has traditionally been based on an acute-care model. In contrast, chronic disease management emphasizes a team approach, medication management and patient adherence, prevention of complications, lifestyle modifications as well as coordination of care among subspecialists. Guidelines for the prevention of T2DM emphasize moving beyond the healthcare system towards integration with other areas, such as government and the media [Lindstromet al. 2010; Paulweber et al. 2010]. There is already evidence that intervention at the public health level could significantly impact rates of T2DM [Elbel et al. 2012; Schwartz et al. 2012]. In the future, coordination of care using case managers, technology that helps patients between medical visits, such as mobile health and telemedicine, and restructuring care using patient-centered medical homes and accountable care organizations may be better suited for T2DM management [Quinn et al. 2011].

How might treatment options look in the year 2025? We can certainly expect that genetic testing will be used to determine whether the patient will develop diabetes and, if so, which of the predisposing genes are involved. Genetic testing is already used to diagnose subtypes of maturity-onset diabetes of the young (MODY), for which six different subtypes resulting from mutations in different genes have been identified. Diagnosing the exact subtype can help the physician select the most appropriate treatment, as well as screening family members who may benefit from support with lifestyle changes [Gardner and Tai, 2012]. In the future, analogous genetic tests for T1DM and T2DM will enable us to offer the patient the appropriate solution, either genetic therapy to ‘repair’ the defective gene or pharmacotherapy to compensate for it. Because there will likely be 50 or more drugs to choose from, the choice of pharmacotherapy will be personalized based on the patient’s genetic profile, which will also indicate predisposition to complications such as kidney disease or retinopathy that can then be treated with a ‘complication-prevention’ drug. An artificial pancreas will be an option for severe cases, or in patients who cannot tolerate other options.

Conclusion

To allow us to cope with periods of famine and feast, humans are adapted to make the most of the energy available to them. What ensured our survival then has become our weakness now, and all predictions indicate the prevalence of T2DM will get worse before it improves. Modern lifestyles allow continual access to food and encourage sedentary behavior, leading to a progressive cycle of overeating and weight gain. Despite efforts at education, lifestyles will likely become yet more sedentary over the next 20 years, facilitated by advances in technology.

For example, concepts for controlling the world around us with only our thoughts would have been science fiction 20 years ago, but are now actively researched [Hochberg et al. 2006]. There seems little doubt that, without interventions, the prevalence of T2DM will increase.

For those who prefer simplistic views, it is easy to blame individuals for having diabetes. Indeed, despite the clear benefits of a healthy lifestyle, changes in long-term behavior and lifestyle are rare. However, as we understand more about our biology, we can appreciate that our environment naturally puts us all at high risk of diabetes. Nowhere is this seen more clearly than in populations that have been exposed to sudden changes in lifestyles as a result of urbanization, such as the Pima Indians in the US, who have far higher rates of T2DM than Pima Indian populations in Mexico [Esparza-Romero et al. 2010].

Is it realistic to ask people to change their lifestyles? The advances in therapy over the past 50 years have provided a remarkable array of options so that treatment can be tailored for each patient, but, even with expert teams of dieticians and diabetes educators, most patients need drug therapy, probably multiple-drug therapy, to achieve recommended HbA1c targets. However, in spite of achieving HbA1c targets, they still retain a residual risk for complications compared with people without diabetes. In the future, we may accept that drugs are needed to allow us to lead modern lifestyles without increasing our risk of diabetes. The scientific community should be applauded for taking the pragmatic approach of searching for interventions that could help individuals, probably the majority, who are unable to maintain healthy lifestyles in the long term. Because our lifestyle means that diabetes will become a normal aspect of life, the research ongoing today is vital to provide tools to counteract the diabetes epidemic.

New Approaches in the Treatment of Hypertension

Abstract

Hypertension is the most common modifiable risk factor for cardiovascular disease and death, and lowering blood pressure with antihypertensive drugs reduces target organ damage and prevents cardiovascular disease outcomes. Despite a plethora of available treatment options, a substantial portion of the hypertensive population has uncontrolled blood pressure. The unmet need of controlling blood pressure in this population may be addressed, in part, by developing new drugs and devices/procedures to treat hypertension and its comorbidities. In this Compendium Review, we discuss new drugs and interventional treatments that are undergoing preclinical or clinical testing for hypertension treatment. New drug classes, eg, inhibitors of vasopeptidases, aldosterone synthase and soluble epoxide hydrolase, agonists of natriuretic peptide A and vasoactive intestinal peptide receptor 2, and a novel mineralocorticoid receptor antagonist are in phase II/III of development, while inhibitors of aminopeptidase A, dopamine β-hydroxylase, and the intestinal Na+/H+ exchanger 3, agonists of components of the angiotensin-converting enzyme 2/angiotensin(1–7)/Mas receptor axis and vaccines directed toward angiotensin II and its type 1 receptor are in phase I or preclinical development. The two main interventional approaches, transcatheter renal denervation and baroreflex activation therapy, are used in clinical practice for severe treatment resistant hypertension in some countries. Renal denervation is also being evaluated for treatment of various comorbidities, eg, chronic heart failure, cardiac arrhythmias and chronic renal failure. Novel interventional approaches in early development include carotid body ablation and arteriovenous fistula placement. Importantly, none of these novel drug or device treatments has been shown to prevent cardiovascular disease outcomes or death in hypertensive patients.

Introduction

Hypertension is the most common modifiable risk factor for cardiovascular disease (CVD) and death; the increased risk associated with blood pressure (BP) elevation can be greatly reduced by treatment with antihypertensive drugs that lower both BP and related target organ damage. A total of 69 drugs in 15 different classes, many of which are also available in single pill combinations, have been approved for the treatment of hypertension in the United States.1 Despite this plethora of treatment options, an estimated 10% to 15% of the general hypertensive population has resistant hypertension, defined as uncontrolled BP on ≥3 antihypertensive drugs of different classes, including a nonpotassium-sparing diuretic, at optimal doses, or requiring ≥4 drugs to achieve control.2,3 In addition, ≈0.5% of hypertensive patients have refractory hypertension,

defined as uncontrolled BP on ≥5 drugs.4

Many more hypertensive patients are uncontrolled because of nonadherence or intolerance to available antihypertensive agents. Recent drug monitoring studies have revealed nonadherence to BP lowering therapy in 25% to 65% of patients with apparent treatment resistant hypertension (TRH).5–9 In 24% to 34.5% of these individuals, who were prescribed 3–5+antihypertensive medications, no antihypertensive medication was detected in blood or urine samples. The unmet need of controlling BP in these high-risk patients may be addressed, in part, by the development of new drugs and devices and procedures that are designed to treat hypertension and comorbidities, such as heart failure (HF), chronic kidney disease, and diabetes mellitus. This review will summarize recent development of novel drugs classes and interventional strategies for the treatment of hypertension and related target organ damage.

Part I. New Drugs

Anti-Aldosterone Agents

Aldosterone is a mineralocorticoid that regulates electrolyte and volume homeostasis in normal subjects and, when elevated, can contribute to the development of hypertension and a variety of related pathologies, including myocardial hypertrophy and fibrosis and HF.10 The principal effector of aldosterone action is the mineralocorticoid receptor (MR), a nuclear transcription factor that is expressed at high levels in the cortical collecting duct of the kidney (Figure 1). Activated MRs stimulate expression of sodium channels, resulting in increased sodium and water reabsorption and potassium loss, leading eventually to a volume expanded form of hypertension. Activation of MRs in extra adrenal tissues, particularly the heart and blood vessels, also promotes the development of hypertension and CVD by upregulating NADPH oxidase and increasing production of reactive oxygen species. This reduces the bioavailability of nitric oxide and leads to endothelial dysfunction and vascular disease.

Mechanism of action of anti-aldosterone agents. Aldosterone synthase inhibitors (ASIs), such as LCI699, inhibit the rate limiting step of aldosterone production. Mineralocorticoid receptor agonists (MRAs), such as finerenone, compete for the binding sites of aldosterone and effectively decrease blood pressure and aldosterone-mediated gene transcription. Both approaches have been shown to be useful in treating aldosterone-mediated hypertension and vascular disease. Aldosterone synthesis, green; cortisol synthesis, red; anti-aldosterone drugs, blue.

Aldosterone is synthesized from 11-deoxycorticosterone in the zona glomerulosa of the adrenal cortex via the action of a mitochondrial cytochrome P450 enzyme, aldosterone synthase, which is encoded by the CYP11B2 gene11 (Figure 1). Aldosterone synthase catalyzes the final 3 rate-limiting steps of aldosterone synthesis (11β-hydroxylation of 11-deoxycorticosterone to form corticosterone, followed by 18-hydroxylation of corticosterone to form 18OH-corticosterone, and 18-oxidation of 18-OH corticosterone to form aldosterone). Cortisol synthesis, which occurs in the zona fasciculata of the adrenal cortex, is mediated by 11β-hydroxylase, which is encoded by the CYP11B1 gene.11,12 CYP11B2 has a high sequence homology with CYP11B1, and both CYP11B2 and CYP11B1 share an 11β-hydroxylase reaction, creating problems for those attempting to design selective aldosterone synthase inhibitors.12–14

Mineralocorticoid Receptor Antagonists

MRs have been therapeutic targets in hypertension treatment for over half a century: the first MR antagonist (MRA), spironolactone, appeared in the early 1960s.15 Although spironolactone monotherapy has modest BP lowering efficacy, it has had a recent resurgence as add-on therapy in patients with resistant hypertension16–19 and in the treatment of HF.20Spironolactone use has been limited by its lack of selectivity for the MR, particularly at higher (>25 mg) doses. Because of its structural similarity to progesterone, spironolactone has significant progestogenic and antiandrogenic activity, leading to troublesome adverse effects in both men and women.21,22 The more selective MRA eplerenone lacks the antiandrogenic effects of spironolactone, but is less potent and has a shorter (3–4 h) half-life, leading to reduced antihypertensive efficacy and a requirement for twice daily dosing.22,23

The search for newer nonsteroidal MRAs that have superior selectivity and affinity for the MR began with the observation that some dihydropyridine calcium channel blockers compete with aldosterone for binding to the ligand binding domain of the MR and decrease aldosterone-mediated recruitment of transcriptional coactivators that are necessary for

MR-directed DNA transcription.22,24,25 Optimization of MRA activity of dihydropyridine compounds led to the development of BAY 94-8862 (finerenone), a nonsteroidal MRA that has greater selectivity than spironolactone for the MR over other steroid hormone receptors, greater affinity than eplerenone for the MR, and no effect on the L-type calcium channel26,27 (Table). Finerenone was designed to have greater cardiac activity than the available steroidal MRAs to improve myocardial function without adversely affecting sodium-potassium homeostasis in the kidney. In preclinical models of hypertension-related HF and renal dysfunction, finerenone resulted in greater cardiorenal target organ protection than steroidal MRAs.28

The mineralocorticoid receptor antagonist tolerability study (ARTS) was designed to assess the safety and tolerability of finerenone in patients with HF with reduced ejection fraction and mild or moderate chronic kidney disease and to select doses for phase III clinical trials.29,30 ARTS (ClinicalTrials.gov: NCT01345656) is a multicenter, randomized, double-blind, parallel-group study divided into 2 parts: Part A tested the effects of finerenone (2.5, 5, or 10 mg once daily) in 65 patients with HF with reduced ejection fraction and mild chronic kidney disease (estimated glomerular filtration rate 60–90 mL/min/1.73 m2); Part B compared finerenone (2.5, 5, or 10 mg daily or 5 mg twice daily) with placebo or open-label spironolactone (25 or 50 mg daily) in 392 patients with HF with reduced ejection fraction and moderate chronic kidney disease (estimated glomerular filtration rate 30–60 mL/min/1.73 m2).30 Finerenone 5 to 10 mg/day was at least as effective as spironolactone 25 or 50 mg/day in decreasing biomarkers of hemodynamic stress (B-type natriuretic peptide [BNP] and amino-terminal proBNP) and albuminuria and was associated with lower incidences of hyperkalemia and worsening renal function. Adverse effects were infrequent and mostly mild. Finerenone may, therefore, offer target-organ protection with a reduced risk of electrolyte disturbances in HF patients. Finerenone is currently under investigation in 2 phase IIb clinical trials: (1) in patients with worsening chronic HF and type 2 diabetes mellitus and chronic kidney disease (ARTS-HF; ClinicalTrials.gov: NCT01807221) and (2) in patients with type 2 diabetes mellitus and diabetic nephropathy (ARTS-DN; ClinicalTrials.gov: NCT01874431).

Aldosterone Synthase Inhibitors

Although MRAs lower BP and protect against hypertension-related target organ damage, they can cause reactive increases in components of the renin–angiotensin–aldosterone system, particularly aldosterone, thus blunting their effectiveness.12,13Further, they do not block, and may even enhance, the nongenomic effects of aldosterone, which include stimulation of cardiac and vascular contractility, worsening of glucose homeostasis, and increasing central sympathetic drive.31–33Awareness of the limitations of MRAs has led to the development of a new class of anti-aldosterone agents, the selective aldosterone synthase inhibitors.22,34,35 LCI699, the first orally active aldosterone synthase inhibitor to be developed for human use, is similar in structure to FAD286, the dextroenantiomer of the nonsteroidal aromatase inhibitor fadrozole36(Table). LCI699 dose-dependently decreases plasma and urine aldosterone concentrations, increases plasma renin activity, and prevents target organ damage in animal models of hypertension and HF.37,38 Similar effects on aldosterone and renin levels have been seen in healthy humans39 and in hypertensive patients.12,40–43

Four phase II studies of LCI699 have been performed in hypertensive patients.12,40–43 The first proof-of-concept study compared LCI699 to placebo in 14 patients with primary aldosteronism.40 LCI699 normalized plasma aldosterone and potassium but also increased 11-deoxycorticosterone and adrenocorticotropin levels and produced only modest reduction (4.2 mm Hg) in 24-hour ambulatory systolic BP. The first randomized, double-blind, placebo-controlled trial of LCI699, performed in 524 patients with primary hypertension, compared the efficacy and safety of different doses of LCI699 with eplerenone.41 All doses of LCI699 produced significant reductions in office systolic BP that were noninferior to those seen with eplerenone. Plasma aldosterone levels were suppressed with LCI699 and increased with eplerenone; both agents were well tolerated, but adrenocorticotropin-stimulated cortisol release was blunted in ≈ 20% of the LCI699 group, likely as a result of inhibition of CYP11B1.

A subsequent study evaluated the effect of LCI699 on the cortisol response to adrenocorticotropin stimulation in 63 treated hypertensive patients to find the maximally tolerated dose in this patient population.42 LCI699 had a dose- and time-

dependent inhibitory effect on both aldosterone- and adrenocorticotropin-stimulated cortisol synthesis, consistent with inhibition of 11-β-hydroxylase (CYP11B1) activity. A fourth study evaluated the safety and efficacy of LCI699, compared with placebo and eplerenone, as add-on therapy in patients with resistant hypertension.43 BP lowering effects of LCI699 were inferior to those of eplerenone and plasma 11-deoxycorticosterone levels increased, confirming inhibition of 11β-hydroxylase and compensatory stimulation of adrenal steroidogenesis. The nonselectivity of LCI699 resulted in off-target inhibition of the 11β-hydroxylase activity of CYP11B1, thus stimulating the hypothalamic–pituitary–adrenal feedback axis and increasing adrenocorticotropin levels and adrenal steroidogenesis to compensate for the reduced cortisol secretion.12In the setting of aldosterone synthase (CYP11B2) inhibition, this resulted in up to a 10-fold increase in the biologically active aldosterone synthase substrate, 11-deoxycorticosterone, which could activate the MR. These effects could account for the disappointing BP reductions seen at higher doses and with twice daily administration of LCI699.

Based on the results of the phase II trials, further development of LCI699 was discontinued, and the investigators outlined the mechanistic properties that would be required for a therapeutically successful aldosterone synthase inhibitor: (1) greater selectivity for aldosterone synthase inhibition on CYP11B2 over 11β-hydroxylase inhibition on CYP11B1; (2) a longer plasma elimination half-life than LCI699; and (3) preferential inhibition of the 18-oxidase step of aldosterone synthesis, thus preventing conversion of the weak mineralocorticoids 18-OH corticosterone and corticosterone to aldosterone.12 A series of novel pyridyl- or isoquinolinyl-substituted indolines and indoles have recently been synthesized using a ligand-based approach. These compounds are as potent and more selective than LCI699 for CYP11B2 over CYP11B114,44 and are currently being tested as treatments for mineralocorticoid-dependent CVD and renal disease.

Activators of the Angiotensin-Converting Enzyme2/Angiotensin(1–7)/MAS Receptor Axis

The classical renin–angiotensin system (RAS) has been studied extensively for decades45 and has yielded numerous effective therapies for hypertension and its complications. More recently, components of the RAS that play counterregulatory roles have been identified, characterized and put forward as therapeutic targets for hypertension and other forms of CVD46–50 (Figure 2). The carboxypeptidase angiotensin-converting enzyme 2 (ACE2) converts the decapeptide angiotensin I (Ang I) to the Ang(1–9) nonapeptide and the octapeptide Ang II to the Ang(1–7) heptapeptide. Ang(1–7) has been studied intensively and shown to activate the G-protein-coupled Mas receptor, triggering a signaling cascade that results in vasodilation, reduction in oxidative stress, and antihypertrophic and antifibrotic effects. ACE2 also mediates degradation of Ang II, likely contributing to the antihypertensive/vasoprotective effects of the counterregulatory RAS pathway.

Figure 2. Drugs targeting the classical and counter regulatory renin angiotensin systems (RAS). Activation of the classical RAS pathway increases BP and target organ damage, and this pathway is the target for many currently available antihypertensive drugs, including angiotensin-converting enzyme (ACE) inhibitors and angiotensin receptor blockers (ARBs). Novel approaches to RAS inhibition, including vaccines targeting angiotensin II (Ang II) and the angiotensin II type 1 (AT1) receptor, are being evaluated in preclinical and clinical trials. In contrast, activation of the more recently described counter regulatory RAS pathway decreases blood pressure (BP) and target organ damage, and drugs that activate this pathway are beginning to be developed as antihypertensive agents. These include ACE2 activators, Ang (1–7) analogs, AT2 receptor agonists, peptide and nonpeptide activators of the Mas receptor, and alamandine complexed with cyclodextrin. Classical RAS, red; counter regulatory RAS, green; drugs, blue. ATR indicates AT1 receptor; MrgD, Mas-related G-protein-coupled receptor, member D; and rhACE2, recombinant human ACE2.

Amplification of ACE2/Ang(1–7)/Mas signaling opposes the effects of the classical RAS and lowers BP and prevents or reverses related target organ damage in hypertensive animal models.48 Interestingly, inhibitors of the classical RAS, including ACE inhibitors and angiotensin receptor blockers (ARBs), increase circulating Ang(1–7) levels, and the Mas antagonist A-779 attenuates the effects of the ACE inhibitors and ARBs, indicating that the 2 RAS axes interact49 and provide further evidence for the therapeutic potential of the ACE2/Ang(1–7)/Mas axis in hypertension.

The more recently described Ang(1–9) has been shown to lower BP and reverse/ameliorate cardiovascular injury in animal models of hypertension by a mechanism that involves activation of the angiotensin type 2 receptor.51 Unlike Ang(1–7), Ang(1–9) does not activate the Mas receptor. The therapeutic potential of angiotensin type 2 receptor activation is being explored in preclinical studies. A selective nonpeptide angiotensin type 2 receptor agonist, compound 21(C21), has been found to have anti-inflammatory, antifibrotic and antiapoptotic properties, but not to lower BP47 (Table). These findings suggest that C21 may be useful in preventing hypertension-induced target organ damage.

Interest in ACE2 as a therapeutic target has led to the synthesis of small molecule ACE2 activators, including XNT52 and DIZE,53 which lower BP, improve myocardial function, and reverse myocardial and perivascular fibrosis in the spontaneously hypertensive rat (SHR; Table). Activation of ACE2 also decreases monocrotaline-induced pulmonary hypertension by a mechanism that involves Mas activation.54,55 As an alternative to pharmacological ACE2 activation, recombinant human ACE2 (rhACE2) has been shown to lower BP in SHR, to have anti-inflammatory effects in a model of lipopolysaccharide-induced lung injury,56 and to slow the progression of diabetic nephropathy in animal models57 (Table). A phase I study in healthy volunteers demonstrated sustained (>24 h) suppression of circulating Ang II levels after a single intravenous injection of rhACE2 with no effect on BP and no major adverse effects.58

Ang(1–7) has been administered in phase I/II studies as a putative antiproliferative and antiangiogenic agent to patients with advanced cancers refractory to standard therapy and as a hematopoietic agent to patients with multilineage cytopenias following chemotherapy.59,60 These studies were limited in scope, and native Ang(1–7) has not been developed further because of its abbreviated half-life in vivo. A cyclic Ang(1–7) analog containing a thioether bridge that makes it resistant to enzymatic digestion and a hydroxypropyl-β-cyclodextrin incorporated Ang(1–7) formulation (HP-β-CD/Ang1-7) have been synthesized and shown to be cardioprotective in animal models of myocardial infarction and insulin resistance/type 2 diabetes61–63 (Table).

As an alternative to Ang(1–7), nonpeptide agonists of the Mas receptor, for example, the imidazole compound AVE0991,64and novel G-protein-coupled receptor activating peptides, for example, CGEN-856S that have high specificity for the Mas receptor,65 have been shown to lower BP and protect the vasculature and kidneys in animal models of hypertension and CVD (Table). The relative merits of Mas receptor activation versus ACE2 stimulation are being debated, but all agree that randomized controlled trials in humans with hypertension and related CVDs are needed to assess the therapeutic potential of activating the ACE2/Ang(1–7)/Mas receptor axis.46,48

A novel member of the Ang peptide family, Ala1-Ang(1–7) (alamandine), has been isolated from human plasma and rat heart.50,66 Alamandine is a product of decarboxylation of the N-terminal Asp residue of Ang II to form Ala, which has been demonstrated in heart, followed by hydrolysis of Ala1-Ang II by ACE2. Alamandine is similar in structure to Ang(1–7) except for replacement of the N-terminal Asp residue by Ala. It has antihypertensive, antifibrotic, and central cardiovascular effects similar to those reported for Ang(1–7), but acts through a different receptor, the Mas-related G-protein-coupled receptor, member D. Alamandine incorporated into a β-cyclodextrin inclusion complex (alamandine/HPβCD) has been shown to be orally active and to reduce BP in SHR and inhibit cardiac fibrosis in isoproterenol-treated rats66 (Table). The oral bioavailability of alamandine/HPβCD has revived prospects for exploring the therapeutic potential of Ang(1–7)-related peptides.

Centrally Acting Aminopeptidase Inhibitors

Activation of the brain RAS plays an important role in the pathogenesis of hypertension in animal models.67,68 Two membrane-bound zinc metalloproteases, aminopeptidase A (APA) and aminopeptidase N, are involved in the metabolism of brain Ang II and III, respectively (Figure 3). APA cleaves the N-terminal Asp from Ang II to form Ang III, and aminopeptidase N cleaves the N-terminal Arg from Ang III to form Ang IV. Ang II and Ang III have similar affinities for Ang II receptors and both peptides stimulate pressor responses by activating sympathetic nervous system activity, inhibiting the baroreflex at the level of the nucleus tractus solitarius and increasing release of arginine vasopressin into the circulation. Studies using selective APA (EC33) and aminopeptidase N (PC18) inhibitors have demonstrated that brain Ang III (not Ang II, as in the periphery) plays a predominant role in BP control in

animal models and have identified APA as a potential therapeutic target for the treatment of hypertension68–71 (Table).

Figure 3. The brain renin angiotensin system (RAS) pathway. Activation of the brain RAS in response to oxidative stress and inflammation increases sympathetic nervous system outflow and arginine vasopressin (AVP) release and inhibits the baroflex, thus raising BP. Angiotensin (Ang) III, which is generated from Ang II by aminopeptidase A (APA), is the predominant pressor peptide in brain in animal models, and APA is a therapeutic target for treatment of hypertension. The APA inhibitor RB150 (QGC 001) has been shown to pass the blood–brain barrier and lower BP in animal models; exploratory studies are underway in humans. Red, classical RAS; light blue, brain RAS pathway; blue, drugs, dotted arrows indicate crosstalk between the systems. APN indicates aminopeptidase N; AT1, angiotensin II type 1; ATR, AT1 receptor; and ROS, reactive oxygen species.

Orally administered RB150, a dimer of EC33, has been shown to enter the brain of SHR and DOCA (deoxycorticosterone acetate)-salt rats to inhibit brain APA activity and block the formation of Ang III and to normalize BP for several hours68,70,71(Table). The RB150-induced depressor response was related to inhibition of arginine vasopressin release, resulting in a diuresis and reduction in volume, and a decrease in sympathetic tone, resulting in reduced vascular resistance. Systemic RAS activity was unaffected, and systemic administration of an ACE inhibitor potentiated the RB150-induced BP decrease, suggesting that centrally acting APA inhibitors might be used in combination with systemic RAS blockers to improve BP control and reduce cardiovascular disease risk in hypertensive patients.70

RB150 (renamed QGC001) has been administered in ascending doses (10–1250 mg) to 56 healthy male volunteers.72QGC001 did not significantly change heart rate or BP at any dose and was safe and well tolerated in this phase Ia study, as well as in a phase Ib study where it was administered in single doses of ≤2000 mg and in multiple doses over a 7 day period.68 A proof-of-concept exploratory study is planned to evaluate the clinical efficacy of RB150/QGC001 for treating hypertension in humans.

Vasopeptidase Inhibitors

The zinc metalloprotease neprilysin (neutral endopeptidase 24.11) is a therapeutic target for hypertension and other forms of CVD because it degrades the natriuretic peptides atrial natriuretic peptide (ANP), BNP, and urodilatin,73 and the increase in circulating natriuretic peptide levels that results from neprilysin inhibition leads to natriuresis, vasodilation, renin–angiotensin–aldosterone system inhibition, reduced sympathetic drive, and antiproliferative and antihypertrophic effects on the heart and vasculature74 (Figure 4). However, neprilysin inhibitors are ineffective in lowering BP, likely because neprilysin also degrades vasoconstrictor peptides, for example, Ang II and endothelin-1.75,76 Thus, combining a neprilysin inhibitor with an RAS blocker or an endothelin-converting enzyme inhibitor offers the theoretical advantage of enhancing the favorable vasodilator/natriuretic effects of ANP and BNP and reducing the deleterious vasoconstrictor effects of Ang II or endothelin-1 on BP and target organ damage.

Figure 4. Vasopeptidase inhibitors. Combining an inhibitor of the natriuretic peptide degrading enzyme neprilysin with an angiotensin receptor blocker (ARB) or an endothelin converting enzyme (ECE) inhibitor in the same molecule offers the theoretical advantage of enhancing the favorable vasodilator/natruiretic effects of the natriuretic peptides and reducing the deleterious vasoconstrictor/proinflammatory effects of angiotensin II (Ang II) and endothelin-1 (ET-1) on blood pressure (BP) and target organ damage. The ARB–neprilysin inhibitor (ARNI), LCZ696, is a single molecule comprising the ARB valsartan and the neprilysin inhibitor pro-drug AHU377 (sacubitril). LCZ696 has been shown to lower BP, particularly in Asian populations, and to prevent death from cardiovascular (CV) causes and hospitalization for heart failure (HF) in patients with reduced left ventricular ejection fraction (LVEF). The ECE–neprilysin inhibitor dagutril has been shown to lower BP in patients with type 2 diabetes mellitus and nephropathy and to reduce pulmonary arterial pressure in patients with HF. Red, classical RAS; orange, natriuretic peptide system; purple, endothelin system; blue, LCZ696; green, dagutril.

Dual-Acting Angiotensin Receptor–Neprilysin Inhibitors

The first-in-class angiotensin receptor–neprilysin inhibitor LCZ696 is a novel single molecule composed of the neprilysin inhibitor prodrug

AHU377 (sacubitril) and the ARB valsartan in a 1:1 ratio77 (Figure 4; Table). In a proof-of-concept randomized, double-blind, placebo-controlled, active comparator trial, graded doses of LCZ696 were compared with graded doses of valsartan and to AHU377 in 1328 patients with mild-to-moderate hypertension.78 LCZ696 produced significantly greater reductions than valsartan in office systolic and diastolic BP and 24-hour ambulatory systolic BP and pulse pressure over the entire dose range tested; AHU377 produced a BP reduction significantly greater than placebo but smaller than either LCZ696 or valsartan. Plasma ANP and cyclic guanosine monophosphate (second messenger for neprilysin activity) levels increased with LCZ696, but the changes in biomarker levels were poorly correlated with BP responses. There were no cases of angioedema, but the trial included only 8% black patients, a group prone to develop angioedema when treated with a combined ACE–neprilysin inhibitor,79 and 3% Asians. To address the lack of information about the efficacy and safety of LCZ696 in Asian persons, a randomized, double-blind, placebo-controlled study was performed in 589 hypertensive patients in 5 Asian countries (Japan, China, Korea, Taiwan, and Thailand).80 Reductions in systolic BP in clinic and nighttime ambulatory settings were 6 to 8 mm Hg greater than those previously reported for Western patients.78 This was attributed to the natriuretic effect of neprilysin inhibition, as valsartan monotherapy has been shown to be relatively ineffective in controlling nighttime BP in Japanese patients.81 No cases of angioedema or other serious adverse effects were noted. The authors pointed out the potential benefit of this BP lowering effect in prevention of stroke in Asian populations.

In light of its modest BP lowering effects in Western populations, LCZ696 is currently under development for treatment of HF, resistant hypertension, and systolic hypertension in the elderly, conditions that are mediated by a combination of vasoconstriction, volume overload, and neurohormonal activation. A Phase II double-blind randomized controlled trial (PARAMOUNT) compared the effects of LCZ696 to those of valsartan alone in 301 patients with HF with preserved ejection fraction.82 LCZ696 induced a greater reduction in systolic BP (9 mm Hg) than valsartan (3 mm Hg), but the changes in biomarkers (NT-proBNP), left atrial volume, renal function, and functional class were independent of the BP lowering effect of LCZ696.83 However, since BP was controlled in the majority of participants at the time of enrollment in PARAMOUNT, a BP-dependent effect of LCZ696 may have been seen in a different population with higher baseline BP.

The Phase III Prospective Comparison of angiotensin receptor–neprilysin inhibitor with ACEI to Determine Impact on Global Mortality and Morbidity in Heart Failure (PARADIGM-HF) trial was a randomized double-blind trial that compared the effects of LCZ696 (200 mg twice daily) to enalapril (10 mg twice daily) in 8442 patients with class II, III, or IV HF and an ejection fraction ≤40%.84,85 The primary outcome was a composite of death from cardiovascular cause or hospitalization for HF. The trial was stopped early (median follow-up 27 months) because of overwhelming benefit. There was a 20% reduction in cardiac death, a 16% reduction in total mortality and a 21% reduction in hospitalization for HF, all highly significant, in the LCZ696 arm compared to the enalapril arm. Further, the symptoms and physical limitations of HF were reduced with LCZ696. Mean systolic BP was slightly but significantly (3.2 mm Hg, $P<0.001$) lower in the LCZ696 group, but this did not account for the outcome benefit. Safety concerns were minor: the LCZ696 group had more symptomatic hypotension, but this rarely required discontinuation of treatment, and more participants in the enalapril group stopped treatment because of other adverse events (most commonly cough or elevated serum potassium) or renal impairment. Angioedema was uncommon in both treatment groups, and the few cases that occurred did not cause airway compromise. However, the study had a run-in phase that excluded persons who were intolerant of an ACE inhibitor or ARB and enrolled few black patients, a group prone to develop angioedema in response to dual vasopeptidase blockade.79 Despite these caveats, the PARADIGM-HF trial has been hailed as revealing the first significant advance in HF therapy in nearly a decade and as offering new thresholds of hope for patients with chronic HF.86

The ongoing prospective comparison of angiotensin receptor neprilysin inhibitor with angiotensin receptor blocker measuring arterial stiffness in the elderly (PARAMETER) study (EUDract ID:2012-002899-14; ClinicalTrials.gov: NCT01692301) compares the effects of LCZ696 versus the ARB olmesartan on aortic stiffness and central aortic hemodynamics in older (aged ≥60 years) patients with systolic hypertension.

87 The central hypothesis of PARAMETER is that by simultaneously enhancing natriuretic peptide effects and inhibiting the RAS, LCZ696 will reduce aortic stiffness and characteristic impedance and improve central hemodynamics, perhaps via mechanisms that are independent of BP reduction. This concept will be examined in greater depth in a parallel study that will determine aortic distensability by MRI and retinal vascular remodeling by scanning laser Doppler flowmetry, respectively (www.ClinicalTrials.gov: NCT01870739). A novel aspect of both studies is assessment of mean aortic pressure and pulse wave velocity on a 24-hour basis using a novel cuff-based oscillometric ambulatory BP monitoring device.88

Dual-Acting Endothelin Converting Enzyme–Neprilysin Inhibitors

Orally active dual inhibitors of neprilysin and endothelin-converting enzyme have been developed, and one of these (daglutril, SLV-306) has been studied in rodent models of diabetes mellitus and in patients with hypertension, HF, and type 2 diabetes mellitus89–93 (Figure 4; Table). Daglutril is a prodrug that is hydrolyzed after oral administration to the active metabolite KC-12615, a mixed inhibitor of neprilysin and endothelin-converting enzyme.89 In diabetic rat models, daglutril and a similar compound have been shown to reduce BP and proteinuria and prevent nephrosclerosis as effectively as the ACE inhibitor captopril.90,94 Daglutril has also been shown to be safe and well tolerated in healthy volunteers,92,95 and biomarker measurements confirmed dual suppression of neprilysin and endothelin-converting enzyme activity in these subjects.92 Data from a multicenter, randomized controlled trial performed in 75 patients with HF showed that daglutril reduced pulmonary and right atrial pressures without affecting systemic arterial pressure, cardiac output, or heart rate.89 Daglutril has also been shown in a small randomized, double-blind, placebo-controlled crossover trial to lower BP but not to reduce albuminuria in patients with type 2 diabetes mellitus and nephropathy.93

Natriuretic Peptide Receptor Agonists

Natriuretic peptide receptor agonists are being developed as an alternative approach to inhibiting the degradation of endogenous natriuretic peptides for the treatment of HF and refractory or resistant hypertension. The natriuretic peptide receptor A (NPR-A) agonist PL-3994 is a synthetic molecule that contains an amino acid mimetic and has reduced affinity for the natriuretic peptide clearance receptor (NPR-C) and increased resistance to neprilysin, resulting in a prolonged half-life after subcutaneous administration96 (Table). A phase I trial of a single subcutaneous dose of PL-3994 in healthy volunteers showed increased natriuresis and diuresis, elevation in plasma cyclic guanosine monophosphate levels, and reduction in systemic BP compared with placebo. A phase II trial in volunteers with hypertension who were receiving ≥1 antihypertensive medications demonstrated a reduction in systemic BP compared with placebo. In particular, PL-3994 appeared to act synergistically with ACE inhibitors, suggesting that it could be administered as an adjunct to standard therapy in patients with refractory or resistant hypertension or HF. No safety concerns were raised in either trial (ClinicalTrials.gov: NCT00686803).

C-ANP4-23 is a ring deleted analog of ANP that is selective for NPR-C and decreases the enhanced expression of Giα proteins that has been implicated in the pathogenesis of hypertension in animal models97 (Table). Intraperitoneal injection of C-ANP4-23 has been shown to decrease BP in SHR by inhibiting enhanced expression of Giα proteins and reducing nitro-oxidative stress, not by modulating the eNOS/cyclic guanosine monophosphate pathway. This study revealed a novel function of NPR-C, which has generally been considered a clearance receptor for natriuretic peptides and has raised the possibility that NPR-C agonists, such as C-ANP4-23, could be useful for the treatment of hypertension and related CVDs.

Soluble Epoxide Hydrolase Inhibitors

Soluble epoxide hydrolase (s-EH) catalyzes the conversion of multiple lipid epoxides to the corresponding dihydroxy lipids.98 Substrates of s-EH include members of the arachidonic acid family, for example, epoxyeicosatrieonic acids, and the effects of s-EH inhibitors have been attributed to increased epoxyeicosatrieonic acid levels.99 Preclinical studies have shown that inhibitors of s-EH lower BP, prevent and reverse pressure overload–induced cardiac hypertrophy, attenuate ischemic and ischemia-reperfusion injury of the brain and heart, prevent atherosclerosis and aneurysm formation, and attenuate insulin resistance in animal models.98–100

AR9281 is a potent and selective inhibitor of human s-EH that has been shown to lower BP, improve vascular function, and reduce renal damage in a rat

model of Ang II-induced hypertension101 and to improve glycemic parameters in a mouse model of diet-induced obesity102 (Table). The metabolic effects of AR9281 were absent in mice with diet-induced obesity due to deletion of the Ephx2 gene, which encodes s-EH, validating the mechanism of the AR9281 effect. Randomized double-blind, placebo-controlled studies in healthy volunteers have shown that AR9281 dose-dependently inhibits s-EH at doses that are well tolerated.103 A randomized double-blind, placebo-controlled dose-ranging phase II study in patients with mild to moderate hypertension and impaired glucose tolerance was terminated in November 2009, and no efficacy results have been reported, suggesting that it was ineffective in lowering BP104 (ClinicalTrials.gov: NCT00847899). However, s-EH inhibitors have other promising therapeutic applications, for example, inflammation, pain, and CVD that warrant future investigation.105

Vasoactive Intestinal Peptide Receptor Agonist

Vasoactive intestinal peptide (VIP) is a neuropeptide with vasodilator and positive inotropic/chronotropic properties that are mediated via the G-protein-coupled receptors VPAC1 and VPAC 2.106 Deficiency in VIP and alterations in properties of VPAC1 and 2 have been described in various forms of cardiopulmonary disease, and VIP is a therapeutic target for hypertension, both systemic and pulmonary, as well as HF. To overcome the abbreviated half-life (<2 min) of VIP, vasomera (PB1046), a stable long-acting form of VIP that is selective for VPAC2, has been developed by fusing an analogue of VIP with an elastin-like polypeptide107 (Table). Selectivity for VPAC2 reduces the potential gastrointestinal side effects associated with activation of VPAC1. Vasomera reduces BP and improves inotropic and lusitropic properties of the heart in animal models of hypertension and HF and has been shown to be safe and well-tolerated after single subcutaneous or intravenous injections in phase I studies in patients with essential hypertension (ClinicalTrials.gov: NCT01523067, NCT01873885). The pharmacodynamic activity of subcutaneous vasomera is supportive of a once weekly dosing regimen that could allow for chronic use in the home setting. Intravenous dosing of vasomera is being evaluated for short-term treatment of HF in the hospital setting.

Intestinal Na+/H+ Exchanger 3 Inhibitor

Excessive sodium intake and impaired sodium excretion plays an important role in the pathogenesis of hypertension and its complications, including HF and chronic kidney disease. Electroneutral Na+/H+ exchangers, for example, NHE2, NHE3, and NHE8, that are expressed in the apical regions of the enterocyte transport sodium from the intestinal lumen into enterocytes.108,109 NHE3 (SLC9A3), the major contributor to intestinal sodium uptake, is inhibited selectively by tenapanor, a compound that does not cross the intestinal barrier (Table). Orally administered tenapanor decreases urinary sodium excretion and increases stool sodium in humans and reverses extracellular volume expansion, lowers BP, and reduces albuminuria and cardiac and renal injury in the 5/6 nephrectomy rat model of sodium-dependent hypertension. Tenapanor also enhances the BP lowering and organ protective effects of the ACE inhibitor enalapril in this model. These findings suggest that reducing sodium transport in the gut could provide a useful alternative or adjunct to dietary sodium reduction or diuretics in the treatment of hypertension and related target organ damage.

Dopamine β-hydroxylase (DβH) Inhibitor

Dopamine β-hydroxylase (DβH), the enzyme that catalyzes the hydroxylation of dopamine to form noradrenaline in the sympathetic nervous system, is a therapeutic target for treatment of hypertension and other cardiovascular disorders characterized by sympathetic activation, for example, HF.110 Inhibition of DβH offers theoretical advantages over adrenergic receptor blockade: (1) it causes gradual sympathetic slowdown instead of acute inhibition and (2) it increases dopamine availability, thus causing renal vasodilation, natriuresis, and diuresis. First, second, and early third generations DβH inhibitors, for example, disulfiram, fusaric acid, and nepicastat, either lacked potency or selectivity for DβH or caused severe CNS-related adverse effects and thus were not clinically useful. Etamicastat (BIA 5–453) is a potent and reversible inhibitor of DβH that does not pass the blood brain barrier and thus is selective for peripheral DβH when administered orally (Table).111 Etamicastat lowers BP in the SHR, but not in normotensive WKY (Wistar Kyoto) rats, and prolongs survival in animal models of HF.112 Studies in healthy men and men with mild to moderate hypertension showed good tolerability and statistically significant dose-dependent decreases in 24-h ambulatory blood pressure (ABP).111 These emerging preliminary results warrant further testing in broader populations.

Vaccines

Vaccines targeting renin for the purpose of treating hypertension have been available for over 50 years.113 Although a renin vaccine successfully lowered BP in animal models, it induced autoimmune disease of the kidneys and further development was suspended.114 An Ang I vaccine also lowered BP in animal models,115,116 but was ineffective in a randomized, double-blind, placebo-controlled clinical trial.117 Further, a vaccine raised in response to an Ang II–derived peptide conjugated to a virus-like particle derived from the bacteriophage Qβ (AngQb) was effective in producing anti-Ang II antibodies and reducing BP in SHR, despite increasing circulating Ang II levels118 (Figure 2; Table). In a placebo-controlled, randomized phase I trial, 12 healthy volunteers received a single injection of AngQb.118 Ang II–specific antibodies were raised in all subjects, and the AngQb antigen was well tolerated.

A subsequent double-blind, randomized, placebo-controlled phase IIa trial tested the effects of immunization with 2 doses of AngQb (also named CYT006-AngQb) on ambulatory BP in 72 patients with mild-to-moderate hypertension.119 Mean ambulatory daytime BP and the early morning BP surge were reduced significantly (by 9/4 and 25/13 mm Hg, respectively) in the high dose group. Changes from baseline in nighttime BP were not significantly different from placebo in either group, and the diurnal pattern of BP response to the vaccine paralleled the diurnal rhythm of RAS activity, with higher levels in daytime than nighttime, and the highest levels of all during the early morning surge in BP. Most observed adverse events were not serious and similar to those seen with other vaccines, that is, mild, transient reactions at the injection site and influenza-like symptoms. The induced antibody response was reversible, with a half-life of ≈4 months, a time course that is compatible with a treatment regimen of 3 to 4 injections per year, which could coincide with regular clinic visits for hypertension management. The authors commented that the immunization strategy could reduce the need for daily dosing of antihypertensive medications, thus enhancing adherence. However, the study was limited by inclusion of a small number of otherwise healthy hypertensive participants, and later stage clinical trials will be needed to evaluate efficacy and safety in a broader hypertensive population.

A subsequent study that used an accelerated immunization schedule in an attempt to induce higher antibody titers, and thereby, greater BP reduction was successful in boosting the antibody titer 5-fold, but resulted in smaller BP reductions.120,121 Antibody affinities were significantly lower, and the amount of Ang II sequestered in the blood of vaccinated persons was significantly less than in the previous study.119 Changes in daytime ambulatory BP correlated with individual antibody affinities and, in particular, with measures of off-rates (how long Ang II is bound to the antibodies). Thus, persons whose antibodies had higher affinity and bound Ang II for a longer period of time showed greater BP reductions. These findings suggest that an accelerated immunization regimen leads to antibody responses with higher titers but lower affinities, perhaps related to disruption in the β-cell antibody affinity maturation process, thereby creating a lower capacity for sequestering Ang II in the blood, resulting in less BP reduction. The authors concluded that a better understanding of how differences in dose and timing of immunizations affect antibody titers, affinities, and BP is crucial for the successful development of an effective vaccine therapy for hypertension. Ongoing phase II trials in patients with mild-to-moderate hypertension are exploring these issues.122

Preclinical studies are evaluating in hypertensive rodent models the safety and efficacy of vaccines against various peptide sequences within the Ang II type 1 receptor, ATRQB-001 and ATR12181,123,124 against Ang II conjugated via the N-terminus to keyhole limpet hemocyanin125 and against a chimeric protein (pHAV–4 Ang IIs) that presents 4 successive repeated Ang II sequences as the functional epitope on the surface of the hepatitis A virus-like particle126 (Figure 2; Table). In all cases, significant BP reductions were achieved, keeping alive the concept that vaccine therapy might be useful in the treatment of human hypertension and its complications.

Novel Approaches to Preeclampsia Treatment

Immunologic approaches are also being explored for the treatment of preeclampsia. Endogenous digitalis-like factors (EDLFs) are a family of circulating Na/K-ATPase inhibitors, including marinobufagenin, that are elevated in preeclampsia and contribute to its pathogenesis by mediating vasoconstriction and vascular fibrosis and inhibiting proliferation and invasion of the cytotrophoblast.127–130 An antidigoxin antibody fragment (Digibind, Ovine Digoxin Immune

Antibody) has been shown to attenuate the inhibitory effects of EDLFs on Na/K-ATPase, to lower BP in animal models of volume-dependent hypertension and to reduce maternal BP and preserve renal function in women with preeclampsia131,132(Table).

A randomized, double-blind, placebo-controlled trial, Digibond Efficacy Evaluation in Preeclampsia, demonstrated beneficial effects on renal function and a trend toward reduction in antihypertensive drug usage but no prolongation of pregnancy or improvement of maternal outcome overall in women with severe preeclampsia.129,133 A secondary analysis of Digibond Efficacy Evaluation in Preeclampsia stratified participants by circulating levels of EDLF activity and found that the beneficial effects of Digibind on renal function and on utilization of antihypertensives, as well as some maternal and fetal/neonatal outcomes, were greater in women who were EDLF positive.129 Digibind production was terminated by its manufacturer in 2010, and DigiFab, affinity purified Fab fragments of antidigoxin antibody, became the only antidigoxin antibody available for clinical use.130 DigiFab has similar effects as Digibind on EDLFs derived from the blood of women with preeclampsia. Ongoing expedited phase II studies are evaluating the safety and efficacy of digoxin-immune Fab for treatment of preeclampsia (Table).

Antithrombin replacement is a novel approach for the treatment of early onset severe preeclampsia that has been shown in small uncontrolled studies to lower BP, reduce proteinuria, and improve coagulation parameters.134 The Prospective Randomized Evaluation of the Safety and Efficacy of Recombinant Antithrombin in Very Preterm Preeclampsia (PRESERVE-1; ClinicalTrials.gov: NCT02059135) is an ongoing phase III randomized placebo controlled trial of recombinant human antithrombin (ATryn) for the treatment of early (24–28 weeks) onset preeclampsia (Table). The primary end point is the increase in gestational age from randomization to delivery; secondary end points include a large number of maternal and fetal outcomes.

Part II. Interventional Treatments

In the 2013 ESH/ESC135 Guidelines for the Management of Arterial Hypertension, interventional strategies are mentioned as therapeutic options for severe TRH. In Part II of this Compendium Review, we focus on 2 main interventional approaches, that is, renal denervation (RDN) and baroreflex activation therapy (BAT) because these interventions are used in clinical practice in some countries. We also discuss briefly other interventional approaches to TRH.

Renal Denervation

This is a rapidly growing research field with numerous published papers dealing with RDN. In this Comprehensive Review, we have focused on clinical aspects and established catheter-based techniques of RDN, but also provide some insights into the emerging field of alternative techniques targeting renal nerves.

Increased sympathetic activity plays an important role in the development, maintenance, and acceleration of arterial hypertension,136,137 especially in TRH. Activation of efferent sympathetic nerves to the kidney stimulates renin release, enhances tubular reabsorption of sodium and water, and decreases renal blood flow.137 In addition, activation of afferent sensory nerves, for example, stimulated by stretch, renal ischemia, hypoxia, or other injury, increases central nervous system sympathetic outflow and, as a consequence, sympathetic activity in key organs, including the heart, kidney, and vasculature, in particular the small resistance vessels.138,139 Catheter-based RDN has been introduced as a new interventional approach targeting sympathetic nerve activity, and hence arterial hypertension in humans.

The first nonrandomized, proof-of-concept study (Symplicity HTN-1) of RDN patients with TRH (systolic BP ≥160 mm Hg) was published in 2009.140 There was a BP reduction of 27/17 mm Hg at 6 months of follow-up, without any significant safety issues. In the subsequent randomized controlled Symplicity HTN-2 study, a 2 week observation period was required and baseline office BP had to be ≥160 mm Hg (or ≥150 mm Hg in diabetics, respectively).141 A BP reduction of 32/12 mm Hg was observed after RDN compared with the control group (1/0 mm Hg) with no outstanding safety concerns. Long-term data (36 months follow-up) from both studies showed that BP reduction was maintained or even became greater, suggesting that clinically meaningful reinnervation did not occur.142,143 However, concerns about the design of the Symplicity HTN-1 and HTN-2 studies have been raised, for example, lack of 24-h ambulatory blood pressure monitoring (ABPM), to confirm true resistant hypertension, (ie, white coat effect was not

definitely excluded) and the unblinded design of the studies.144

To overcome these deficiencies and after the advice of the Food and Drug Administration of the United States, the prospective, single-blind, randomized, sham-controlled Symplicity HTN-3 trial was performed.145 The primary safety end point was met, but the prespecified primary efficacy end point (reduction in office BP) was not reached. There was significant BP reduction from baseline in both groups (P<0.001), but between the RDN and sham-control group, neither office (−2.29 mm Hg, P=0.026) nor 24-h ABPM (−1.96 mm Hg, P=0.98) was significantly different at 6 months of follow-up.145

After publication of the primary findings of Symplicity HTN-3, it became clear that the results are difficult to interpret because of procedural shortcomings. Post hoc analysis of the imaging taken during RDN showed that only 19 patients had complete 4 quadrant ablations (covering 360° of the renal artery) for both renal arteries and only 68 for one renal artery, respectively.146 These data indicate that 253 of the participants randomized to the active treatment arm of the study did not have circumferential ablation of both renal arteries, calling into question the completeness of RDN in the trial. Office systolic BP changes in the group with incomplete RDN of both renal arteries were −14.2±24 (N=253); in those with ≥1 complete RDN, −16.1±23 (N=68); and in those with complete RDN of both arteries, −24.3±23 mm Hg (N=19). In addition, 60 of the 111 operators performed just 1 or 2 RDN procedures, further questioning the quality of the RDN procedures in the trial.146Although each procedure was supervised by an experienced proctor and performed per protocol instructions, the denervation achieved was thus incomplete in most cases, owing to an insufficient number of ablations, lack of 4-quadrant ablations, or other technical features that might explain the failure to lower BP significantly.147

In contrast to previous trials, mainly conducted in Europe and Australia, approximately one-quarter of patients enrolled in the Symplicity HTN-3 study were African American and there appeared to be an interaction with race and change in office BP (P for interaction =0.09). In African Americans, office systolic BP decreased by 15.5 in the RDN group and 17.8 mm Hg in the control group (P=0.641). In Non-African Americans (nearly all were white), office systolic BP was reduced by 15.2±24 mm Hg in the RDN group and 8.57±25 mm Hg in the sham-control group, with a significant difference between the 2 groups of 6.63 mm Hg (95% confidence interval, −11.81 to −1.44; P=0.012).145 Of note, randomization into the RDN and control groups was stratified by race (African American versus Non-African American), that is, the subgroup analysis is solid because randomization was maintained, in contrast to all other subgroup analyses included in the Symplicity HTN-3 article.145 Differences in the response to BP treatment according to race are well known, and hence many guidelines recommend differential antihypertensive drug strategies in African Americans versus other racial groups.148,149

Further debate about the efficacy of RDN is related to the numeric differences between office BP reduction (eg, Symplicity HTN-2 32/12 mm Hg, n=49) and 24-h ABP reduction (eg, Symplicity HTN-2 11/7 mm Hg, n=20).141 In a pooled analysis, it was shown that in patients with TRH, both office BP and, to a lesser extent, 24-h ABP were significantly reduced, whereas in patients with white coat hypertension, only office BP was significantly reduced.150 This discrepancy in BP reduction between office BP and 24-h ABP has been observed repeatedly. In the largest reported clinical study, a registry-based analysis, it was found that the disproportionate decreases in office BP versus 24-h ABP were related to the pretreatment BP (which is higher with office BP readings compared with 24-h ABPM) and that the changes in office and 24-h ABPM were not related in a 1:1 fashion.151

Most recently, 2 randomized (not sham) controlled trials of RDN in patients with TRH have been published. In the PRAGUE-15 trial, the antihypertensive effects of RDN were compared with those of intensified pharmacological treatment, including spironolactone.152,153 The reductions in office BP and ABP were comparable in the 2 groups, but 39% of the patients in the pharmacological therapy group experienced adverse effects, for example, hyperkalemia and antiandrogen effects. In the French DENER-HTN study, 106 patients with TRH were randomized to RDN (N=53) or intensified drug treatment (N=53).154 Baseline-adjusted changes in daytime and nighttime ABP from baseline to 6 month follow-up were significantly greater in the RDN group than in the control group: systolic daytime BP −5.9 mm Hg (P=0.0329), systolic nighttime BP −6.3 mm Hg (P=0.0296), diastolic daytime BP −3.1 mm Hg (P=0.092), and diastolic

nighttime BP −3.2 mm Hg (P=0.051).154Both trials included patients whose hypertension was not truly treatment resistant because office BP and ABP were lowered significantly after uptitrating pharmacological therapy in the intensified drug treatment groups.

Although current knowledge of the effects of RDN is based largely on studies performed with the Symplicity Flex catheter, multi-electrode approaches may reduce the need for catheter manipulation and decrease procedure time. The effects of RDN with various catheters on BP are generally similar, and most of the published trials were uncontrolled (Figure 5).

Figure 5. Recent studies of the effects of renal denervation with various catheters on blood pressure.Adapted from Ott and Schmieder211 with permission of the publisher. Copyright ©2014, Springer. Authorization for this adaptation has been obtained both from the owner of the copyright in the original work and from the owner of copyright in the translation or adaptation.

An unresolved but critical issue in assessing the clinical utility of RDN is to identify a reliable predictor of BP response. Analyses that have focused mainly on clinical characteristics of the patients and technical aspects of the catheters and procedures have failed to identify consistent and reliable predictors, with the exception of baseline systolic BP.141,150Baseline BP is positively related to the amount of BP reduction post-RDN according to Wilder's law155 and regression to the mean effect.

Other clinical effects of RDN, which are at least in part beyond BP reduction, have been reported. These include improvement in glucose metabolism,156 beneficial effect on end-organ damage, for example, left ventricular hypertrophy,157arterial stiffness158 and albuminuria,159 attenuation of decline in renal function, and improvement in functional status in patients with congestive HF.

Non-catheter-based approaches of targeting the renal nerves have also been developed. In an adult swine model, a 3-needle delivery device was introduced and 3 different doses of ethanol (each n=3) were injected through the vessel wall into the peri-adventitial tissue in a circumferential manner. There was a dose-dependent decrease in renal parenchymal norepinephrine concentration (0.15 mL/artery, 54%; 0.3 mL/artery, 78%; and 0.6 mL/artery, 88%; all P<0.05) after 2 weeks. Histopathologic examination revealed no evidence of device or ethanol-induced intimal injury.160 An alternative ethanol-based approach was reported in one patient with TRH and end-stage renal disease. Percutaneous ethanol delivery was guided via computer tomography, and ethanol was injected around the renal arteries. After 1 month, office BP was reduced from baseline 172/84 to 143/70 mm Hg. No data about 24-h ABPM, which is considered gold-standard, was provided.161

The feasibility of a noninvasive approach to RDN by extracorporeal high-intensity focused ultrasound has been tested in a canine model. Compared with baseline, BP and renal parenchymal noradrenaline concentration were reduced (day 6, −50.1%; day 28, −55.4%; both P<0.001), whereas no significant change was observed in the sham-control group. Histopathologic examination demonstrated nerve fiber disruption at day 28 after RDN.162 The WAVE I trial tested Kona Medical's ultrasound-based Surround Sound® Renal Denervation System (Kona Medical, Menlo Park, CA) in 24 patients with TRH who were taking an average of 4.5 antihypertensive medications and had a mean baseline BP of 190/100 mm Hg. Eighteen focused lesions were applied over 13 minutes to each artery, but for targeting and tracking reasons, a 5F intravascular renal artery catheter had to be used. Office systolic BP was reduced by 29 mm Hg at 6 months after externally focused ultrasound RDN. The WAVE II study, using an optimized treatment protocol (14 externally focused lesions over 3 minutes were applied per side) has been completed, but not yet reported. The WAVE III study used an improved protocol of energy delivery without the need to have a guiding catheter inserted, thus achieving a fully noninvasive RDN approach in humans. First results (n=22), presented at the 2014 Transcatheter Cardiovascular Therapeutics (TCT) meeting, showed an office BP reduction of 29.6/11.8 mm Hg at 3 months after ultrasound RDN.163 The WAVE IV trial, a prospective, randomized sham-controlled study, in which office and ABP are to be obtained after 6 months, is underway (www.ClinicalTrials.gov: NCT02029885).

Baroreflex Activation Therapy

Knowledge of baroreflex regulation of BP goes back to ancient times. The arteries of the neck were named karotides, based on the Greek rout karos (heavy sleep) and karoun (to choke, to stupefy) because pressing on these arteries produced sedation. In the 20th century, the role of the carotid baroreflex was demonstrated for short-term BP regulation, but it was assumed to play no role in long-term BP control.

However, based on several important studies in animals, interest in the role of the carotid sinus baroreceptor on long-term BP control has returned,164–166 and a surgical implantable device has been developed to administer BAT via electrical stimulation of the carotid baroreceptors.167 The prospective nonrandomized DEBut-HT trial was a multicenter European feasibility trial for the early-generation device (Rheos System; CVRx Inc., Minneapolis, MN) performed in 45 patients with TRH (systolic BP ≥160/90 mm Hg, despite ≥3 antihypertensive drugs, including a diuretic). In this proof-of-concept study, there was a reduction of 21±4/12±2 mm Hg (n=37) in office BP at 3 months, with further decreases of 30±6/20±4 (n=26) at 1 year and 33±48/22±26 mm Hg (n=17) at 2 years (all P=<0.005), respectively. There was also a statistically significant reduction in 24-h ABP at 1 year follow-up. In contrast, no BP change was observed in 10 control patients who declined device implantation.167 In total, 8 serious adverse events (7 procedure-related and 1 device-related) were reported, a number comparable to published complication rates with carotid surgery.168,169 A substudy of 12 patients from the DEBut-HT trial demonstrated that muscle sympathetic nerve activity and BP were decreased after activation of BAT and increased without activation, providing evidence that reduction of sympathetic outflow is the primary mechanism for BP reduction with BAT.170

The double-blind, randomized, parallel-design Rheos Pivotal trial enrolled 256 patients with TRH. One month after Rheos device implantation, patients were randomized in a 2:1 manner to immediate BAT (device on) or delayed BAT (device remained off for 6 months). The prespecified acute primary efficacy end point (proportion of patients achieving BP reduction of ≥10 mm Hg after 6 months with a superiority margin of 20%) was not met, and the secondary efficacy end point (mean change in systolic BP after 6 months) failed statistical significance (group A [device on]: −16±29 versus group B [device off]: −9±29 mm Hg; P=0.08). There was an unexpected difference in systolic BP between preimplant (−1 month) and immediately postimplant (month 0) time points, prompting an additional post hoc analysis of the data. BP reductions from preimplant levels to 6 months postimplant were 26±30 mm Hg in the device on group versus 17±29 mm Hg (P=0.03) in the device off group. The sustained primary efficacy end point, defined as BP reduction of ≥10 mm Hg from months 0 to 12, with ≥50% of BP reduction seen at month 6 (primary end point) was reached. The procedural primary safety end point was not met, mainly because of surgical complications (4.8%) and transient (4.4%) or residual (4.8%) nerve injuries, but the prespecified criteria of both BAT and device safety were met.171 After completion of the Rheos Pivotal Trial, participants continued in an open-label, nonrandomized follow-up for an average of 28±9 months. A mean BP reduction of 36/16 mm Hg (P<0.001) was observed in the selected group of long-term responders (n=245, 76%), defined by achieved systolic BP ≤140 mm Hg (≤130 mm Hg for diabetic or renal disease patients) or systolic BP reduction of ≥20 mm Hg from device activation.172

A second-generation system of BAT (Barostim neo™) has been designed to address shortcomings of the original device. A single (instead of 5) electrode is implanted at one carotid site, thus reducing the operating field (and hence possible complications). Moreover, the battery is smaller, with an extended life span (≈3 years). In a single-arm open-label study enrolling 30 patients with TRH (based on systolic BP ≥140 mm Hg although on ≥3 antihypertensive drugs, including a diuretic), a BP reduction of 26.0±4.4/12.4±2.5 mm Hg was observed after 6 months and 3 perioperative and 1 long-term procedure-related complications occurred.173 Upcoming studies (eg, Barostim Hypertension Pivotal Trial, NCT01679132) will clarify the future of this approach to BP reduction.

Animal studies indicate that BAT directly affects autonomic regulation of the heart. Analysis of data from 34 patients pooled from different studies that used BAT demonstrated improvement in left atrial and ventricular structure and function (assessed by echocardiography). Left atrial dimensions and left ventricular mass, wall thickness, and stroke work were reduced, although left ventricular ejection fraction increased.174 The effects of BAT on metabolic parameters (eg, glucose metabolism) and hypertensive organ damage have not yet been examined.

Carotid Body Ablation

Studies in animal models175 and human subjects176 have revealed enhanced carotid body (CB) sensitivity in hypertension, but the mechanisms of this abnormality are not known. CB hypersensitivity has been shown to precede the development of hypertension in SHR175 and in patients with white-coat hypertension.176 In a small, randomized, crossover, placebo-controlled study, deactivation of

CB chemoreceptors by hyperoxia (respiration with 100% oxygen) attenuated the enhanced muscle sympathetic nerve activity in untreated hypertensive men, but no change was observed in controls.177 It has also been shown that hyperoxia decreases BP acutely in patients with hypertension, but not in normotensive controls.178 These data point to a potential pathogenetic role of tonic chemoreceptor drive in the development of sympathetic overactivity in hypertension.177

Surgical removal of the CB has been performed in humans for reasons other than hypertension (eg, bronchial asthma and chronic obstructive pulmonary disease [COPD]). A BP fall from 170 to 130 mm Hg was observed 5 days postop and sustained for 6 months after bilateral CB surgery in hypertensive patients, whereas no BP lowering effect was seen in normotensive patients, and a rise in BP was documented in hypotensive patients after bilateral CB resection.179,180 To date, no study addressing the effect of uni- or bilateral CB resection for hypertension in humans has been completed, but first-in-man studies are ongoing.

Arteriovenous Fistula

A novel mechanistic approach to BP reduction is used by the ROX coupler system (ROX Medical Inc., San Clemente, CA). This self-expanding device creates a 4 mm arteriovenous fistula (AVF) between the iliac artery and vein, generating a sustained calibrated shunt volume (≈ 800 mL/min) within a short period of time (≈1 h). Detailed technical information about deployment of the device is given elsewhere.181 Several mechanisms are hypothesized to cause BP reduction after creation of an AVF.182 Reduction in total systemic vascular resistance, despite an increment in cardiac output, is considered to be the key mechanism. Enhanced tissue oxygen delivery caused by increased arterial oxygen content may reduce peripheral and renal chemoreceptor activation and thus decrease sympathetic activity. Reductions in systemic vascular compliance and effective arterial volume may also improve arterial compliance, contributing to a reduced cardiac workload, despite increased cardiac output.182 Expected adverse effects are induction of venous stenosis and thrombosis and potential worsening/development of right ventricular failure.

The Rox coupler system was originally developed for the treatment of patients with COPD. Early positive results extended the indication to patients with concomitant arterial hypertension. A subset of 24 COPD patients (NCT00832611 and NCT00992680) with an office systolic BP ≥130 mm Hg when on antihypertensive treatment was retrospectively analyzed after the ROX coupler procedure was performed. Compared with baseline (145±12/86±13 mm Hg), systolic and diastolic BP were significantly reduced after 6 (130±18/71±13 mm Hg, P<0.01) and 12 months (132±18/67±13 mm Hg, P<0.01), respectively.181 No clinical meaningful BP reduction was seen in normotensive COPD patients after creation of an AVF using the ROX coupler.

Based on this first evidence of efficacy of AVF in patients with COPD and coexisting arterial hypertension, the concept was further tested in a small prospective, nonrandomized study enrolling 8 patients with TRH, but without COPD. Compared with baseline, both office BP (175±19/87±14 versus 158±26/74±19 mm Hg) and 24-h ABP (152±17/82±15 versus 142±18/69±14 mm Hg) decreased at 6 months post creation of AVF. Subsequently, the European prospective, open-label, multi-center ROX CONTROL-HTN (NCT01642498) study was initiated to evaluate the ROX Coupler used along with standard drug therapy in 100 patients with TRH without COPD. In the ROX coupler group, office BP decreased by 26.3/20.1 mm Hg (control group 3.7/2.44 mm Hg) and ambulatory BP by 13.5/13.5 mm Hg (control group 0.5/0.1 mm Hg) after 6 months. Reductions were of similar magnitude in those with previous renal denervation.183 Procedural complications related to arteriovenous coupler placement occurred in N=13 (31%) with venous stenosis occurring in N=12 (29%) of the 42 patients treated.183 In relation to worsening of hypertension, 5 hospital admissions for hypertensive crisis were reported in 3 (8%) of the 39 control patients, compared with none in the arteriovenous coupler group (P=0.0225).

Neurovascular Decompression

Animal studies have shown that pulsatile compression of the rostral ventrolateral medulla at the root-entry zone of cranial nerves IX and X increases both BP and sympathetic outflow,184,185 and clinical data suggest that neurosurgical decompression of the rostral ventrolateral medulla (used for neurological disorders) leads to BP reduction.186 A relationship between relief of hypertension and neurovascular decompression was demonstrated by Geiger et al, who observed

improvement in BP control (7 out of 8 patients) 3 months after neurovascular decompression.187 Sympathetic nerve activity was significantly reduced after microvascular decompression in parallel with the BP decrement. Long-term effects were less promising, however, because hypertension relapsed, and 18 months post intervention, sympathetic nerve activity had increased to preoperative levels.188,189 Because no long-term clinically significant BP reduction has been demonstrated in a randomized controlled study and special postprocessing software for the analysis of MRI images of the rostral ventrolateral medulla (that are not commonly available) are required to qualify a patient for the procedure, microvascular decompression for treatment of TRH is restricted to compassionate use in patients with severe TRH and proven neurovascular compression using advanced imaging techniques.190

Renal Artery Stenting (Revascularization)

Clinical indications for percutaneous transluminal angioplasty with stenting for renal artery stenosis are controversial. Recent clinical findings from large prospective randomized controlled trials revealed little or no benefit for BP control, preservation of kidney function, or prevention of cardiovascular or renal events, calling into question broad use of renal artery stenting in hypertensive patients with renal artery stenosis.191–193 In the ASTRAL trial, renal arterial revascularization did not result in a clinically relevant reduction in BP, but did cause a high incidence (17%) of adverse procedure-related complications.192However, methodological questions have been raised regarding the inclusion criteria. To enroll a patient in the trial, physicians had to be uncertain whether the patient would profit from the intervention, thus excluding those patients with a clear indication for renal artery stenting and creating a selection bias. For example, 40% of the enrolled patients had <70% narrowing of the renal artery. The STAR trial showed that the primary end point, ≥20% decrement of estimated creatinine clearance, did not differ between medical therapy alone and medical therapy combined with revascularization.191 However, ≈30% of patients allocated to combined therapy did not undergo revascularization because at the time of angiography, the degree of stenosis was <50%.191 In the CORAL study of patients with atherosclerotic renal artery stenosis, hypertension, and chronic kidney disease,193 reduction in systolic BP over time was greater (−2.3 mm Hg; 95% confidence interval, −4.4 to −0.2; P=0.03) in the revascularization group, but this did not result in prevention of cardiovascular or renal events over a median follow-up of 43 months.

All of the studies of renal artery revascularization have been criticized on grounds that they did not critically evaluate the hemodynamic relevance of the renal artery stenosis.194,195 With the exception of subtotal occlusion of the renal artery, the angiographic degree of renal artery stenosis is a poor reflection of hemodynamic relevance.196 Hemodynamic relevance (to be suspected if stenosis is >80%) can be assessed by intraarterial pressure measurement or duplex sonography. A diminished resistance index in the cortical tissue reveals hemodynamic relevance, but measurement of blood flow velocity alone is not valid.197,198 In the CORAL study, translesional renal artery pressure gradients were obtained, but are not yet published.199 The ongoing controversy about the utility of renal revascularization is portrayed in many publications of pooled data, meta-analyses, and long-term follow-up data. In the absence of more convincing evidence of benefit (Figure 6),200 it may be wise not to stent as a primary therapeutic option in patients with atherosclerotic renal artery stenosis unless hemodynamic relevance can be demonstrated or rapid deterioration in kidney function or worsening BP is evident.201–203

Figure 6. Renal artery revascularization: updated meta-analysis with the CORAL trial summary estimates of cardiovascular outcomes for revascularization vs medical therapy.

In contrast to atherosclerotic renal artery stenosis, a systematic review and meta-analysis of patients with fibromuscular dysplasia as cause of renal artery stenosis revealed that percutaneous transluminal angioplasty alone (without stenting) improves BP control or even cures hypertension.204 Further, BP outcome was inversely associated with age. Hence, the European consensus on the diagnosis and management of fibromuscular dysplasia proposes revascularization for hypertension because of fibromuscular dysplasia, especially in patients with recent onset hypertension or TRH.205

Future

Published studies of interventional BP lowering treatments were performed almost exclusively in patients with severe TRH, defined as systolic BP ≥160 mm Hg (or ≥150 mm Hg in diabetics), despite treatment with an average of 5 different

antihypertensive drugs. However, an expansion to broader populations of TRH (office BP ≥140/90 mm Hg) is being discussed.206 A small uncontrolled study of patients with true moderate TRH (office BP ≥140/90 mm Hg and 24-h ABP ≥130/80 mm Hg, despite treatment with ≥3 antihypertensive drugs, including a diuretic) showed reductions in office (13/7 mm Hg) and 24-h ABP (14/7 mm Hg) at 6 months after RDN, despite a decrease in BP medication. In 51% of these patients, office BP was controlled to <140/90 mm Hg after RDN.207

It is generally recognized that several pivotal steps must be taken before adopting RDN as a procedure for BP treatment outside the research setting. First, prospective, randomized, sham-controlled studies have to show the efficacy of RDN in lowering BP in TRH. In this regard, multi-electrode systems may provide more complete, and hence effective, RDN. Second, reliable tools have to be developed to assess the completeness of RDN. Third, because the BP response to RDN varies greatly from patient to patient,141,208,209 a reliable clinical predictor of BP response to RDN is needed to improve patient selection. Fourth, the efficacy of RDN in improving clinical outcomes has to be demonstrated. It is likely that global registry data will be needed to reach this final critical goal.

Liver transplantation for chronic liver disease: advances and controversies in an era of organ shortages

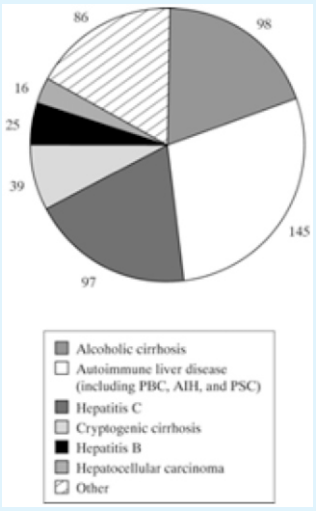
Abstract

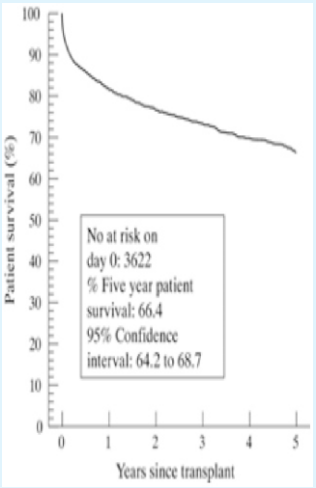
Since liver transplantation was first performed in 1968 by Starzl et al, advances in case selection, liver surgery, anaesthetics, and immunotherapy have significantly increased the indications for and success of this operation. Liver transplantation is now a standard therapy for many end stage liver disorders as well as acute liver failure. However, while demand for cadaveric organ grafts has increased, in recent years the supply of organs has fallen. This review addresses current controversies resulting from this mismatch. In particular, methods for increasing graft availability and difficulties arising from transplantation in the context of alcohol related cirrhosis, primary liver tumours, and hepatitis C are reviewed. Together these three indications accounted for 42% of liver transplants performed for chronic liver disease in the UK in 2000. Ethical frameworks for making decisions on patients' suitability for liver transplantation have been developed in both the USA and the UK and these are also reviewed.

Thomas Starzl et al first reported successful human orthotopic liver transplantation in 1968 in Pittsburgh, USA.1 Since then liver transplantation has become one of the standard therapeutic options for advanced chronic liver disease and selected patients with acute liver failure (most commonly due to paracetamol overdose or viral infection). In the year 2000, 678 liver transplants were performed for chronic liver disease in the UK. The indications for these transplants are shown in fig 1.

Figure 1

Indications for primary liver transplantation in the UK in 2000; AIH, autoimmune hepatitis, PBC, primary biliary cirrhosis; PSC, primary sclerosing cholangitis. (Statistics prepared by UK Transplant from the National Transplant Database maintained on behalf of transplant services in the UK and Republic of Ireland.)





Improvements in surgical techniques and immunosuppression have markedly increased the success rates of liver transplantation. Much of the morbidity and mortality associated with transplantation is concentrated in the first postoperative month when the risks of rejection, sepsis, and surgical complications are highest, particularly after acute liver failure. However, after this the outlook for most patients is excellent. As shown in fig 2, one and five year patient survival after transplant is 81% and 66% respectively. Furthermore many patients can return to a normal level of social and physical functioning with a vastly improved quality of life.2

Kaplan-Meier estimates of five year patient survival after non-urgent liver transplants in the UK, 1 January 1994 to 31 December 2000. (Statistics prepared by UK Transplant from the National Transplant Database maintained on behalf of transplant services in the UK and Republic of Ireland.)

The success of liver transplantation has lead to increasing numbers of referrals. However, at the same time the availability of cadaveric organs has diminished (partially due to improvements in road safety), resulting in the number of potential recipients for liver transplantation exceeding organ supply with attendant deaths of patients on waiting lists. We review areas of controversy and new approaches developing in response to this mismatch.

IMPROVING THE RATIO OF TRANSPLANT SUPPLY AND DEMAND

There are three approaches to improving the ratio of liver availability to potential recipients. First, to maximise efficiency of liver distribution between centres; second, to examine ways of expanding the donor pool; and third, to impose limits on use of livers for certain indications. Xenotransplantation is not currently an option for human liver transplantation.

Organ distribution protocols minimise inequalities in supply and demand between regions and therefore improve the efficiency of organ utilisation. This is most important for patients with acute liver failure where the "window" between listing for transplant and death may only be one to three days. In such cases patients in the UK are assigned to a "super-urgent" list and receive the first suitable liver available from any centre in the UK.

Four methods to increase the donor pool of livers for transplantation have been proposed. First, legislative changes may increase the numbers of patients donating organs. Changing the current system, which requires the potential donors to register approval of the use of their organs before death (an "opt in" system) to one where patients are assumed not to object to donation unless otherwise noted (an "opt out" system) has received considerable media coverage. At present the legislative and ethical barriers to this have not been overcome in the UK.

The second method for increasing organ availability has been to use "marginal" livers that would previously have been regarded as unsuitable for transplantation. Three categories of "marginal" livers have been considered. Livers from donors who have suffered brief cardiopulmonary arrest have not generally been used for transplantation because of the potential for damage to the grafted liver. However, Totsuka et al recently reported that despite early biochemical abnormalities, 90 day graft survival was similar to that from conventional donors, suggesting that these should be more widely used.3 Similarly, livers showing macroscopic evidence of steatosis (fatty liver) were previously thought unsuitable for transplantation because of an increased risk of primary graft non-function. However, this increased risk is mainly restricted to livers with severe steatosis (microscopic steatosis in over 60% of hepatocytes).4 Increasingly transplant centres are using livers with mild (less than 30%) or moderate (30%–60%) steatosis, although there is still a small increased risk of primary non-function with moderate steatosis. Finally, livers from hepatitis C positive donors may be transplanted into hepatitis C positive recipients. As discussed below, graft reinfection is universal in patients who are hepatitis C RNA positive pre-transplant. Hence, provided that the donor liver is not significantly fibrotic before transplantation, postoperative survival may be unimpaired. Recent reports from the USA suggest survival is at least equal to that of conventional donors.5,6 Hepatitis C positive donors are not currently used in Europe.

The third method for improving liver supply has been to share a single organ between two recipients by surgically splitting the donor liver. This approach originated in paediatric liver transplant programmes where extreme shortages of child donors led to use of surgically reduced adult livers (primarily the left lateral lobe).7 The remaining segment (an extended right lobe) of liver could then be used in an adult recipient. This graft regenerates over three to six months to close to normal adult size. Subsequent work has defined the optimal method for organ splitting together with estimates of the minimum ratio of organ section size to recipient weight allowing organ sharing between two adults. Current practice in the USA favours in situ (that is before removal from the native donor blood supply) splitting of the donor liver along the anatomical boundary between left and right lobes.8 The transplanted liver section must be at least 50% of the recipient's standard liver volume (a measure of the expected healthy liver volume based on recipient's height and weight) to provide adequate function for patient survival before liver regeneration.9Although the success rates of these methods are improving, the

adult recipient still has survival below that expected with whole organ transplantation8 and accordingly these methods are not in widespread use in the UK. Furthermore few British centres have the facilities to perform two transplant operations simultaneously. However, these techniques are gaining favour in the USA where waiting lists are longer and the excess mortality due to decreased operative success rates may be outweighed by reduced mortality on the waiting list.

The final method for increasing organ availability has been to transplant segments of livers from living donors. These methods were primarily derived in Japan where legislation previously precluded cadaveric donation and over 400 such operations have been performed to date.10 The surgical methods adopted are similar to liver splitting, although the right lobe is now favoured for transplantation.11 As the graft comes from a healthy donor, a slightly reduced volume ratio (40%) is acceptable.12 There are ethical problems with this approach however.13 Deaths among living related donors have recently been recorded along with a substantial morbidity rate (estimated at 4%).11 Furthermore, issues regarding donor consent and the exclusion of coercion by the recipient (whether intentional or not) are unclear. Perhaps the greatest potential for these techniques would be in the treatment of acute liver failure,9 however the potential for unintentional coercion here is highest given the short period available to obtain consent.14Given these unresolved problems, living related liver segment donation is used only occasionally in the UK, mainly in paediatric patients.

The final method for matching organ availability and requirements is to limit the indications for transplantation and thus reduce demand. Ideally transplantation would be limited to those patients with the highest predicted mortality with conservative therapy and the highest long term survival after transplant. Three indications for transplantation, alcoholic liver disease (ALD), recurrent hepatitis C, and hepatocellular carcinoma, have been questioned from this perspective.

LIVER TRANSPLANTATION FOR ALCOHOLIC LIVER DISEASE

ALD is the commonest cause of chronic liver failure in Europe and North America and is one of the most controversial indications for transplantation. A survey of attitudes to liver transplantation reported that ALD is the least popular reason for transplantation both among the public and family physicians.15 However, the sheer burden of alcoholic cirrhosis means that ALD accounts for a substantial and increasing proportion of all liver transplantation (fig 3).

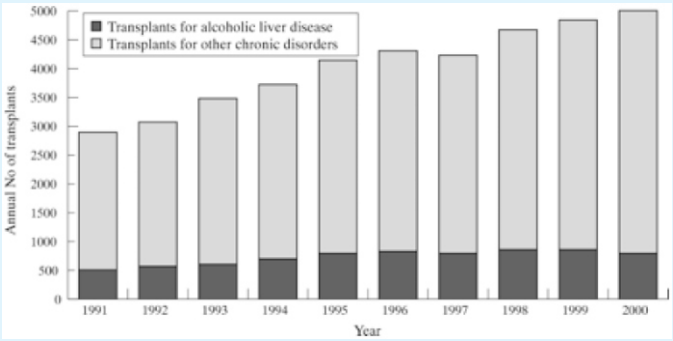


Figure 3

Proportion of liver transplants due to alcoholic liver disease in the USA, 1990 to 2000. (Adapted from data in Belle et al16 and Neuberger and Tang17.)

The concerns about offering transplantation for ALD stem from reluctance to "waste" a liver on a "self induced" disease and the potential for recurrent alcoholic abuse after transplantation. From a clinical perspective ALD is a very good indication for transplantation with a similar or better prognosis after transplantation to most other liver diseases except cholestatic liver disease.16

Two questions underlie much of the controversy regarding transplantation for ALD. How can one predict who is likely to relapse after transplantation and how can we prevent relapse in these patients?

Studies of recidivism after transplantation are difficult to compare because of differences both in definitions of recidivism (ranging from any alcohol use to continuous heavy alcohol usage) and in methods of follow up (from self reported telephone surveys to intensive counselling with laboratory measures of alcohol related parameters). Accordingly rates of recidivism have varied greatly (from 9%–80%17) with the majority of estimates being around 20%–30%. Recidivism is considered undesirable because of recurrence of liver disease, decreased compliance with immunosuppression,18 and loss of support for transplantation programmes with the attendant loss of organ donations.

In contrast to "recidivism", recurrence of ALD has been defined as heavy drinking together with appropriate histological changes.19There are very few studies of the incidence of recurrent ALD. Lee examined a series of 29 liver biopsies from patients with "excessive" postoperative alcohol consumption

and abnormal liver function tests.19 Although 83% of biopsies showed steatosis, only 28% were fibrotic and a further 23% (six patients) had progressed to cirrhosis. Five of the patients with cirrhosis also had concurrent hepatitis C making it difficult to be sure whether the cirrhosis was due to alcohol. This study was also likely to over-estimate the frequency of recurrent ALD as no biopsies were taken from patients with recurrent drinking and normal liver function tests. However, combining these results with estimates of recidivism, the rate of recurrent fibrotic ALD after transplantation is probably less than 15%, which is similar to other transplant indications.

The commonest method for limiting recurrence is use of the "six month rule"(that is requiring patients to be abstinent for six months before listing for transplantation). This rule is standard in the UK and many European transplant centres.17 The stated purpose of this rule is several fold. First, abstinence is said to be associated with decreased recidivism post-transplant. However, the evidence for this is limited. Bird et al examined the outcome of transplantation in 24 patients with ALD.20 All three patients who were drinking heavily before the transplant had laboratory evidence of recurrent alcohol abuse after transplant (although this was denied by all three patients, highlighting the difficulty in obtaining reliable estimates of recidivism). In comparison, only one of 21 long term abstinent patients returned to alcohol use. Kumar et al reported recidivism (identified though a telephone survey) among three of seven patients (43%) abstinent for less than six months compared to three of 45 patients (7%) abstinent for longer.21 In contrast, Pereira et al did not find any relationship between length of abstinence and rates of recidivism.22 The evidence that the six month rule promotes post-transplant abstinence is therefore slight. 23

The second reason for advising at least six months of abstinence is to improve the immediate postoperative outcome. Evidence to support this is again limited. Only one study has reported reduced post-transplant survival in persistent drinkers (one year survival 68% v 85%).24 Furthermore, although alcoholic hepatitis at the time of transplantation is said to carry an extremely poor prognosis, the only study to examine the outcome of patients with alcoholic hepatitis on explant histology reported similar survival to patients with "pure" cirrhosis.25

The final (and probably most persuasive) argument for continuing to apply the six month rule is to give patients a chance to recover spontaneously and avoid transplantation. Many patients present to liver units with very advanced liver disease (as defined by either the Child-Pugh26 or Maddrey27 score) but will improve substantially with prolonged abstinence. The five year survival of patient with severe alcoholic cirrhosis (that is Child-Pugh stage C) with continued abstinence is over 50%,28 which compares favourably with liver transplantation. Transplantation can then be considered for patients whose synthetic function has not improved after abstinence. Unfortunately, this approach does not guide the management of patients who continue to deteriorate to a life threatening degree within six months of ceasing to drink or who have a very occasional "slip" in this period.

Predicting which patients will return to excessive drinking is difficult. Recurrence is more likely where patients are truly alcohol dependent (as defined by DSM 4 or International Classification of Diseases, 10th revision), or have coexisting substance misuse,29have had multiple previous failures at abstinence,30 have major psychiatric disorders (including depression),31,32 or post-traumatic stress disorder.33 Finally lack of social support is associated with increased relapse.34

No single approach has been shown to prevent relapses. Both Weinrieb23 and Beresford29 suggest that they can be minimised by continued supportive counselling and good relationships between staff and patients, supplemented by careful patient selection and treatment of associated psychiatric conditions.

In summary, ALD carries a similar prognosis to other liver diseases. Although the rate of recidivism is around 30%, the risk of recurrent liver disease is probably much lower. The six month rule may be justified by allowing patients a chance to improve without transplantation, but evidence that it improves transplant outcome (whether medically or by minimising recidivism) is questionable.

TRANSPLANTATION AND RECURRENT HEPATITIS C

There are no overall population studies of the prevalence of hepatitis C in the UK. It affects 0.6% of blood donors35 and has been estimated to affect around 1% of the population. Infection frequency is much higher in other countries such as Italy (up to 3%)36and Egypt (up to 40%).37 Altogether 75% of patients exposed to hepatitis C fail to clear the virus spontaneously and become chronically infected.

After 20 years of chronic infection, approximately 20% of patients develop cirrhosis, whereafter there is a 3% annual risk of hepatocellular carcinoma. Progression to cirrhosis is commoner with increasing length of infection, male sex, and alcohol use over 70 g (seven units) per week.38 Although testing for hepatitis C has been possible for approximately 10 years, highly effective therapy with interferon and ribavirin has only been available for two years and interferon monotherapy (which is approximately half as effective) was only recommended in the UK in 2000.39 Although in the future combination antiviral therapy may substantially reduce the number of patients with hepatitis C related cirrhosis and hepatocellular carcinoma, this is currently the commonest indication for liver transplantation in Europe and the USA.40

Hepatitis C imposes a large burden both on transplant services and hepatology services generally. As a proportion of patients cannot tolerate interferon related side effects and therapy is less effective in cirrhotic patients,41,42 the majority of patients remain RNA positive (that is have active viral replication) at the time of transplantation. Although recurrence of peripheral viraemia and liver infection is virtually universal in RNA positive patients after transplantation (presumably from virus replicating in lymphocytes),43,44 five year survival is similar to other accepted indications for transplantation. Hepatitis C is a well accepted indication for primary transplantation.

Recurrent hepatitis C may be defined as continued RNA positivity postoperatively together with histological changes consistent with viral liver damage. This affects up to 50% of grafts two years after transplantation.45,46 Although rapidly progressive cholestatic hepatitis is described,47 the majority of patients with histologically recurrent disease develop a slowly progressive chronic hepatitis. This follows an indolent course with only 6% of grafts being lost to recurrent disease after five years.48 Progression of graft hepatitis appears to be worse with certain hepatitis C genotypes (particularly 1a49 and 1b48), increased immunosuppression (especially monoclonal antilymphocyte preparations and steroids),50,51 and possibly certain donor HLA types.52 Of these only the immunosuppression regimen is amenable to clinical variation. Our unit currently treats hepatitis C transplant patients with tacrolimus monotherapy. Corticosteroids are used initially and rapidly weaned over one month. The choice of calcineurin inhibitor (for example cyclosporin or tacrolimus) does not appear to affect recurrence rates.51,53

Antiviral therapy after transplantation should theoretically reduce recurrent hepatitis. However, the immunostimulatory effects of interferon might also increase rejection episodes. Although there have been no controlled trials of interferon monotherapy, small series have not shown it to be beneficial. For example, Feray et al reported their experience with 14 patients.54 Four patients showed biochemical improvement but none achieved viral clearance and five suffered rejection. A preliminary report of a randomised controlled trial of interferon and ribavirin combination therapy suggests more success (21% long term viral response with no increase in rejection). However, 41% of patients in this trial could not tolerate active treatment.55 The new immunosuppressant mycophenylate mofetil prevents rejection through an azathioprine- like mechanism. However this agent can also inhibit inosine monphosphate dehydrogenase (the likely mechanism for ribavirin's effect). Preliminary reports suggest that patients treated with mycophenylate mofetil have lower hepatitis C virus RNA levels after transplantation although whether this translates into reduced histological recurrence is unclear.56

Although graft loss due to recurrent hepatitis only affects a small proportion of patients, the sheer number of transplants for hepatitis C means that absolute number of organs being lost is substantial. Retransplantation has a worse prognosis than initial transplantation and the rate and severity of recurrence of hepatitis in the second graft mirrors that in the first.51 The three year survival after retransplantation is only 54%.51,57 In the UK and the USA the feasibility of transplantation for recurrent hepatitis is seriously questioned given the donor: recipient mismatch.

HEPATOCELLULAR CARCINOMA AND LIVER TRANSPLANTATION

There are many theoretical attractions for treating hepatocellular carcinoma with liver transplantation. These include “complete removal” of malignant tissue, “cure” of the underlying liver disease (most hepatocellular carcinomas occur in cirrhotic livers), and removal of a diseased liver which has undergone “field change” predisposing it to further metachronous tumours. However the observed survival does not match these expectations due to

tumour progression while waiting for transplantation,58 intraoperative micrometastasis,59 60 and drug induced postoperative impairment of recipient tumour immunosurveillance. Three year survival rates after transplantation for hepatocellular carcinoma range from 18% to 69%,61–64 and are the lowest of any indication for primary transplantation. Given this, there is increasing debate, particularly given current organ shortages whether patients with hepatocellular carcinoma should be treated with surgical resection or liver transplantation.

The "evidence base" gives little guidance to the optimum therapy. There are no randomised trials comparing transplantation and resection. Of six large series,61–66 four have favoured transplantation and two resection. Interpretation of these series is hindered by selection bias between the therapy arms. Furthermore all six series excluded patients who were listed for transplantation but removed from waiting lists because of tumour progression. These "drop outs" have a poorer prognosis (two year survival 54%) and account for up to 22% of referrals. Survival figures corrected for this (that is intention to treat survival) show little advantage of transplantation over resection.58

As expected, transplantation works best in patients with small locally non-advanced tumours. However these are also exactly the patients who do best with resection or other locally curative therapy such as radiofrequency ablation. Current UK Transplant Special Support Authority guidelines suggest that transplantation should only be considered in patients with a 50% five year survival postoperatively. In practice this means that transplant may be considered for patients with three or less nodules of tumour all of which are less than 3 cm in diameter, or a single nodule less than 5 cm in diameter with no evidence of extrahepatic spread. The individual risks of resection versus transplantation may then be considered in patients fulfilling these criteria. Transplantation may be preferred in patients who are likely to do poorly with resection (due to advanced cirrhosis or portal hypertension58) or patients likely to have premalignant "field change" in the remaining liver segment (for example patients with viral cirrhosis or haemachromatosis). Conversely resection may offer a better chance of long term survival in patients with mild cirrhosis.

Preoperative assessment is vital when considering surgical options. Our unit currently assesses all patients with serum alpha fetoprotein, computed tomography of chest and abdomen, magnetic resonance imaging and angiography of abdomen, lipiodol angiography (combined with local chemotherapy (see below)), and laparoscopy.

Transcutaneous liver biopsy should not be used in the assessment of patients with hepatocellular carcinoma as this may disseminate localised potentially "curable" disease. Tumour seeding on the track of the biopsy has recently been reported in up to 5% of patients60 and tumour RNA may be detected peripherally in all biopsied patients.59 If the nature of a nodule is uncertain we currently perform biopsy at the time of assessment laparoscopy.

Resection rather than transplantation is generally the best option for the small group of patients with hepatocellular carcinoma developing in non-cirrhotic livers. A recent systematic review of 77 cases estimated that three and five year survival after transplant were just 29.8% and 11.2%.67

Fibrolamellar cancer is a rare primary liver tumour. It tends to affect younger patients than hepatocellular carcinoma and to develop de novo in non-cirrhotic livers. Furthermore it is slow growing and patients may have reasonable long term survival even following recurrence. The largest series of patients with fibrolamellar carcinoma reported 70% 10 year survival with resection and 28% with transplantation.68 Although better survival after transplantation has been reported in other series (for example 55% five year survival69), resection probably remains the most effective treatment for fibrolamellar tumours.

Chemotherapy may improve survival and minimise tumour progression in patients with hepatocellular carcinoma waiting for transplantation. Although, again there are no randomised controlled trials of this therapy, comparison to historical controls does seem to suggest a benefit. This seems particularly effective when given locally by transarterial chemoembolisation where five year survival rates of up to 79% have been reported.68–70 The roles of intraoperative and postoperative chemotherapy are less clear.

As waiting times substantially affect the success of transplantation,58 hepatocellular carcinoma may be a suitable indication for living related resection and donation. However, as above, this technique is not widely available in the UK and donors are only available for 15% of patients.71

REFERRAL FOR TRANSPLANTATION

The American Medical Association Committee on Ethical Issues has outlined acceptable and unacceptable criteria for selection for liver transplantation (see box 1).72 Neuberger and James, in association with a large committee of stakeholders (including lay members and patient representatives), supplemented these criteria in the UK.73 Neuberger and James outlined four fundamental concepts based "on the fact that the liver resource is limited rather than cost benefit [considerations]". First, local and national guidelines for transplantation should be agreed by all stakeholders including patient representatives. Second, patients should primarily be selected on the basis of poor quality of life and/or anticipated very limited life expectancy without transplantation (usually less than one year), thus restricting transplantation to the severest cases of liver disease. Third, patients should not be offered transplantation if there is a less than 50% expected survival five years after surgery, thus limiting transplant to patients with maximum potential for benefit. Finally, livers should be allocated to maximise outcome "in preference to allowing every possible recipient to have a chance of an organ". These concepts emphasise the importance of choosing patients to maximise benefit rather than merely on severity of patient illness.

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