German Air Force Centre of Aerospace Medicine

Molecular pathology
in aircraft accident investigations

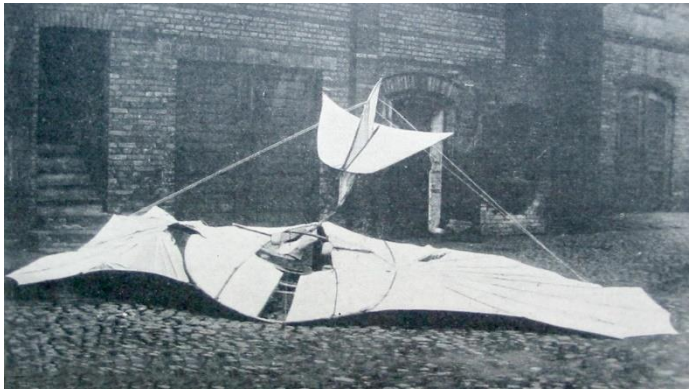
Dr. Michael J. Schwerer, Lt. Col.
Anatomic Pathologist, Forensic Pathologist,
Aeromedical Examiner (class 1, military),
Branch I 4 – Aircraft Accident Investigation,
Fuerstenfeldbruck, Germany

In co-operation with:
Institute of Forensic Medicine
Ludwig-Maximilians-University,
Munich, Germany
Head: Prof. Dr. M. Graw

Disclosure statement

- I have no financial relationships to disclose.
- I will not discuss any off-label use and/or investigational use in my presentation.

Background



Otto Lilienthal
† 1896

Actual Airliner Crash -
No fatalities

Background

International Civil Aviation Organization

Founded on
7th December 1944

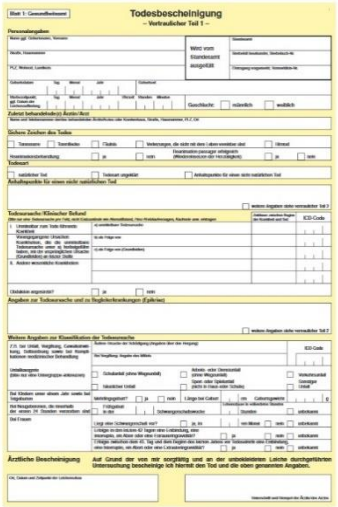
ICAO Annex 13:
Aircraft Accident and Incident Investigation

Background

The ICAO Annex 13 „Aircraft accident and incident investigation“ requires the assessment of aircraft mishaps to increase flight safety and to prevent future accidents.

- Included in European law: VOEU996/2010.
- Included in German national law: Flugunfalluntersuchungsgesetz; FlUUG 1998.

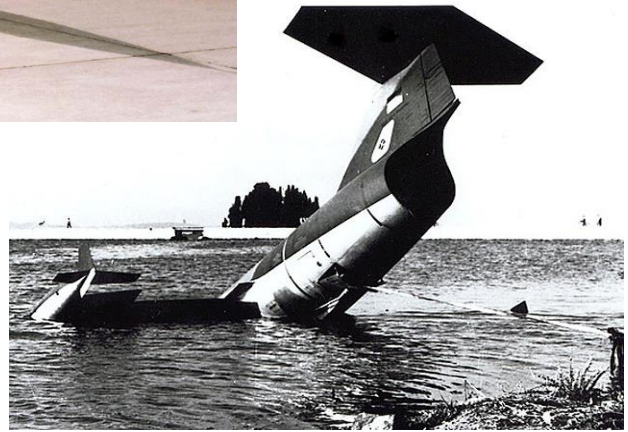
Technical investigation carried out by the „Bundesstelle für Flugunfalluntersuchung“ as an independent federal agency.


Todesbescheinigung
– Vertraulicher Teil 1 –

Medical investigation carried out in institutes for forensic pathology by order of public prosecutors.

Background

German Air Force
F104G
„Starfighter“



- 900 aircraft ordered
- 292 aircraft accidents
- 116 pilots killed

Aircraft accident investigation

Luftwaffe
Wir. Dienen. Deutschland.

Forensic medical examination includes:

- Crash site investigation
- External inspection and forensic autopsy
- Identification using DNA-analysis
- Toxicology to confirm or exclude illicit substances

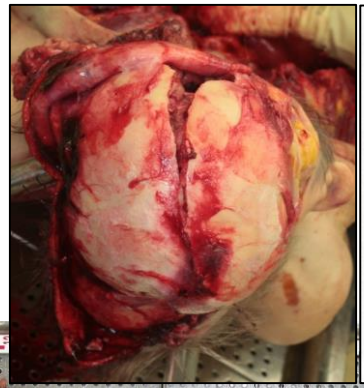
The basic questions in the reconstruction of the mishap are:

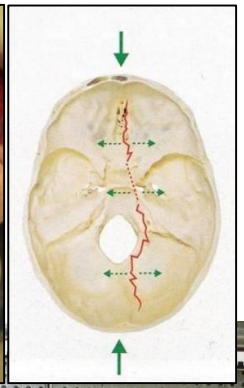
- Was the pilot alive at the moment of loss of control?
- Occured a sudden incapacitation of the pilot?
- Did any kind of pre-existing disease cause the loss of control?

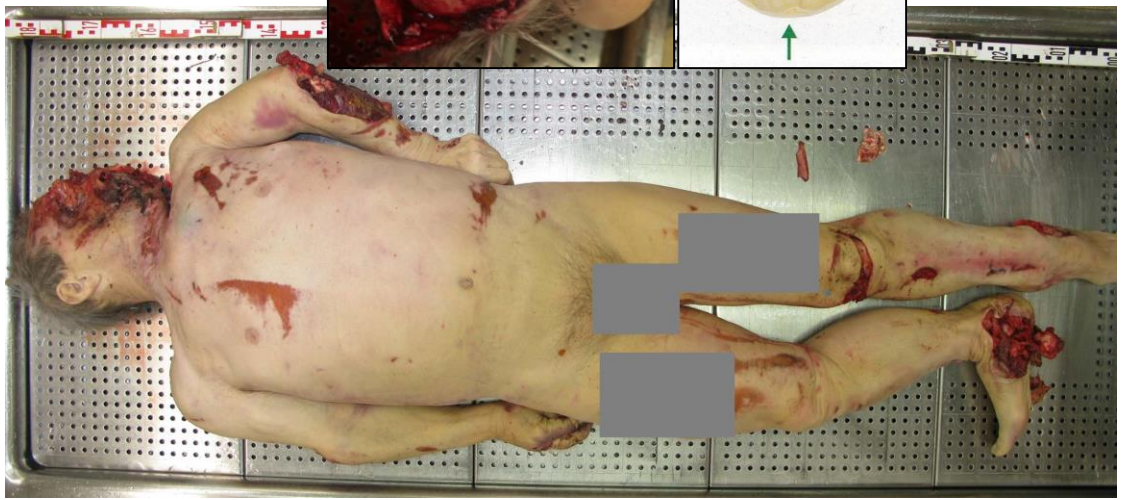
(Forensic) Pathology in mishap investigation

Luftwaffe
Wir. Dienen. Deutschland.

These questions remain unanswered in autopsy alone in the majority of cases.







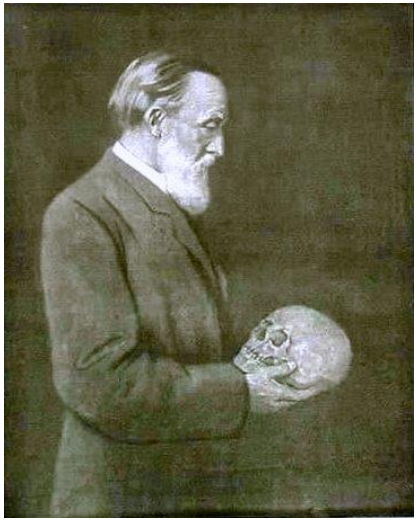
The basic questions in the reconstruction of the mishap are:

- Was the pilot alive at the moment of loss of control?
- Occured a sudden incapacitation of the pilot?
- Did any kind of pre-existing disease cause the loss of control?

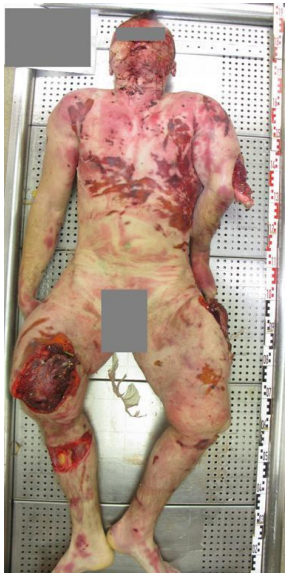
(Forensic) Pathology in mishap investigation

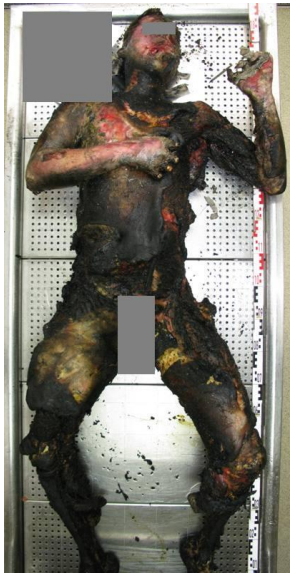
Luftwaffe
Wir. Dienen. Deutschland.

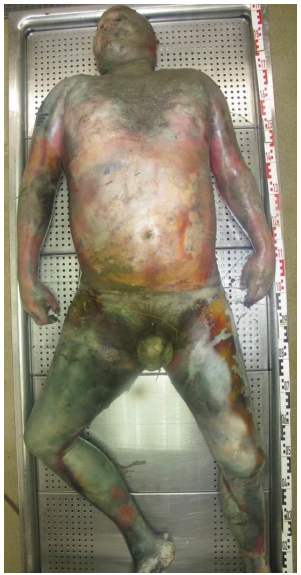
Additional techniques usually involve microscopy/histology.



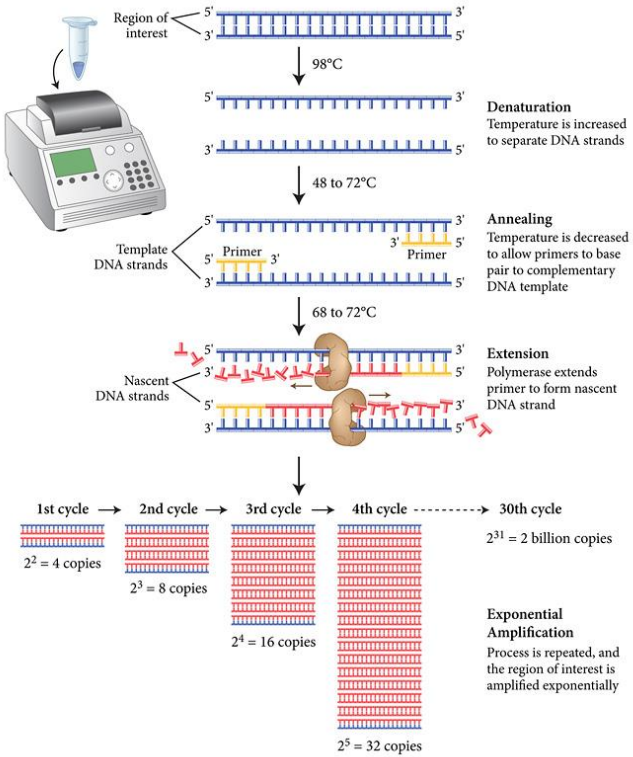
However, these methods require sufficient qualitiy of available tissues.

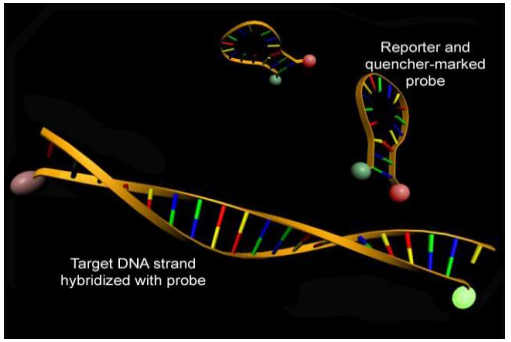




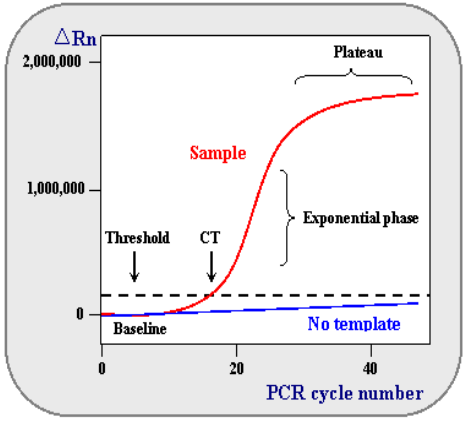


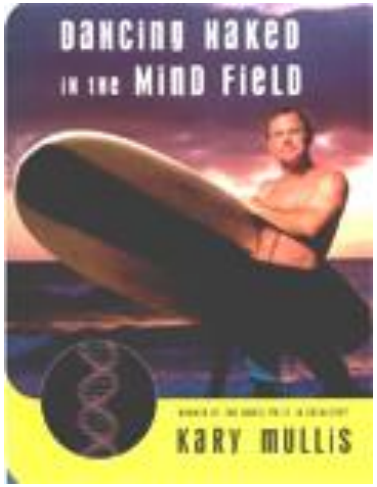
(Molecular) Pathology in mishap investigation



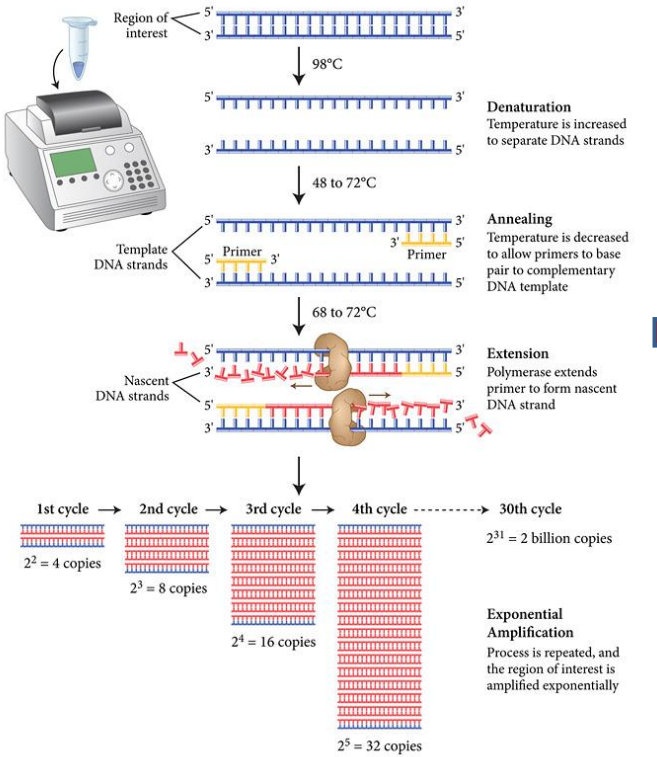


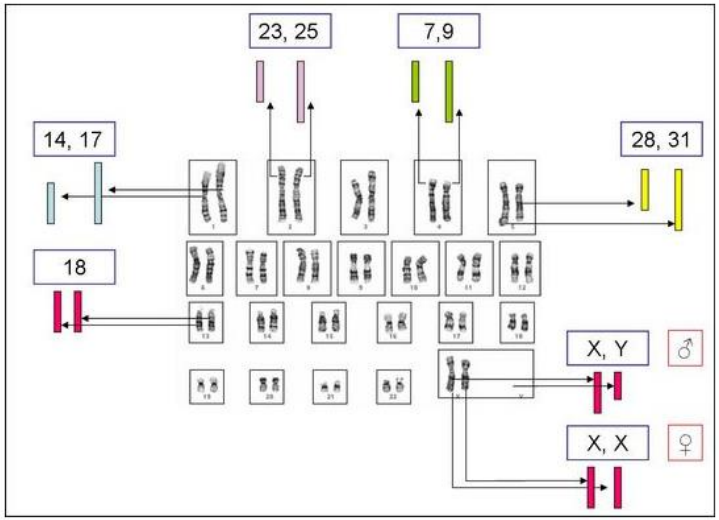
Model of real time quantitative PCR plot

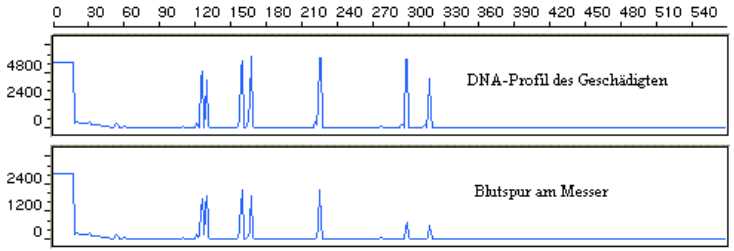




(Molecular) Pathology in mishap investigation

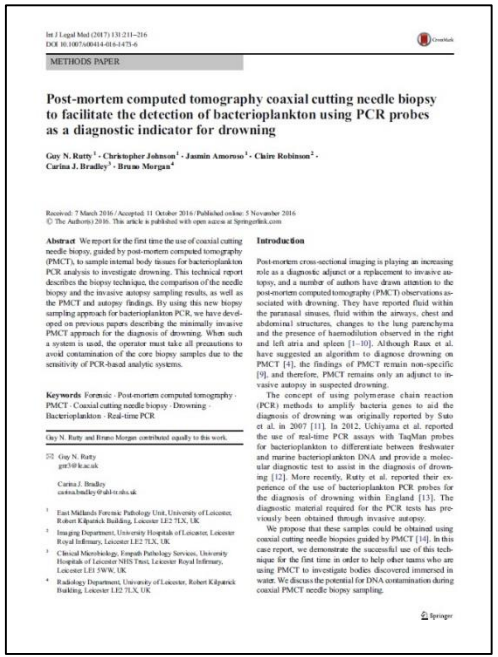






Aims and methods

A literature review was carried out to look for new techniques in clinical pathology, which can also be used in the investigation of aircraft accidents.



Int J Legal Med (2017) 131:211–216
DOI 10.1007/s00414-016-1473-6

METHODS PAPER

Post-mortem computed tomography coaxial cutting needle biopsy to facilitate the detection of bacterioplankton using PCR probes as a diagnostic indicator for drowning

Guy N. Rutty[1] · Christopher Johnson[1] · Jasmin Amoroso[1] · Claire Robinson[2] · Carina J. Bradley[3] · Bruno Morgan[4]

Received: 7 March 2016 / Accepted: 11 October 2016 / Published online: 5 November 2016
© The Author(s) 2016. This article is published with open access at Springerlink.com

Abstract We report for the first time the use of coaxial cutting needle biopsy, guided by post-mortem computed tomography (PMCT), to sample internal body tissues for bacterioplankton PCR analysis to investigate drowning. This technical report describes the biopsy technique, the comparison of the needle biopsy and the invasive autopsy sampling results, as well as the PMCT and autopsy findings. By using this new biopsy sampling approach for bacterioplankton PCR, we have developed on previous papers describing the minimally invasive PMCT approach for the diagnosis of drowning. When such a system is used, the operator must take all precautions to avoid contamination of the core biopsy samples due to the sensitivity of PCR-based analytic systems.

Keywords Forensic · Post-mortem computed tomography · PMCT · Coaxial cutting needle biopsy · Drowning · Bacterioplankton · Real-time PCR

Guy N. Rutty and Bruno Morgan contributed equally to this work.

✉ Guy N. Rutty
gnr3@le.ac.uk

Carina J. Bradley
carina.bradley@phls.nhs.uk

[1] East Midlands Forensic Pathology Unit, University of Leicester, Robert Kilpatrick Building, Leicester LE2 7LX, UK
[2] Imaging Department, University Hospitals of Leicester, Leicester Royal Infirmary, Leicester LE2 7LX, UK
[3] Clinical Microbiology, Empath Pathology Services, University Hospitals of Leicester NHS Trust, Leicester Royal Infirmary, Leicester LE1 5WW, UK
[4] Radiology Department, University of Leicester, Robert Kilpatrick Building, Leicester LE2 7LX, UK

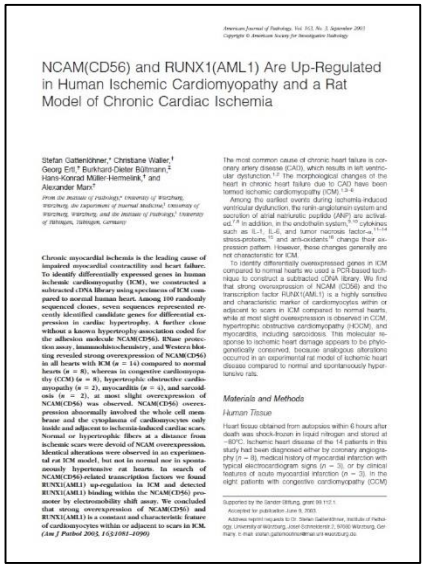
Introduction

Post-mortem cross-sectional imaging is playing an increasing role as a diagnostic adjunct or a replacement to invasive autopsy, and a number of authors have drawn attention to the post-mortem computed tomography (PMCT) observations associated with drowning. They have reported fluid within the paranasal sinuses, fluid within the airways, chest and abdominal structures, changes to the lung parenchyma and the presence of haemodilution observed in the right and left atria and spleen [1–10]. Although Raux et al. have suggested an algorithm to diagnose drowning on PMCT [4], the findings of PMCT remain non-specific [9], and therefore, PMCT remains only an adjunct to invasive autopsy in suspected drowning.

The concept of using polymerase chain reaction (PCR) methods to amplify bacteria genes to aid the diagnosis of drowning was originally reported by Suto et al. in 2007 [11]. In 2012, Uchiyama et al. reported the use of real-time PCR assays with TaqMan probes for bacterioplankton to differentiate between freshwater and marine bacterioplankton DNA and provide a molecular diagnostic test to assist in the diagnosis of drowning [12]. More recently, Rutty et al. reported their experience of the use of bacterioplankton PCR probes for the diagnosis of drowning within England [13]. The diagnostic material required for the PCR tests has previously been obtained through invasive autopsy.

We propose that these samples could be obtained using coaxial cutting needle biopsies guided by PMCT [14]. In this case report, we demonstrate the successful use of this technique for the first time in order to help other teams who are using PMCT to investigate bodies discovered immersed in water. We discuss the potential for DNA contamination during coaxial PMCT needle biopsy sampling.

Springer



American Journal of Pathology, Vol. 163, No. 3, September 2003
Copyright © American Society for Investigative Pathology

NCAM(CD56) and RUNX1(AML1) Are Up-Regulated in Human Ischemic Cardiomyopathy and a Rat Model of Chronic Cardiac Ischemia

Stefan Gattenlöhner,* Christiane Waller,† Georg Ertl,† Burkhard-Dieter Büttmann,‡ Hans-Konrad Müller-Hermelink,* and Alexander Marx*

From the Institute of Pathology, University of Würzburg, Würzburg, the Departments of Internal Medicine,† University of Würzburg, Würzburg, and the Institute of Pathology,‡ University of Tübingen, Tübingen, Germany*

Chronic myocardial ischemia is the leading cause of impaired myocardial contractility and heart failure. To identify differentially expressed genes in human ischemic cardiomyopathy (ICM), we constructed a subtracted cDNA library using specimens of ICM compared to normal human heart. Among 100 randomly sequenced clones, seven sequences represented recently identified candidate genes for differential expression in cardiac hypertrophy. A further clone without a known hypertrophy-association coded for the adhesion molecule NCAM(CD56). RNase protection assay, immunohistochemistry, and Western blotting revealed strong overexpression of NCAM(CD56) in all hearts with ICM ($n = 14$) compared to normal hearts ($n = 8$), whereas in congestive cardiomyopathy (CCM) ($n = 8$), hypertrophic obstructive cardiomyopathy ($n = 2$), myocarditis ($n = 4$), and sarcoidosis ($n = 2$), at most slight overexpression of NCAM(CD56) was observed. NCAM(CD56) overexpression abnormally involved the whole cell membrane and the cytoplasma of cardiomyocytes only inside and adjacent to ischemia-induced cardiac scars. Normal or hypertrophic fibers at a distance from ischemic scars were devoid of NCAM overexpression. Identical alterations were observed in an experimental rat ICM model, but not in normal nor in spontaneously hypertensive rat hearts. In search of NCAM(CD56)-related transcription factors we found RUNX1(AML1) up-regulation in ICM and detected RUNX1(AML1) binding within the NCAM(CD56) promoter by electromobility shift assay. We concluded that strong overexpression of NCAM(CD56) and RUNX1(AML1) is a constant and characteristic feature of cardiomyocytes within or adjacent to scars in ICM. (*Am J Pathol 2003, 163:1091–1098*)

The most common cause of chronic heart failure is coronary artery disease (CAD), which results in left ventricular dysfunction.[1,2] The morphological changes of the heart in chronic heart failure due to CAD have been termed ischemic cardiomyopathy (ICM).[1,2–4]

Among the earliest events during ischemia-induced ventricular dysfunction, the renin-angiotensin system and secretion of atrial natriuretic peptide (ANP) are activated.[2,5] In addition, in the endothelin system,[5–10] cytokines such as IL-1, IL-6, and tumor necrosis factor-α,[11–14] stress-proteins,[15] and anti-oxidants[16] change their expression pattern. However, these changes generally are not characteristic for ICM.

To identify differentially overexpressed genes in ICM compared to normal hearts we used a PCR-based technique to construct a subtracted cDNA library. We find that strong overexpression of NCAM (CD56) and the transcription factor RUNX1(AML1) is a highly sensitive and characteristic marker of cardiomyocytes within or adjacent to scars in ICM compared to normal hearts, while at most slight overexpression is observed in CCM, hypertrophic obstructive cardiomyopathy (HOCM), and myocarditis, including sarcoidosis. This molecular response to ischemic heart damage appears to be phylogenetically conserved, because analogous alterations occurred in an experimental rat model of ischemic heart disease compared to normal and spontaneously hypertensive rats.

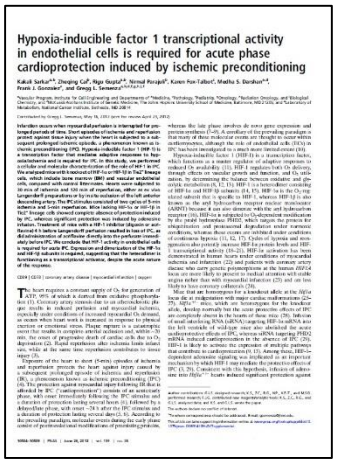
Materials and Methods

Human Tissue

Heart tissues obtained from autopsies within 6 hours after death was shock-frozen in liquid nitrogen and stored at −80°C. Ischemic heart disease of the 14 patients in this study had been diagnosed either by coronary angiography ($n = 9$), medical history of myocardial infarction with typical electrocardiogram signs ($n = 3$), or by clinical features of acute myocardial infarction ($n = 2$). In the slight patients with congestive cardiomyopathy (CCM)

Supported by the Sander-Stiftung, grant 99.112.1.
Accepted for publication June 9, 2003.

Address reprint requests to Dr. Stefan Gattenlöhner, Institute of Pathology, University of Würzburg, Josef-Schneider-Str. 2, 97080 Würzburg, Germany. E-mail: stefan.gattenloehner@mail.uni-wuerzburg.de.



Hypoxia-inducible factor 1 transcriptional activity in endothelial cells is required for acute phase cardioprotection induced by ischemic preconditioning

Kaluk Sarkar*†, Zhenjing Cai*, Raja Gupta*†, Nirmal Parajuli*, Karen Fox-Talbot*, Mathia S. Dardzhan*†, Pramila J. Gonsalves*, and Gregg L. Semenza*†‡§¶‖#

*Vascular Program, Institute for Cell Engineering and Departments of †Medicine, ‡Pathology, §Pediatrics, ¶Oncology, ‖Radiation Oncology, and #Biological Chemistry, and **Molecular Sciences Institute of General Medicine, The Johns Hopkins University School of Medicine, Baltimore, MD 21205; and ††Laboratory of Metabolism, National Cancer Institute, Bethesda, MD 20814*

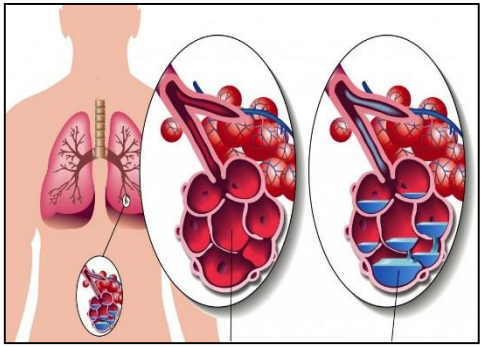
Contributed by Gregg L. Semenza, May 18, 2011 (sent for review April 25, 2011)

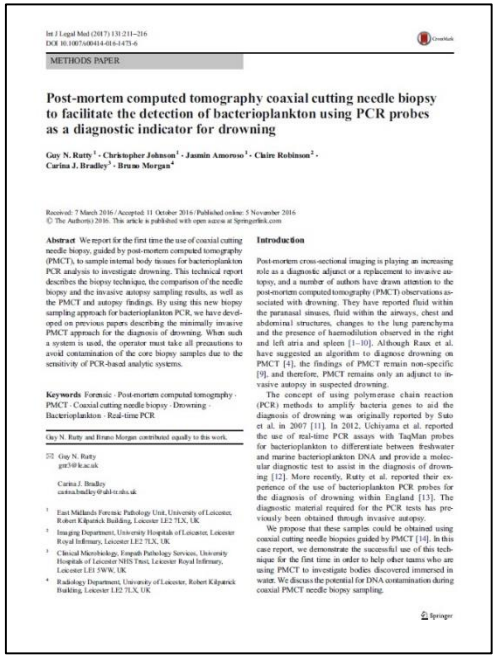
Own results from the implementation of these new techniques in forensic pathology practice are demonstrated.

Diagnosis of pathogens

Luftwaffe
Wir. Dienen. Deutschland.

Demonstration of the presence of *Aeromonas species* in skin wounds in clinical pathology and in veterinary medicine.





Int J Legal Med (2017) 131:211–216
DOI 10.1007/s00414-016-1473-6

METHODS PAPER

Post-mortem computed tomography coaxial cutting needle biopsy to facilitate the detection of bacterioplankton using PCR probes as a diagnostic indicator for drowning

Guy N. Rutty[1] · Christopher Johnson[1] · Jasmin Amoroso[1] · Claire Robinson[2] · Carina J. Bradley[3] · Bruno Morgan[4]

Received: 7 March 2016 / Accepted: 11 October 2016 / Published online: 5 November 2016

Abstract We report for the first time the use of coaxial cutting needle biopsy, guided by post-mortem computed tomography (PMCT), to sample internal body tissues for bacterioplankton PCR analysis to investigate drowning. This technical report describes the biopsy technique, the comparison of the needle biopsy and the invasive autopsy sampling results, as well as the PMCT and autopsy findings. By using this new biopsy sampling approach for bacterioplankton PCR, we have developed on previous papers describing the minimally invasive PMCT approach for the diagnosis of drowning. When such a system is used, the operator must take all precautions to avoid contamination of the core biopsy samples due to the sensitivity of PCR-based analytic systems.

Keywords Forensic · Post-mortem computed tomography · PMCT · Coaxial cutting needle biopsy · Drowning · Bacterioplankton · Real-time PCR

Guy N. Rutty and Bruno Morgan contributed equally to this work.

✉ Guy N. Rutty
gnr3@le.ac.uk

Carina J. Bradley
carina.bradley@uhl-tr.nhs.uk

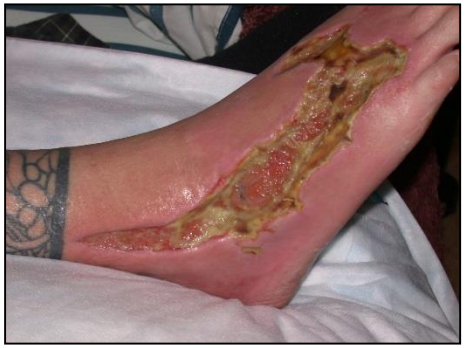
[1] East Midlands Forensic Pathology Unit, University of Leicester, Robert Kilpatrick Building, Leicester LE2 7LX, UK
[2] Imaging Department, University Hospitals of Leicester, Leicester Royal Infirmary, Leicester LE2 7LX, UK
[3] Clinical Microbiology, Empath Pathology Services, University Hospitals of Leicester NHS Trust, Leicester Royal Infirmary, Leicester LE1 5WW, UK
[4] Radiology Department, University of Leicester, Robert Kilpatrick Building, Leicester LE2 7LX, UK

Introduction

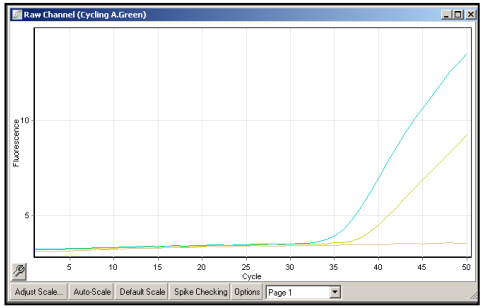
Post-mortem cross-sectional imaging is playing an increasing role as a diagnostic adjunct or a replacement to invasive autopsy, and a number of authors have drawn attention to the post-mortem computed tomography (PMCT) observations associated with drowning. They have reported fluid within the paranasal sinuses, fluid within the airways, chest and abdominal structures, changes to the lung parenchyma and the presence of haemodilution observed in the right and left atria and spleen [1–10]. Although Raux et al. have suggested an algorithm to diagnose drowning on PMCT [4], the findings of PMCT remain non-specific [9], and therefore, PMCT remains only an adjunct to invasive autopsy in suspected drowning.

The concept of using polymerase chain reaction (PCR) methods to amplify bacteria genes to aid the diagnosis of drowning was originally reported by Sato et al. in 2007 [11]. In 2012, Uchiyama et al. reported the use of real-time PCR assays with TaqMan probes for bacterioplankton to differentiate between freshwater and marine bacterioplankton DNA and provide a molecular diagnostic test to assist in the diagnosis of drowning [12]. More recently, Rutty et al. reported their experience of the use of bacterioplankton PCR probes for the diagnosis of drowning within England [13]. The diagnostic material required for the PCR tests has previously been obtained through invasive autopsy.

We propose that these samples could be obtained using coaxial cutting needle biopsies guided by PMCT [14]. In this case report, we demonstrate the successful use of this technique for the first time in order to help other teams who are using PMCT to investigate bodies discovered immersed in water. We discuss the potential for DNA contamination during coaxial PMCT needle biopsy sampling.

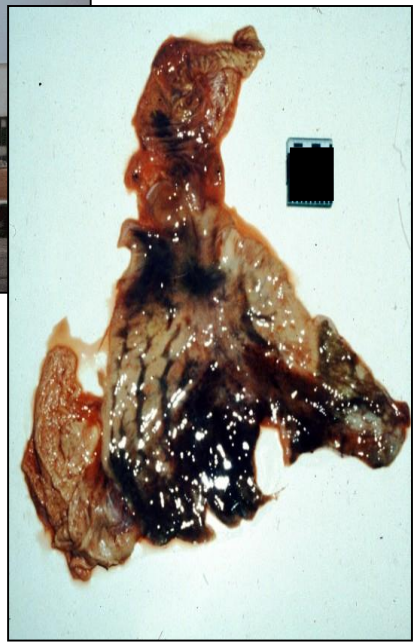


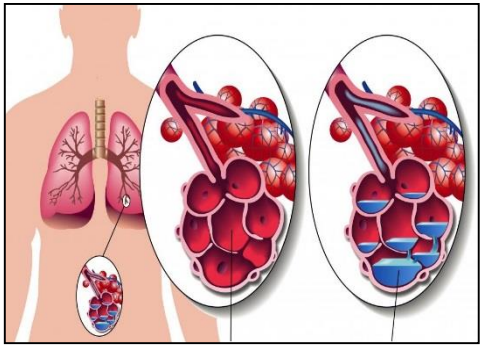
Identification of the same bacteria in the peripheral blood/ deep muscles of the lower limbs is diagnostic for drowning.



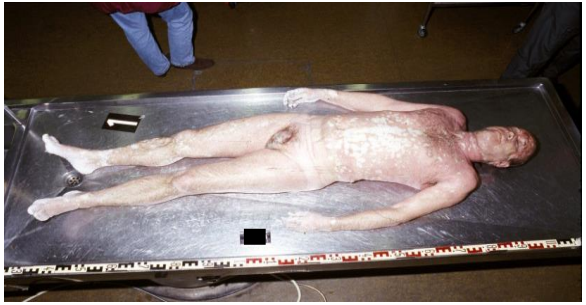
Diagnosis of pathogens

„Cold case“ – Aircraft accident in the Lake Constance with 7 persons killed in 1994.

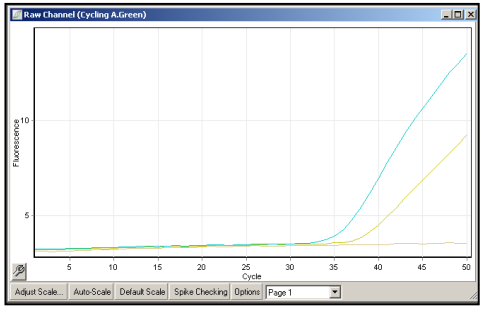




Identification of the same bacteria in the peripheral blood/ deep muscles of the lower limbs is diagnostic for drowning.



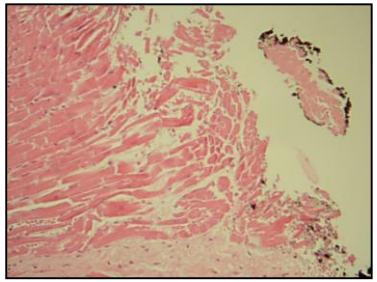
No macroscopic or histologic signs of drowning.



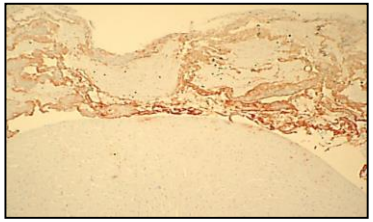
Diagnosis of manifest heart disease

NCAM(CD56) and RUNX1(AML1) Are Up-Regulated in Human Ischemic Cardiomyopathy and a Rat Model of Chronic Cardiac Ischemia

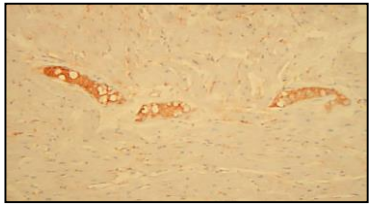
Stefan Gattenlöhner,* Christiane Waller,† Georg Ertl,† Burkhard-Dieter Büttmann,‡ Hans-Konrad Müller-Hermelink,† and Alexander Marx†



Hematoxylin-eosin - staining

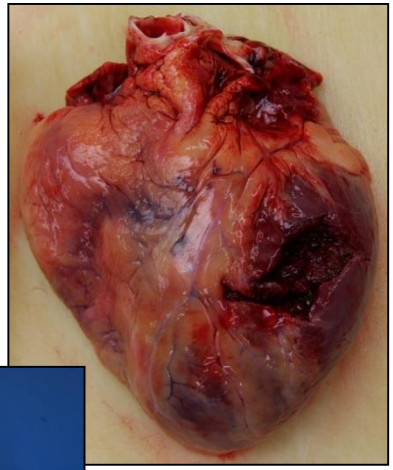


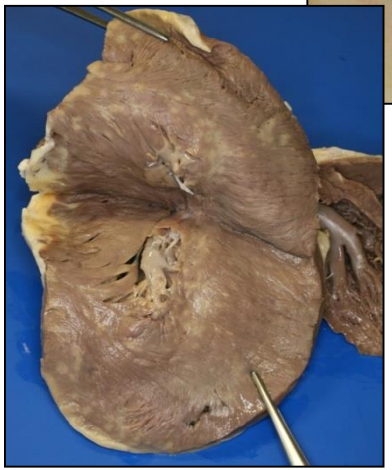
Fibronectin - IHC



C_{5b-9} - IHC

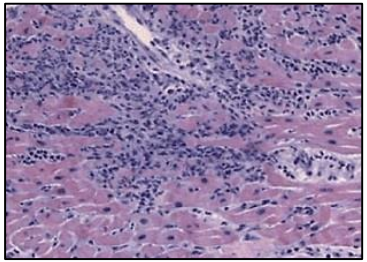
Hypertrophic heart with penetrating injury from rib fracture.

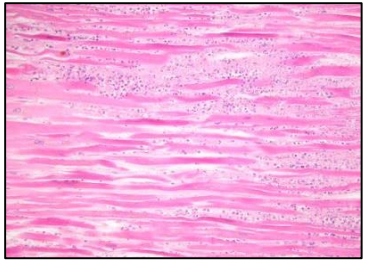


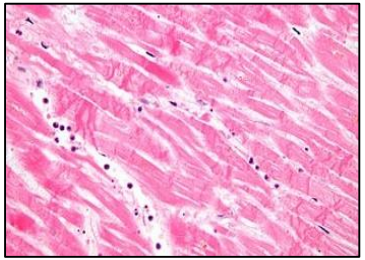


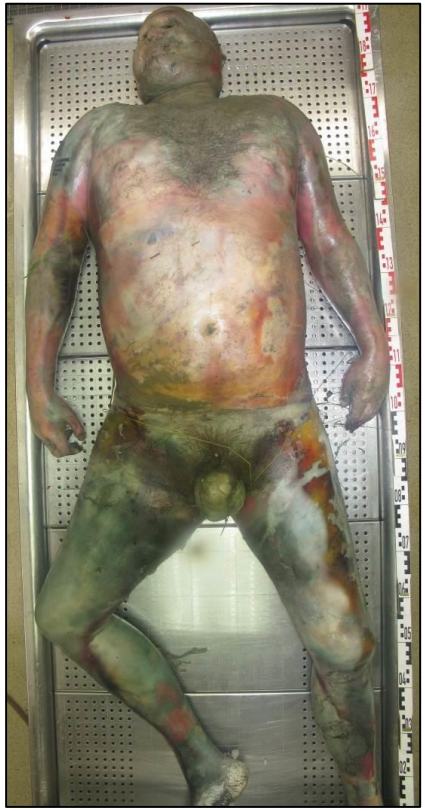
White scar tissue from infarction (formalin-fixed organ).

Diagnosis of latent heart disease

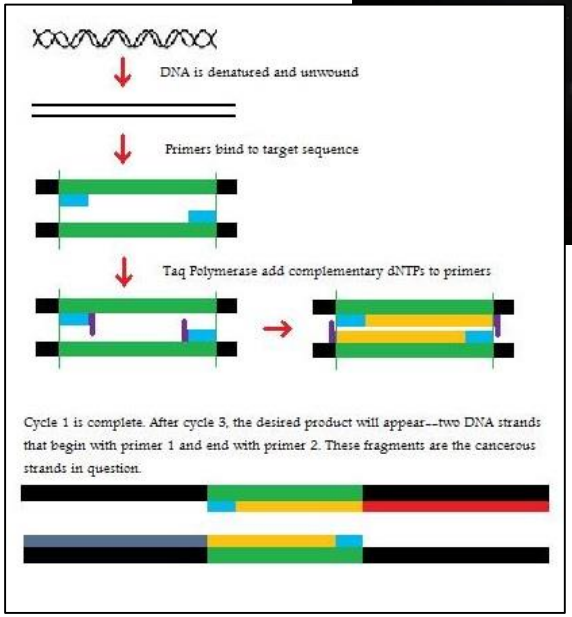








DNA degrades into short fragments.

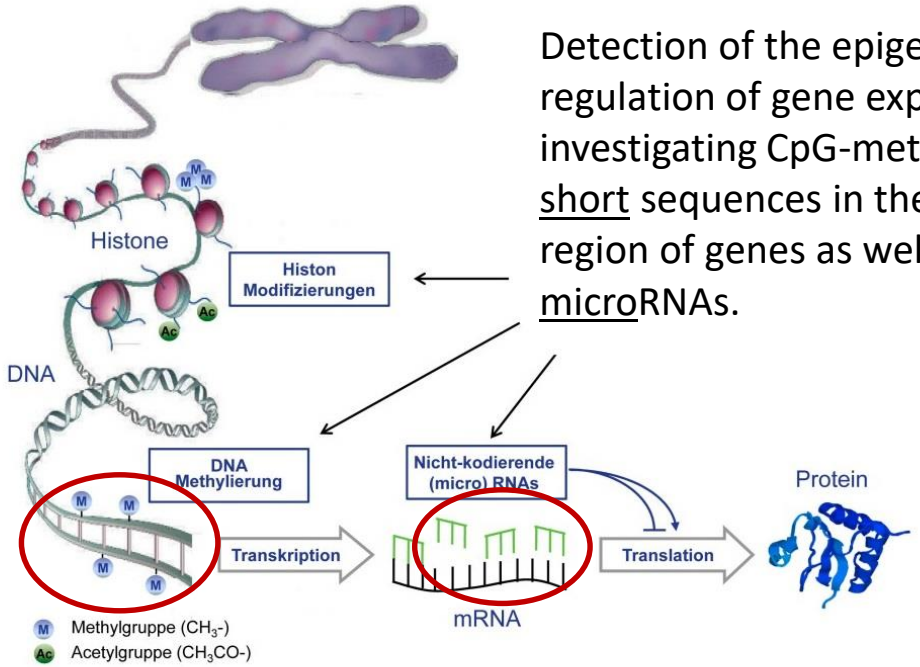


When the fragments are too short, primer annealing to the target sequence and strand elongation is impossible.

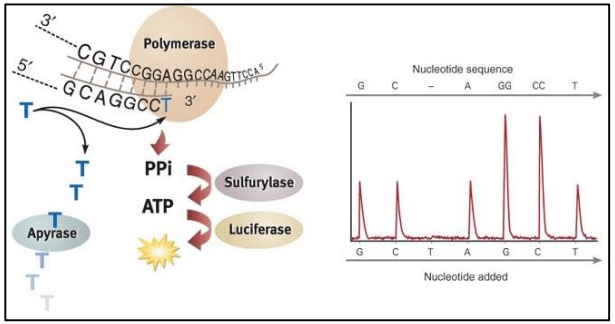
Diagnosis of latent heart disease

Luftwaffe
Wir. Dienen. Deutschland.

One solution investigating material with degraded DNA:



Detection of the epigenetic regulation of gene expression investigating CpG-methylation in short sequences in the promotor region of genes as well as short microRNAs.

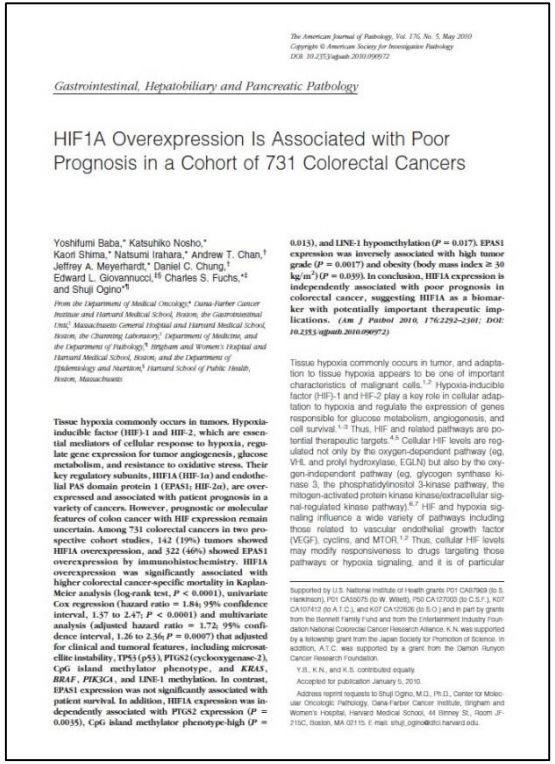


Methylation analysis using pyrosequencing.

MicroRNA analysis employing SybrGreen-based real-time PCR.

Diagnosis of latent Heart Disease

Luftwaffe
Wir. Dienen. Deutschland.



The American Journal of Pathology, Vol. 176, No. 5, May 2010
Copyright © American Society for Investigative Pathology
DOI: 10.2353/ajpath.2010.090972

Gastrointestinal, Hepatobiliary and Pancreatic Pathology

HIF1A Overexpression Is Associated with Poor Prognosis in a Cohort of 731 Colorectal Cancers

Yoshifumi Baba,* Katsuhiko Nosho,* Kaori Shima,* Natsumi Irahara,* Andrew T. Chan,† Jeffrey A. Meyerhardt,* Daniel C. Chung,† Edward L. Giovannucci,‡§ Charles S. Fuchs,*‖ and Shuji Ogino*¶

From the Department of Medical Oncology, Dana-Farber Cancer Institute and Harvard Medical School, Boston, the Gastrointestinal Unit,† Massachusetts General Hospital and Harvard Medical School, Boston, the Channing Laboratory,‡ Department of Medicine, and the Department of Pathology,¶ Brigham and Women's Hospital and Harvard Medical School, Boston, and the Departments of Epidemiology and Nutrition,§ Harvard School of Public Health, Boston, Massachusetts*

Tissue hypoxia commonly occurs in tumors. Hypoxia-inducible factor (HIF)-1 and HIF-2, which are essential mediators of cellular response to hypoxia, regulate gene expression for tumor angiogenesis, glucose metabolism, and resistance to oxidative stress. Their key regulatory subunits, HIF1A (HIF-1α) and endothelial PAS domain protein 1 (EPAS1; HIF-2α), are overexpressed and associated with patient prognosis in a variety of cancers. However, prognostic or molecular features of colon cancer with HIF expression remain uncertain. Among 731 colorectal cancers in two prospective cohort studies, 142 (19%) tumors showed HIF1A overexpression, and 322 (46%) showed EPAS1 overexpression by immunohistochemistry. HIF1A overexpression was significantly associated with higher colorectal cancer-specific mortality in Kaplan-Meier analysis (log-rank test, $P < 0.0001$), univariate Cox regression (hazard ratio = 1.84; 95% confidence interval, 1.37 to 2.47; $P < 0.0001$) and multivariate analysis (adjusted hazard ratio = 1.72; 95% confidence interval, 1.26 to 2.36; $P = 0.0007$) that adjusted for clinical and tumoral features, including microsatellite instability, TP53 (p53), PTGS2 (cyclooxygenase-2), CpG island methylator phenotype, and *KRAS*, *BRAF*, *PIK3CA*, and LINE-1 methylation. In contrast, EPAS1 expression was not significantly associated with patient survival. In addition, HIF1A expression was independently associated with PTGS2 expression ($P = 0.0035$), CpG island methylator phenotype-high ($P = 0.015$), and LINE-1 hypomethylation ($P = 0.017$). EPAS1 expression was inversely associated with high tumor grade ($P = 0.0017$) and obesity (body mass index ≥ 30 kg/m²) ($P = 0.039$). In conclusion, HIF1A expression is independently associated with poor prognosis in colorectal cancer, suggesting HIF1A as a biomarker with potentially important therapeutic implications. (*Am J Pathol 2010, 176:2292–2301; DOI: 10.2353/ajpath.2010.090972*)

Tissue hypoxia commonly occurs in tumor, and adaptation to tissue hypoxia appears to be one of important characteristics of malignant cells.[1,2] Hypoxia-inducible factor (HIF)-1 and HIF-2 play a key role in cellular adaptation to hypoxia and regulate the expression of genes responsible for glucose metabolism, angiogenesis, and cell survival.[1–3] Thus, HIF and related pathways are potential therapeutic targets.[4,5] Cellular HIF levels are regulated not only by the oxygen-dependent pathway (eg, VHL and prolyl hydroxylase, EGLN) but also by the oxygen-independent pathway (eg, glycogen synthase kinase 3, the phosphatidylinositol 3-kinase pathway, the mitogen-activated protein kinase kinase/extracellular signal-regulated kinase pathway).[6,7] HIF and hypoxia signaling influence a wide variety of pathways including those related to vascular endothelial growth factor (VEGF), cyclins, and MTOR.[1,2] Thus, cellular HIF levels may modify responsiveness to drugs targeting those pathways or hypoxia signaling, and it is of particular

Supported by U.S. National Institute of Health grants P01 CA87969 (to S. Hankinson), P01 CA55075 (to W. Willett), P50 CA127003 (to C.S.F.), K07 CA107413 (to K.O.), and K07 CA72826 (to S.O.) and in part by grants from the Bennett Family Fund and from the Entertainment Industry Foundation National Colorectal Cancer Research Alliance. K.N. was supported by a fellowship grant from the Japan Society for Promotion of Science. In addition, A.T.C. was supported by a grant from the Damon Runyon Cancer Research Foundation.

Y.B., K.N., and K.S. contributed equally.

Accepted for publication January 5, 2010.

Address reprint requests to Shuji Ogino, M.D., Ph.D., Center for Molecular Oncologic Pathology, Dana-Farber Cancer Institute, Brigham and Women's Hospital, Harvard Medical School, 44 Binney St., Room JF-215C, Boston, MA 02115. E-mail: shuji_ogino@dfci.harvard.edu.

Hypoxia-inducible factor 1 transcriptional activity in endothelial cells is required for acute phase cardioprotection induced by ischemic preconditioning

Kakali Sarkar[a,b], Zheqing Cai[b], Rigu Gupta[a,b], Nirmal Parajuli[b], Karen Fox-Talbot[c], Madhu S. Darshan[a,d], Frank J. Gonzalez[e], and Gregg L. Semenza[a,b,c,d,f,g,1,2]

[a]Vascular Program, Institute for Cell Engineering and Departments of [b]Medicine, [c]Pathology, [d]Pediatrics, [e]Oncology, [f]Radiation Oncology, and [g]Biological Chemistry, and [h]Motzkin-Rothberg Institute of Genetic Medicine, The Johns Hopkins University School of Medicine, Baltimore, MD 21205; and [1]Laboratory of Metabolism, National Cancer Institute, Bethesda, MD 20814

Contributed by Gregg L. Semenza, May 18, 2012 (sent for review April 23, 2012)

Infarction occurs when myocardial perfusion is interrupted for prolonged periods of time. Short episodes of ischemia and reperfusion protect against tissue injury when the heart is subjected to a subsequent prolonged ischemic episode, a phenomenon known as ischemic preconditioning (IPC). Hypoxia-inducible factor 1 (HIF-1) is a transcription factor that mediates adaptive responses to hypoxia/ischemia and is required for IPC. In this study, we performed a cellular and molecular characterization of the role of HIF-1 in IPC. We analyzed mice with knockout of HIF-1α or HIF-1β in Tie2+ lineage cells, which include bone marrow (BM) and vascular endothelial cells, compared with control littermates. Hearts were subjected to 30 min of ischemia and 120 min of reperfusion, either ex vivo (Langendorff preparations) or by in situ occlusion of the left anterior descending artery. The IPC stimulus consisted of two cycles of 5-min ischemia and 5-min reperfusion. Mice lacking HIF-1α or HIF-1β in Tie2+ lineage cells showed complete absence of protection induced by IPC, whereas significant protection was induced by adenosine infusion. Treatment of mice with a HIF-1 inhibitor (digoxin or acriflavine) 4 h before Langendorff perfusion resulted in loss of IPC, as did administration of acriflavine directly into the perfusate immediately before IPC. We conclude that HIF-1 activity in endothelial cells is required for acute IPC. Expression and dimerization of the HIF-1α and HIF-1β subunits is required, suggesting that the heterodimer is functioning as a transcriptional activator, despite the acute nature of the response.

CD39 | CD73 | coronary artery disease | myocardial infarction | oxygen

The heart requires a constant supply of O_2 for generation of ATP, 95% of which is derived from oxidative phosphorylation (1). Coronary artery stenosis due to an atherosclerotic plaque results in reduced perfusion and myocardial ischemia, especially under conditions of increased myocardial O_2 demand, as occurs when heart work is increased in response to physical exertion or emotional stress. Plaque rupture is a catastrophic event that results in complete arterial occlusion and, within ~20 min, the onset of progressive death of cardiac cells due to O_2 deprivation (2). Rapid reperfusion after ischemia limits infarct size, while at the same time reperfusion contributes to tissue injury (3).

Exposure of the heart to short (5-min) episodes of ischemia and reperfusion protects the heart against injury caused by a subsequent prolonged episode of ischemia and reperfusion (IR), a phenomenon known as ischemic preconditioning (IPC) (4). The protection against myocardial injury following IR that is afforded by IPC ("cardioprotection") consists of an acute/early phase, with onset immediately following the IPC stimulus and a duration of protection lasting several hours (6), followed by a delayed/late phase, with onset ~24 h after the IPC stimulus and a duration of protection lasting several days (5, 6). According to the prevailing paradigm, molecular events during the early phase consist of posttranslational modifications of preexisting proteins, whereas the late phase involves de novo gene expression and protein synthesis (7–9). A corollary of the prevailing paradigm is that many of those molecular events are thought to occur within cardiomyocytes, although the role of endothelial cells (ECs) in IPC has been investigated to a much more limited extent (10).

Hypoxia-inducible factor 1 (HIF-1) is a transcription factor, which functions as a master regulator of adaptive responses to reduced O_2 availability (11). HIF-1 regulates both O_2 delivery, through effects on vascular growth and function, and O_2 utilization, by determining the balance between oxidative and glycolytic metabolism (8, 12, 13). HIF-1 is a heterodimer consisting of HIF-1α and HIF-1β subunits (14, 15). HIF-1α is the O_2-regulated subunit that is specific to HIF-1, whereas HIF-1β is also known as the aryl hydrocarbon receptor nuclear translocator (ARNT) because it also dimerizes with the aryl hydrocarbon receptor (16). HIF-1α is subjected to O_2-dependent modification by the prolyl hydroxylase PHD2, which targets the protein for ubiquitination and proteasomal degradation under normoxic conditions, whereas these events are inhibited under conditions of continuous hypoxia (11, 12, 17). Cycles of hypoxia and reoxygenation also potently increase HIF-1α protein levels and HIF-1 transcriptional activity (18–23). HIF-1α activation has been demonstrated in human hearts under conditions of myocardial ischemia and infarction (22) and patients with coronary artery disease who carry genetic polymorphisms at the human *HIF1A* locus are more likely to present to medical attention with stable angina rather than with myocardial infarction (23) and are less likely to have coronary collaterals (24).

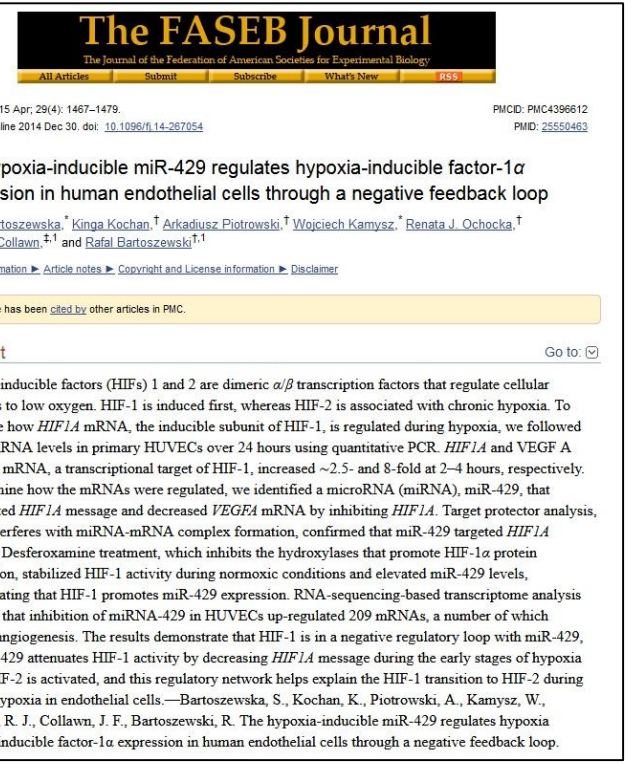
Mice that are homozygous for a knockout allele at the *Hif1a* locus die at midgestation with major cardiac malformations (25–27). *Hif1a*$^{+/-}$ mice, which are heterozygous for the knockout allele, develop normally but the acute protective effects of IPC are completely absent in the hearts of these mice (28). Infusion of small interfering RNA (siRNA) targeting HIF-1α mRNA into the left ventricle of wild-type mice also abolished the acute cardioprotective effects of IPC, whereas siRNA targeting PHD2 mRNA induced cardioprotection in the absence of IPC (29). HIF-1 is likely to activate the expression of multiple pathways that contribute to cardioprotection (9, 13). Among these, HIF-1–dependent adenosine signaling was implicated as an important mechanism by which HIF-1 may mediate the protective effects of IPC (5, 29). Consistent with this hypothesis, infusion of adenosine into *Hif1a*$^{+/-}$ hearts induced significant protection against

Author contributions: G.L.S. designed research; K.S., Z.C., R.G., N.P., K.F.-T., and M.S.D. performed research; F.J.G. contributed new reagents/analytic tools; K.S., Z.C., R.G., and G.L.S. analyzed data; and K.S. and G.L.S. wrote the paper.

The authors declare no conflict of interest.

[2]To whom correspondence may be addressed. E-mail: gsemenza@jhmi.edu.

This article can be accessed in supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1208077109/-/DCSupplemental.



The FASEB Journal
The Journal of the Federation of American Societies for Experimental Biology
All Articles | Submit | Subscribe | What's New | RSS

FASEB J. 2015 Apr; 29(4): 1467–1479.
Published online 2014 Dec 30. doi: 10.1096/fj.14-267054
PMCID: PMC4396612
PMID: 25550463

The hypoxia-inducible miR-429 regulates hypoxia-inducible factor-1α expression in human endothelial cells through a negative feedback loop

Sylwia Bartoszewska,[*] Kinga Kochan,[†] Arkadiusz Piotrowski,[†] Wojciech Kamysz,[*] Renata J. Ochocka,[†] James F. Collawn,[‡,1] and Rafal Bartoszewski[†,1]

Author information ► Article notes ► Copyright and License information ► Disclaimer

Abstract

Go to:

Hypoxia-inducible factors (HIFs) 1 and 2 are dimeric α/β transcription factors that regulate cellular responses to low oxygen. HIF-1 is induced first, whereas HIF-2 is associated with chronic hypoxia. To determine how *HIF1A* mRNA, the inducible subunit of HIF-1, is regulated during hypoxia, we followed *HIF1A* mRNA levels in primary HUVECs over 24 hours using quantitative PCR. *HIF1A* and VEGF A (*VEGFA*) mRNA, a transcriptional target of HIF-1, increased ~2.5- and 8-fold at 2–4 hours, respectively. To determine how the mRNAs were regulated, we identified a microRNA (miRNA), miR-429, that destabilized *HIF1A* message and decreased *VEGFA* mRNA by inhibiting *HIF1A*. Target protector analysis, which interferes with miRNA-mRNA complex formation, confirmed that miR-429 targeted *HIF1A* message. Desferoxamine treatment, which inhibits the hydroxylases that promote HIF-1α protein degradation, stabilized HIF-1 activity during normoxic conditions and elevated miR-429 levels, demonstrating that HIF-1 promotes miR-429 expression. RNA-sequencing-based transcriptome analysis indicated that inhibition of miRNA-429 in HUVECs up-regulated 209 mRNAs, a number of which regulate angiogenesis. The results demonstrate that HIF-1 is in a negative regulatory loop with miR-429, that miR-429 attenuates HIF-1 activity by decreasing *HIF1A* message during the early stages of hypoxia before HIF-2 is activated, and this regulatory network helps explain the HIF-1 transition to HIF-2 during chronic hypoxia in endothelial cells.—Bartoszewska, S., Kochan, K., Piotrowski, A., Kamysz, W., Ochocka, R. J., Collawn, J. F., Bartoszewski, R. The hypoxia-inducible miR-429 regulates hypoxia hypoxia-inducible factor-1α expression in human endothelial cells through a negative feedback loop.

Diagnosis of latent heart disease

Luftwaffe
Wir. Dienen. Deutschland.

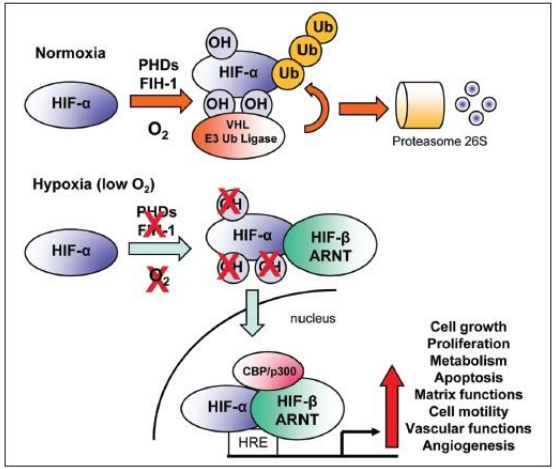
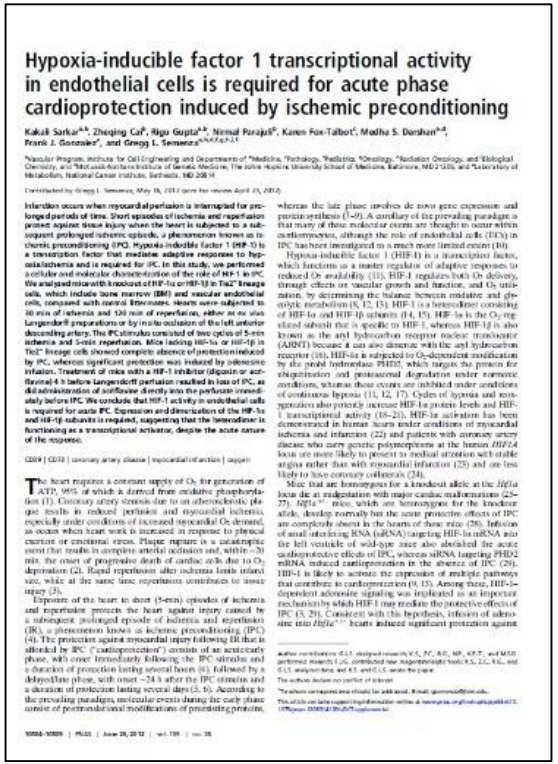


Figure 2. Regulation of hypoxia-inducible factor (HIF) activity. Under normoxic conditions, HIF-α subunits are polyubiquitinated at 2 proline residues within the oxygen-dependent degradation domain (ODDD) by a family of enzymes known as prolyl hydroxylases (PHDs). This promotes recognition by the VHL E3 ubiquitin ligase complex and subsequent degradation of HIF-α via the 26S proteasome. In addition, hydroxylation of a C-terminal asparagine residue of HIF-α by factor-inhibiting HIF-1 (FIH-1) prevents binding of cofactors required for HIF activity. Hypoxia inhibits the activity of the PHD and FIH-1 enzymes, allowing HIF-α proteins to escape recognition by VHL, be stabilized, and translocate to the nucleus. There, they dimerize with HIF-1β/ARNT and bind hypoxia response elements (HREs) within the promoters of target genes. Together with the co-activator proteins p300 and CBP, the HIF complex activates the transcription of a panel of genes required for the response to hypoxia. OH = hydroxylation; Ub = ubiquitin.



Hypoxia-inducible factor 1 transcriptional activity in endothelial cells is required for acute phase cardioprotection induced by ischemic preconditioning

Kakali Sarkar[a,b], Zhuqing Cai[a], Rigu Gupta[a,b], Nirmal Parajuli[a], Karen Fox-Talbot[c], Madhu S. Darshan[a,d], Frank J. Gonzalez[e], and Gregg L. Semenza[a,b,c,d,e,f,1]

[a]Vascular Program, Institute for Cell Engineering and Departments of [b]Medicine, [c]Pathology, [d]Pediatrics, [e]Oncology, [f]Radiation Oncology, and [g]Biological Chemistry, and [h]McKusick-Nathans Institute of Genetic Medicine, The Johns Hopkins University School of Medicine, Baltimore, MD 21205; and [e]Laboratory of Metabolism, National Cancer Institute, Bethesda, MD 20814

Contributed by Gregg L. Semenza, May 18, 2012 (sent for review April 23, 2012)

Infarction occurs when myocardial perfusion is interrupted for prolonged periods of time. Short episodes of ischemia and reperfusion protect against tissue injury when the heart is subjected to a subsequent prolonged ischemic episode, a phenomenon known as ischemic preconditioning (IPC). Hypoxia-inducible factor 1 (HIF-1) is a transcription factor that mediates adaptive responses to hypoxia/ischemia and is required for IPC. In this study, we performed a cellular and molecular characterization of the role of HIF-1 in IPC. We analyzed mice with knockout of HIF-1α or HIF-1β in Tie2+ lineage cells, which include bone marrow (BM) and vascular endothelial cells, compared with control littermates. Hearts were subjected to 30 min of ischemia and 120 min of reperfusion, either ex vivo using Langendorff preparations or by in situ occlusion of the left anterior descending artery. The IPC stimulus consisted of two cycles of 5-min ischemia and 5-min reperfusion. Mice lacking HIF-1α or HIF-1β in Tie2+ lineage cells showed complete absence of protection induced by IPC, whereas significant protection was induced by adenosine infusion. Treatment of mice with a HIF-1 inhibitor (digoxin or acriflavine) 4 h before Langendorff perfusion resulted in loss of IPC, as did administration of acriflavine directly into the perfusate immediately before IPC. We conclude that HIF-1 activity in endothelial cells is required for acute IPC. Expression and dimerization of the HIF-1α and HIF-1β subunits is required, suggesting that the heterodimer is functioning as a transcriptional activator, despite the acute nature of the response.

CORE | CD132 | coronary artery disease | myocardial infarction | oxygen

The heart requires a constant supply of O_2 for generation of ATP, 95% of which is derived from oxidative phosphorylation (1). Coronary artery stenosis due to an atherosclerotic plaque results in reduced perfusion and myocardial ischemia, especially under conditions of increased myocardial O_2 demand, as occurs when heart work is increased in response to physical exertion or emotional stress. Plaque rupture is a catastrophic event that results in complete arterial occlusion and, within ~30 min, the onset of progressive death of cardiac cells due to O_2 deprivation (2). Rapid reperfusion after ischemia limits infarct size, while at the same time reperfusion contributes to tissue injury (3).

Exposure of the heart to short (5-min) episodes of ischemia and reperfusion protects the heart against injury caused by a subsequent prolonged episode of ischemia and reperfusion (IR), a phenomenon known as ischemic preconditioning (IPC) (4). The protection against myocardial injury following IR that is afforded by IPC ("cardioprotection") consists of an acute/early phase, with onset immediately following the IPC stimulus and a duration of protection lasting several hours (6), followed by a delayed/late phase, with onset ~24 h after the IPC stimulus and a duration of protection lasting several days (5, 6). According to the prevailing paradigm, molecular events during the early phase consist of posttranslational modifications of preexisting proteins, whereas the late phase involves de novo gene expression and protein synthesis (7–9). A corollary of the prevailing paradigm is that many of those molecular events are thought to occur within cardiomyocytes, although the role of endothelial cells (ECs) in IPC has been investigated to a much more limited extent (10).

Hypoxia-inducible factor 1 (HIF-1) is a transcription factor, which functions as a master regulator of adaptive responses to reduced O_2 availability (11). HIF-1 regulates both O_2 delivery, through effects on vascular growth and function, and O_2 utilization, by determining the balance between oxidative and glycolytic metabolism (8, 12, 13). HIF-1 is a heterodimer consisting of HIF-1α and HIF-1β subunits (14, 15). HIF-1α is the O_2-regulated subunit that is specific to HIF-1, whereas HIF-1β is also known as the aryl hydrocarbon receptor nuclear translocator (ARNT) because it can also dimerize with the aryl hydrocarbon receptor (16). HIF-1α is subjected to O_2-dependent modification by the prolyl hydroxylase PHD2, which targets the protein for ubiquitination and proteasomal degradation under normoxic conditions, whereas these events are inhibited under conditions of continuous hypoxia (11, 12, 17). Cycles of hypoxia and reoxygenation also potently increase HIF-1α protein levels and HIF-1 transcriptional activity (18–23). HIF-1α activation has been demonstrated in human hearts under conditions of myocardial ischemia and infarction (22) and patients with coronary artery disease who carry genetic polymorphisms at the human *HIF1A* locus are more likely to present to medical attention with stable angina rather than with myocardial infarction (23) and are less likely to have coronary collaterals (24).

Mice that are homozygous for a knockout allele at the *Hif1a* locus die at midgestation with major cardiac malformations (25–27). *Hif1a*$^{-/-}$ mice, which are heterozygous for the knockout allele, develop normally but the acute protective effects of IPC are completely absent in the hearts of these mice (28). Infusion of small interfering RNA (siRNA) targeting HIF-1α mRNA into the left ventricle of wild-type mice also abolished the acute cardioprotective effects of IPC, whereas siRNA targeting PHD2 mRNA induced cardioprotection in the absence of IPC (29). HIF-1 is likely to activate the expression of multiple pathways that contribute to cardioprotection (9, 13). Among these, HIF-1–dependent adenosine signaling was implicated as an important mechanism by which HIF-1 may mediate the protective effects of IPC (5, 28). Consistent with this hypothesis, infusion of adenosine into *Hif1a*$^{+/-}$ hearts induced significant protection against

Author contributions: K.S., designed research; K.S., Z.C., R.G., N.P., K.F.-T., and M.S.D. performed research; K.S. contributed new reagents/analytic tools; K.S., Z.C., R.G., and G.L.S. analyzed data; and K.S. and G.L.S. wrote the paper.

The authors declare no conflict of interest.

*To whom correspondence may be addressed. E-mail: gsemenza@jhmi.edu.

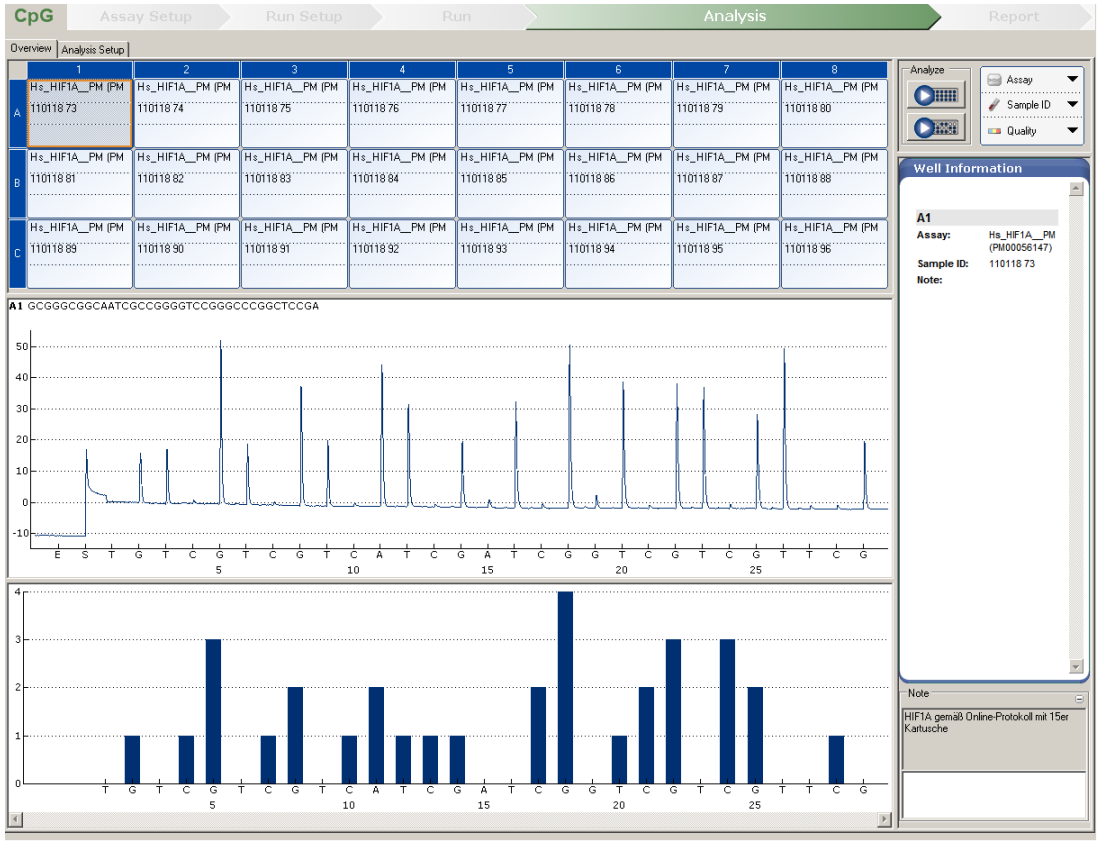
This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1208077109/-/DCSupplemental.

Table 1. Hypoxia-Inducible Factor (HIF) Transcriptionally Induced Genes

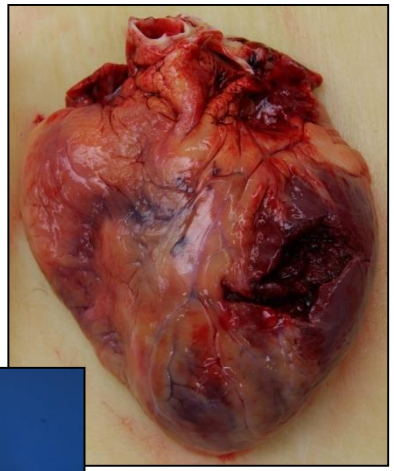
Functions	Hypoxia/HIF Target Genes
Proliferation/survival	IGF-BP1/2/3, IGF2, CCD1, TGF-α/β, P21, Cyclin G2, NOS2
Cell metabolism	
Glucose	PDK, PFK, PGK, LDHA, GLUT-1/3, hexokinase-1/2, enolase-1, GAPDH, ALDA, ALDC, PKM, TPI
Iron	Transferrin, transferrin-R, ceruloplasmin
pH	Carbonic anhydrase-9
Nucleotide	Adenylate kinase-3, ecto-5′-nucleotidase
Amino acid	Transglutaminase2
Apoptosis	P53, BNIP3, NIX, Bax, RTP801/REDD1, Ref-1, Bcl-2, NFκB, HSP70, Bid
Migration/invasion	CXCR4, MMP-2, Lox, PAI-1, c-MET, LRP1, MIC2/CD99, fibronectin, UPAR, collagen type V, AMF/GPI, CATHD, integrin-linked kinase, integrins
Transcriptional regulation	DEC1, DEC2, ETS-1, NUR77
Cytoskeletal structure	KRT14, KRT18, KRT19, vimentin

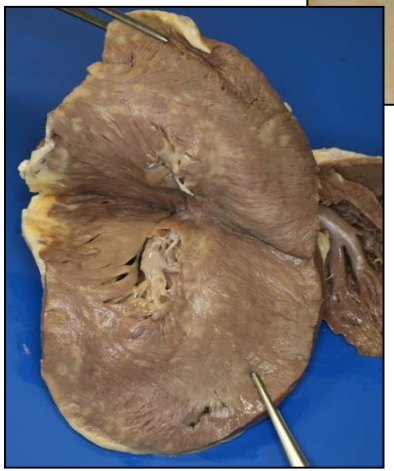
ALDA = aldolase A; ALDC = aldolase C; AMF = autocrine motility factor; Bcl-2 = B-cell leukemia/lymphoma 2; BNIP3 = Bcl-2 nineteen kilodalton interacting protein 3; CATHD = cathepsin D; CCD1 = coiled-coil-DIX1; CXCR4 = CXC chemokine receptor 4; DEC1/2 = differentiated embryo-chondrocyte expressed gene 1/2; GLUT-1/3 = glucose transporter1/3; GAPDH = glyceraldehyde-3-P-dehydrogenase; HSP70 = heat shock protein 70; IGF2 = insulin-like growth factor 2; IGF-BP1/2/3 = IGF factor binding protein 1/2/3; KRT14/18/19 = keratin 14/18/19; LDHA = lactate dehydrogenase A; LRP1 = LDL receptor–related protein 1; Lox = lysyl oxydase; MDR1 = multidrug resistance 1; MIC2 = microneme protein 2; MMP2 = matrix metalloproteinase 2; NFκB = nuclear factor kappa B; NOS2 = nitric oxide synthase 2; NUR77 = nuclear receptor 77; PDK = pyruvate dehydrogenase kinase; PFK = phosphofructokinase; PGK = phosphoglycerate kinase; PKM = pyruvate kinase M; REDD1 = regulated in development and DNA damage responses 1; Ref-1 = redox factor–1; TGF-α = transforming growth factor–α; TGF-β = transforming growth factor–β; TPI = triosephosphate isomerase; UPAR = urokinase plasminogen activator receptor.

Diagnosis of latent heart disease



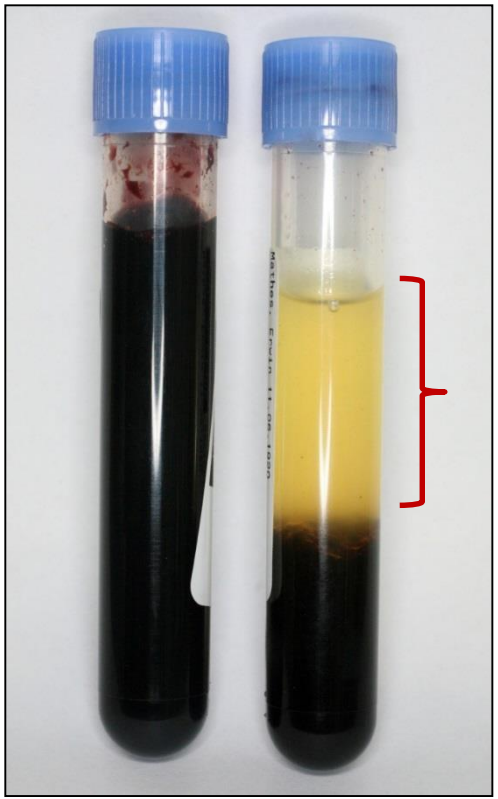
Hypertrophic heart with penetrating injury from rib racture.





White scar tissue from infarction (formalin-fixed organ).

Diagnosis of latent heart disease

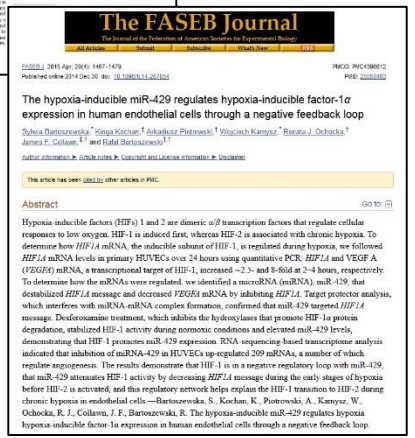


Applicable in living patients to detect e.g. tumor markers or other disease-related spill of DNA or microRNA in the bloodstream.

„Liquid biopsy technique“

Applicable also in blood samples from deceased persons. Informative e.g. of disease related markers present prior to an accident event.

Hypoxia-inducible factor 1 transcriptional activity in endothelial cells is required for acute phase cardioprotection induced by ischemic preconditioning



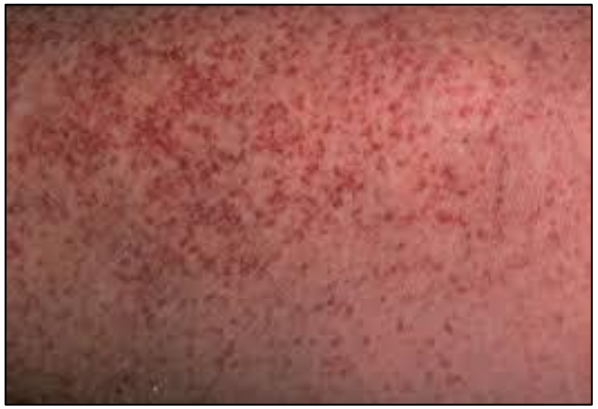
The FASEB Journal

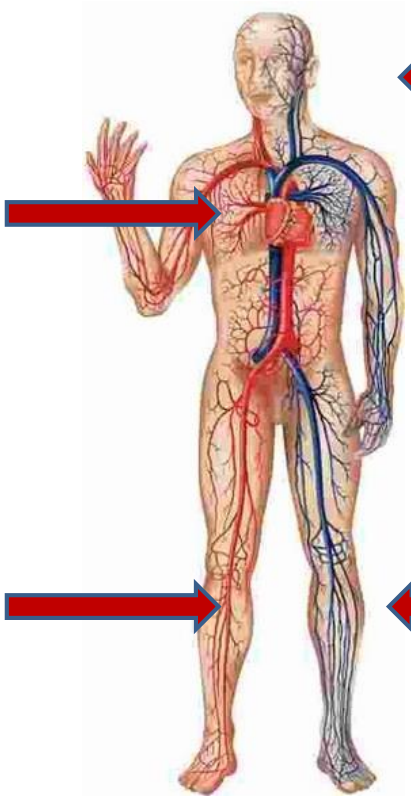
The hypoxia-inducible miR-429 regulates hypoxia-inducible factor-1α expression in human endothelial cells through a negative feedback loop

Abstract

Further applications

Markers for lung damage, e.g. in fighter pilots using positive pressure breathing.



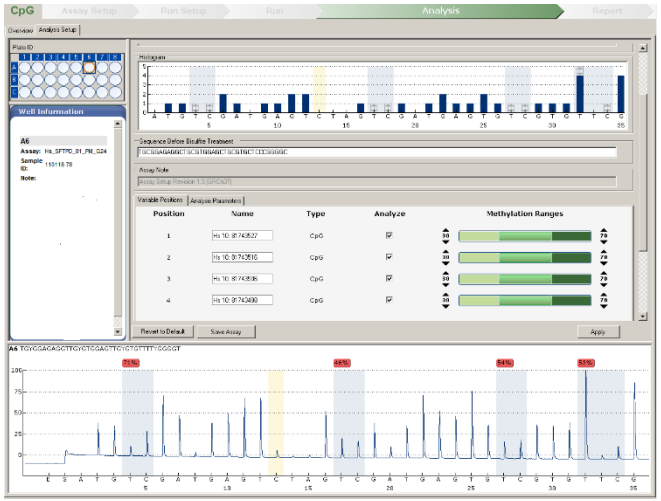


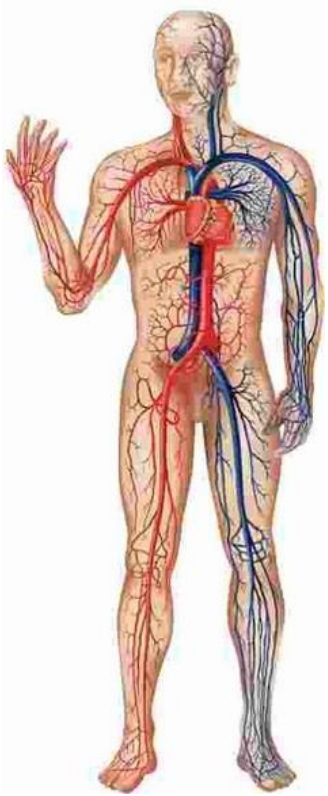
Markers for other aeromedical flieds of interest including

- Circadian rhythm and performance
- Fatigue
- Mood disorders
- White matter lesions

Markers for vascular damage, e.g. in flyers wearing anti-G-suits.

CpG-methylation in SFTP-promotor



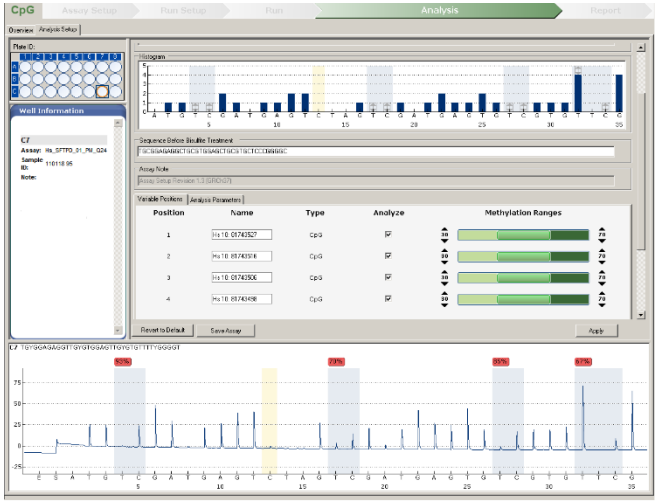


Methylation in lung tissue:

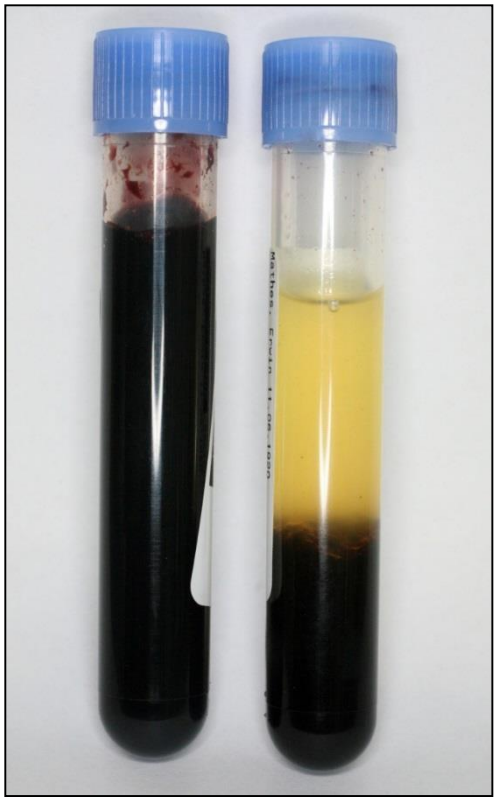
- 71%
- 46%
- 54%
- 53%

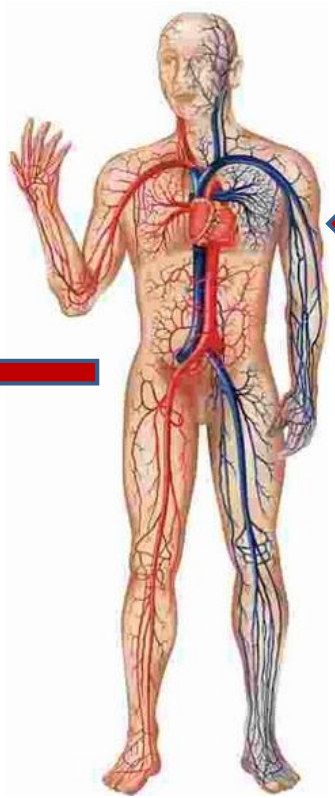
Methylation in muscle tissue:

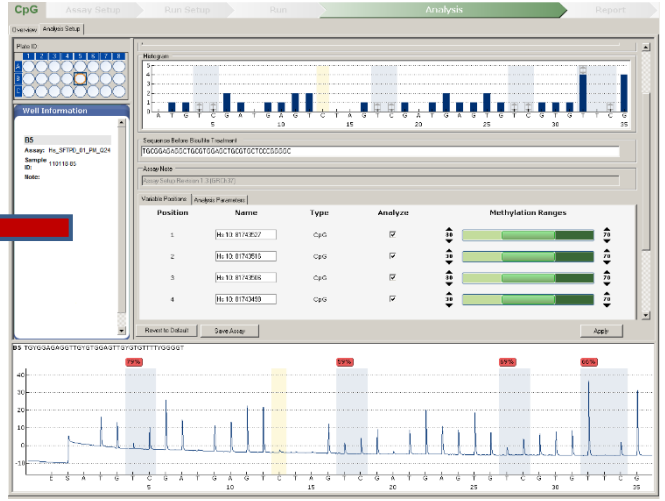
- 93%
- 70%
- 85%
- 67%



CpG-methylation in SFTP-promotor



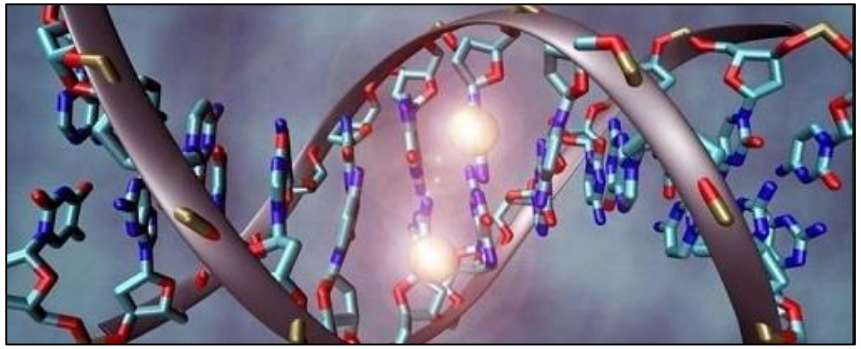




Methylation in liquid biopsy:

- 79%
- 59%
- 69%
- 66%

Summary



With these recently developed techniques molecular pathology made ist way into (scientific) aerospace medicine.

?

Thank you very much for your attention.