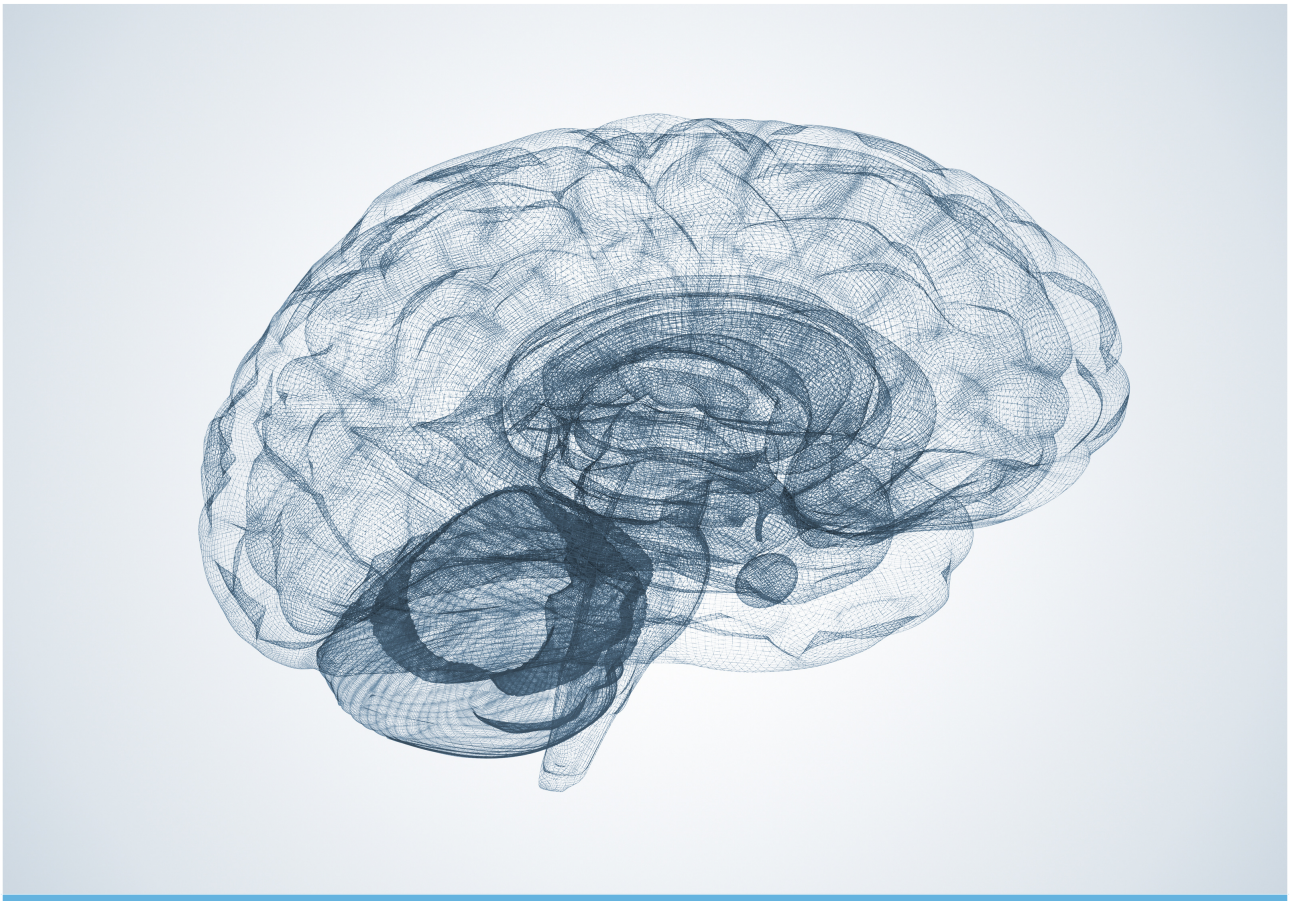

Université de Liège
Faculté de Médecine

CONTRIBUTION TO THE OUTCOME PREDICTION AFTER CARDIAC ARREST

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Thèse présentée en vue de l'obtention du grade de Docteur en Sciences Médicales
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"When the wind of change blows, some build walls, others windmills"

Chinese proverb

"It always seems impossible until it's done"

Nelson Mandela

To my family: Françoise, Mika, Laura, and my parents, Josée and Jean-André

In memory of Marc Stammet, my uncle

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ABBREVIATIONS

AED	automated external defibrillator
aEEG	amplitude integrated EEG
AMI	acute myocardial infarction
AUC	area under the curve
BBB	blood-brain barrier
BIS	bispectral index
BLS	basic life support
CA	cardiac arrest
cEEG	continuous EEG
CHL	Centre Hospitalier de Luxembourg
CI	confidence interval
circRNA	circular RNA
CK-BB	creatine kinase-BB isoenzyme
CK-MB	creatine kinase-MB isoenzyme
CNER	comité national d'éthique de recherche Luxembourg
CPC	cerebral performance category
CPR	cardiopulmonary resuscitation
CT scan	computed tomography scanner
DWI	diffusion weighted imaging
ECG	electrocardiogramme
ECLIA	electro-chemo-luminescent-immuno-assay
ECLS	extra coporeal life support
ECMO	extra coporeal membrane oxygenation
EEG	electroencephalography
EMG	electromyography
EMS	emergency medical service
ERC	European Resuscitation Council
FPR	false positive rate
GFAP	glial fibrillary acidic protein
GOS	Glasgow outcome scale
GOSE	Glasgow outcome scale extended
ICU	intensive care unit
IDI	integrated discrimination improvement
ILCOR	International Liaison Committee on Resuscitation
lncRNA	long non coding RNA
LRC	Luxembourg Resuscitation Council
miR	micro RNA
miRNA	micro RNA
MRI	magnetic resonance imaging

mRNA	messenger RNA
mRS	modified Rankin scale
NFL	neurofilament light chain
NMB	neuromuscular blockade
NRI	net reclassification improvement
NSE	neuron specific enolase
OHCA	out-of-hospital cardiac arrest
PCAS	post cardiac arrest syndrome
PCR	polymerase chain reaction
PCT	procalcitonin
PEA	pulseless electric activity
PLR	pupillary light reflex
Pol II	polymerase II
PSE	postanoxic status epilepticus
qPCR	quantitative polymerase chain reaction
RISC	RNA induced silencing complex
RNA	ribonucleic acid
ROC	receiver operating characteristics
ROSC	return of spontaneous circulation
S100	protein S100
SAMU	service d'aide médicale urgente
SAPS II	simplified acute physiology score II
SCD	sudden cardiac death
SSEP	somatosensory evoked potentials
TH	therapeutic hypothermia
TTM	target temperature management
VF	ventricular fibrillation
VT	ventricular tachycardia
WLST	withdrawal of life supporting therapies
Youden	Youden index (best compromise between sensitivity and specificty)

ACKNOWLEDGEMENTS - REMERCIEMENTS

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SUMMARY

With this doctoral thesis we aimed to contribute to the improvement of outcome prediction after cardiac arrest. Cardiac arrest represents a high burden on the individual patients as well as on our society. With an in-hospital death rate of approximatively 50 percent of initially successfully resuscitated patients, there is a need for adequate outcome prediction.

By taking the advantage of an established biobank with blood samples from 700 patients included in the "Target Temperature Management trial" (TTM), we were able to test 2 biomarkers used in outcome prediction, neuron specific enolase (NSE) and the neuron enriched protein S100. We could show that NSE is the more robust outcome predictor when it is measured serially after cardiac arrest. An increase of NSE over time, associated with high absolute values, is strongly predictive of poor outcome. S100 has also a good predictive capacity, but because of a steady decrease over time in all outcome groups only the 24 hours time point was the most sensitive. Interestingly, the level of targeted temperature did not have an influence on the levels and the prediction capacities of both studied biomarkers.

Within our local observational study cohort (NorthPole project), we performed an analysis of the outcome prediction capacities of the bispectral index (BIS), a simplified neurophysiological tool. We were able to demonstrate that BIS is not only capable of detecting patients with a poor outcome, but this can also be done very early. Indeed, when mean BIS values over the first 12.5 or even 6.5 hours are low respectively very low, then outcome is very likely to be poor. This finding is important since it illustrates that BIS has the potential for very early outcome prediction or triage of patients.

As a consequence of our previous work, we combined BIS and biomarkers and were able to show that adding S100 (or NSE) to BIS improved outcome prediction substantially, whereas adding both biomarkers to BIS did not further improve adequacy compared to one biomarker plus BIS. This approach is interesting since it combines a biomarker to a neurophysiological test and thus comprises two different brain damage entities for prognostication.

Finally, we also investigated the role of a new kind of biomarkers: miRNA. We were the first to describe a biosignature after cardiac arrest based on miRNA in our proof of concept study. Using a micro array analysis including 695 miRNA we determined two miRNA that were differentially expressed. With an in vitro model of neuronal cell cultures and quantitative PCR, we were able to detect miR-122 and miR-21 as outcome predictors after cardiac arrest.

With this work, we could improve the accuracy of outcome prediction by the routinely used biomarkers NSE and S100. We could furthermore show that the level of target temperature management does not significantly influence these biomarkers. BIS is a promising and simple neurophysiological technique that bears the potential to predict outcome earlier than 24 hours after cardiac arrest. A sensible combination of different and selected outcome predictors may further increase prediction accuracy. Novel biomarkers are under investigation and they may, besides an improvement in outcome prediction, also bear potential therapeutic implications because of their inherent regulatory functions in gene expression.

RÉSUMÉ

Avec ce travail nous avons voulu contribuer à l'amélioration de la prédiction neurologique après arrêt cardiaque. L'arrêt cardiaque représente un lourd fardeau, à la fois pour le patient et son entourage, ainsi que pour la société. Avec une mortalité intra-hospitalière des patients initialement réanimés avec succès qui avoisine les 50 pourcent, il y a un besoin de disposer de moyens de prédiction fiables.

Nous avons pu bénéficier des échantillons de biobanque des quelque 700 patients ayant fait partie de l'étude « Target Temperature Management » (TTM) pour tester deux biomarqueurs utilisés dans la prédiction neurologique, la « neuron specific enolase » (NSE) et la « neuron enriched protein S100 » (S100). Nous avons pu démontrer que la NSE est le meilleur des deux biomarqueurs quand on le mesure en série après un arrêt cardiaque. Une augmentation des valeurs de NSE au fil du temps, associée à des valeurs absolues élevées est un fort indicateur de mauvais pronostic neurologique. La protéine S100 a également une bonne capacité prédictive, mais en raison d'une diminution des valeurs de S100 au fil du temps dans tous les groupes, seulement la valeur à 24 heures était la plus adaptée pour la prédiction. Nous avons également pu démontrer que le niveau de la gestion de la température corporelle n'avait aucune influence significative sur les biomarqueurs.

Grâce à notre projet local NorthPole, nous avons pu étudier les capacités de prédiction d'une approche neurophysiologique simplifiée, le BIS (bispectral index). Nous avons démontré que le BIS est non seulement capable de détecter les patients avec un mauvais pronostic, mais qu'il permet également de faire cela précocement. En effet, si les valeurs moyennes de BIS restent basses, voire très basses au cours des premières 12.5 respectivement 6.5 heures après l'admission en réanimation, ceci est associé à un mauvais pronostic. Ceci est important puisque peu de facteurs prédictifs sont valables avant 24 heures et que le BIS a donc un potentiel pour devenir un outil de prédiction précoce ou de triage des patients.

Suite à nos travaux préliminaires nous avons combiné le BIS à des biomarqueurs et nous avons pu démontrer la valeur ajoutée de cette approche. La combinaison entre un biomarqueur (S100 ou NSE) au BIS a permis d'améliorer substantiellement la capacité de prédiction tandis que rajouter les deux biomarqueurs au BIS n'a pas permis d'améliorer davantage l'adéquation de la prédiction. Cette approche est intéressante puisqu'elle combine un biomarqueur avec une mesure neurophysiologique simple et comprend donc deux entités distinctes pour la prédiction : l'une lésionnelle et l'autre fonctionnelle.

Enfin, nous avons investigué le rôle d'une nouvelle catégorie de biomarqueurs, les microRNA (miRNA). Nous avons été les premiers à décrire une biosignature basée sur des miRNA après arrêt cardiaque dans cette étude de faisabilité. Grâce à l'étude par microarrays comprenant 695 miRNA, nous avons pu identifier deux miRNA qui ont été exprimés de façon différentielle en fonction du devenir neurologique : le miR-21 et le miR-122. Avec un modèle in vitro sur des cultures de cellules neuronales et ensuite par une analyse PCR quantitative, nous avons pu déterminer un pouvoir prédictif de ces deux miRNA.

Avec cette thèse, nous avons pu contribuer à l'amélioration des moyens pronostiques après arrêt cardiaque grâce aux biomarqueurs disponibles en routine, NSE et S100. En plus, nous avons montré que le niveau de la température ciblée n'influe pas de façon significative les biomarqueurs. Le BIS est un outil neurophysiologique simple et prometteur en ce qui concerne la prédiction neurologique précoce, avant 24 heures après l'arrêt cardiaque. Une combinaison censée entre différents prédicteurs permettrait d'améliorer encore davantage l'adéquation de la prédiction. Les nouveaux biomarqueurs qui sont actuellement le sujet d'une recherche intensive pourraient, en outre de leur capacité de prédiction, aussi avoir un rôle thérapeutique potentiel inhérent à leur fonction de régulation de l'expression génétique.

1. INTRODUCTION:

1.1 Cardiac arrest

Cardiac arrest (CA), the cessation of the pumping function of the heart, is probably what best describes death for the general public. With the stopping of the heart beats and the subsequent interruption of blood flow and oxygen delivery to the organs, death will become definite and irreversible within minutes if no resuscitation measures are undertaken. Nevertheless, even if the heart stops to beat or rather stops to efficiently pump blood through the organism, death is not immediate. During a variable period of time, mostly depending on external conditions, life can be ”insufflated” again into a dying body. Modern resuscitation efforts can contribute to save many lives. The most important issue when resuscitating a person is to act within the window where the apparent death is not yet irreversible or severe organ damage is not yet definite, as within minutes oxygen stores are depleted and cellular death begins. The brain is the most vulnerable organ to oxygen deprivation and permanent neuronal damage can already be expected within minutes. As such, death is only definite once the brain activity has completely and irreversibly ceased. The period in between a vital organ dysfunction, like cardiac arrest, and death remains a grey zone. There is rather a process of dying until death has become irreversible and only within the first moments of this process of dying, resuscitation is possible. Xavier Bichat, 18th century French pathologist, stated that “Life is the set of functions that resist to death”.

Until the 1960’s, French legal texts interestingly define death by the cessation of the function of the heart and the lungs. But with the emerging “technicity” of medicine and the advent of cardio-pulmonary resuscitation, this definition did not stand anymore, as the borders between life and death became blurred. Furthermore, in legal terms, death is not defined (except in the particular case before organ donation), but only “observed” and its certification is left upon the “men of art”, the medical doctor. Only after 1968, French law introduced the concept of “brain death” and the notion of “coma dépassé” (exceeded coma). Prof Christian Cabrol summarized the definition of death by these words: “there is just one death, brain death, may it be primary or secondary to cardiac arrest”.

In terms of outcome prediction or prognostication of outcome after cardiac arrest, the major issue is to identify amongst these initially successfully resuscitated patients, those that will either die or have severe neurological impairment and those with the best chances of low neurological sequels and good recovery.

1.2 Epidemiology of cardiac arrest

In high income countries, sudden out-of-hospital cardiac arrest is a very common cause of death. SCD is a disease of the modern lifestyle including its many risk factors like “malnutrition” and lack of exercise. Most recent estimates state some 275 000 up to more than 400 000 deaths in the European Union per year that are due to SCD (Atwood, Eisenberg et al. 2005, Berdowski, Berg et al. 2010, European_Commission 2012). Even though, the latter figure might be exaggerated as it probably comprises all out of hospital cardiac arrests, even those where no cardio-pulmonary resuscitation (CPR) attempts have been performed, these figures are dramatic as they represent half up to nearly the whole population of the Grand-Duchy of Luxembourg that would be extinguished in just one year…

Regardless of the continent, these data are comparable even though there are wide variations due to the reporting, treatment and health care resources. As such, sudden cardiac arrest is definitely one of the leading causes of death worldwide.

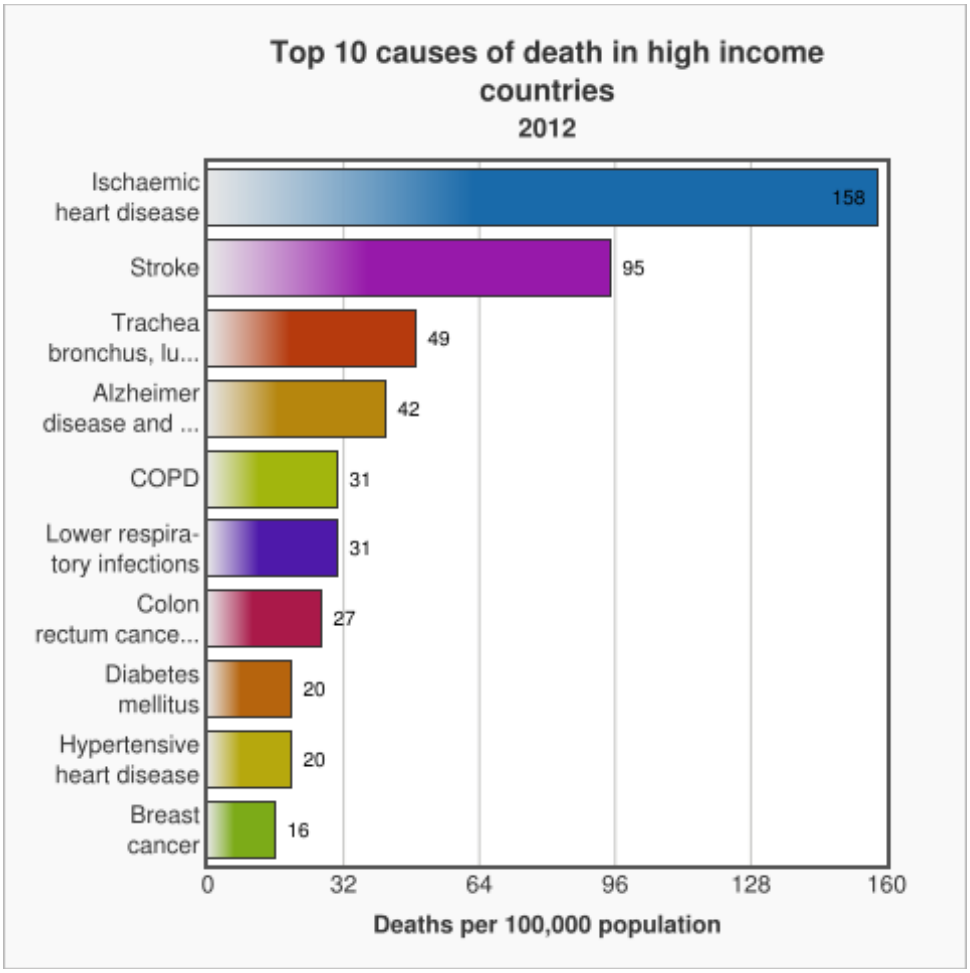


Figure 1: From www.who.int : Fact sheet, the top 10 causes of death in high income countries.

The main etiology of cardiac arrest in developed countries is represented by acute cardiac ischemia. A cardiac cause, defined as no other obvious cause of cardiac arrest, is held responsible for up to 83% of all sudden OHCA admitted to a hospital (Bro-Jeppesen, Kjaergaard et al. 2009). Prevalence of ventricular fibrillation at EMS arrival is estimated around 30-40% in all SCD cases in Europe, North America and Australia (Berdowski, Berg et al. 2010) and around 60% in OHCA from a cardiac cause.

1.3 Initial treatment of cardiac arrest

In early years of modern medicine, resuscitation attempts were more or less experimental or even mystic by “insufflating life” into a dying body. Only since the early 1950’s with the works of Peter Safar and Vladimir Negovsky, modern resuscitation started. In fact, the first guidelines and scientific research in this area were launched in that time by Safar.

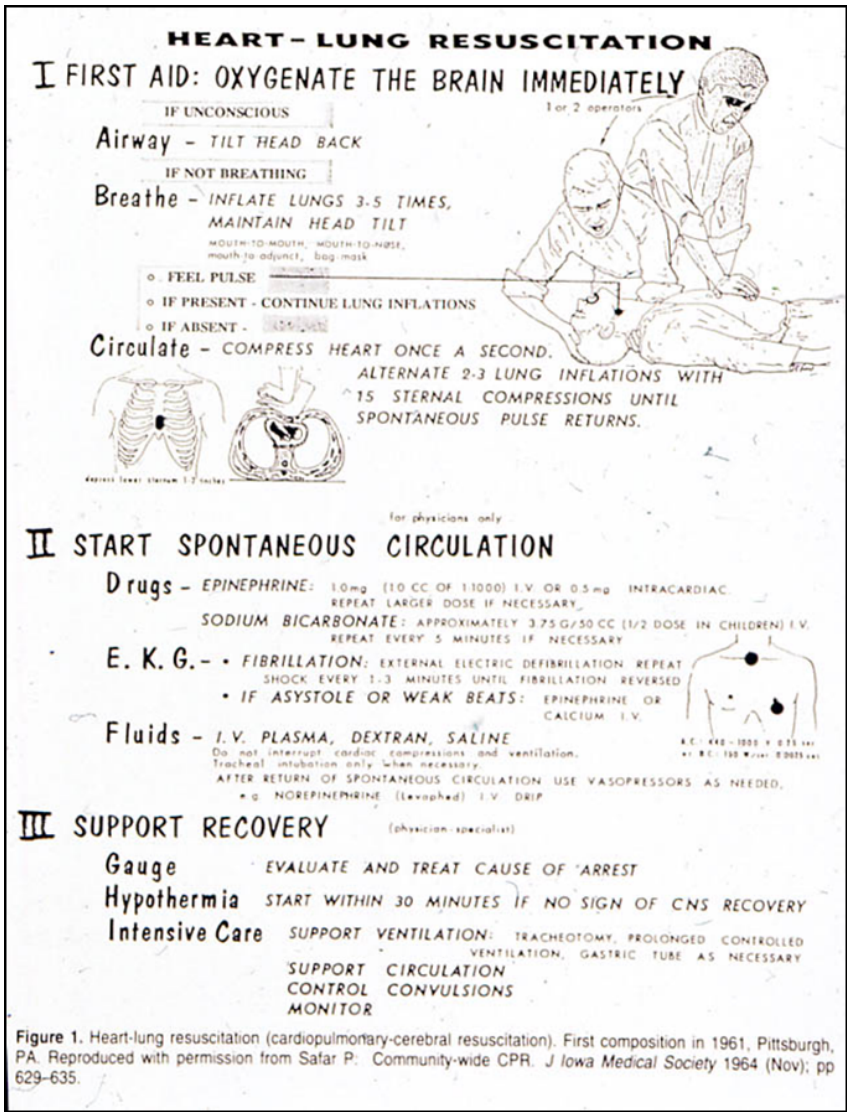


Figure 1. Heart-lung resuscitation (cardiopulmonary-cerebral resuscitation). First composition in 1961, Pittsburgh, PA. Reproduced with permission from Safar P: Community-wide CPR. *J Iowa Medical Society* 1964 (Nov); pp 629–635.

Figure 2: first published CPR algorithm by Peter Safar, 1964

His suggested mnemonic “A-B-C…” approach has since then become the standard for the diagnosis and treatment many life-threatening situations, including cardiac arrest and multiple trauma. Because of the simplicity and ease of teaching, these guidelines have been adopted by many scientific societies throughout the world. Nowadays, the ILCOR, representing the most relevant resuscitation societies of the world, has become the leading force in establishing new and up to date guidelines for resuscitation. The last version of the guidelines have been published in October 2015. Since 2005, these guidelines are based on structured evidence based evaluations how to best perform CPR according to the latest knowledge of science. Since then, also the algorithm for chest compressions has changed to the actual 30 compressions followed by 2 rescue breaths. These guidelines aim at simplification to increase adherence. The ultimate goal is to rapidly restore a minimal circulation to allow for sufficient brain oxygenation to reduce the hypoxic ischemic brain injury. This can be most easily and readily achieved by basic life support (BLS) measures like chest compressions and (mouth-to-mouth) ventilation as advocated in the actual cardio (cerebro) pulmonary resuscitation (CPR) guidelines (Perkins, Handley et al. 2015).

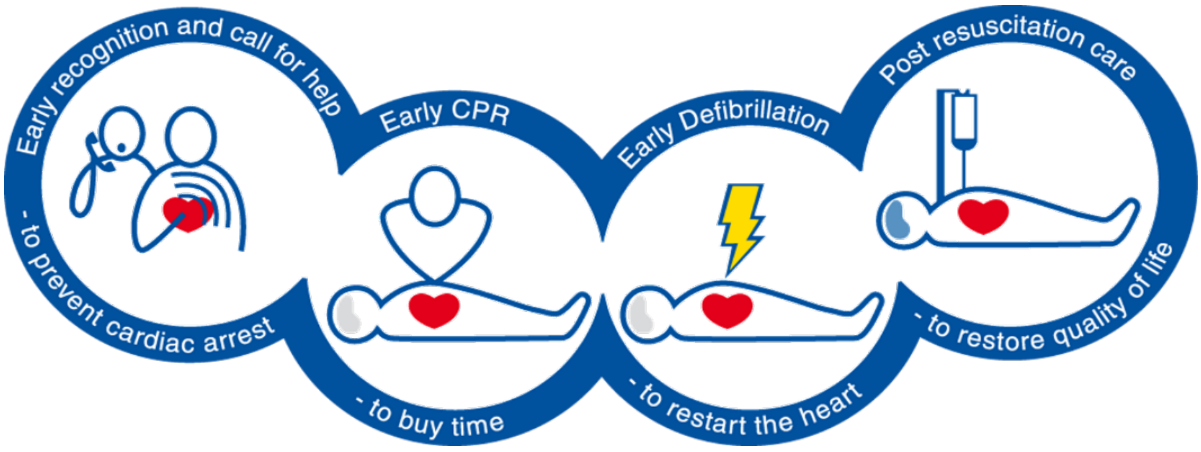


Figure 3: Chain of survival after cardiac arrest. From (Monsieurs, Nolan et al. 2015)

Most important features of these guidelines are represented by the 2005 introduced "new" chain of survival that depicts each of the 4 crucial links of successful resuscitation. As a chain, every weakness of a single link can have dramatic consequences on the global outcome of the patient. Proper training and implementation of adequately applied resuscitation techniques, adapted to the level of the caregiver (ranging from lay people to highly specialized physicians) are cornerstones of an increase in survival of cardiac arrest patients, with, most importantly, no or only light neurological sequels. In particular, the guidelines focus on prevention and in case of failure of the preventive measures, basic CPR skills as well as the use of automated external defibrillators (AED) are put upfront. Indeed, these simple measures that can be performed by any lay person are the cornerstones of the survival chain. Our group could also show that patients benefitting from pre hospital life support by lay people, before arrival of the emergency medical services had much improved survival (Stammet, Collas et al. 2012).

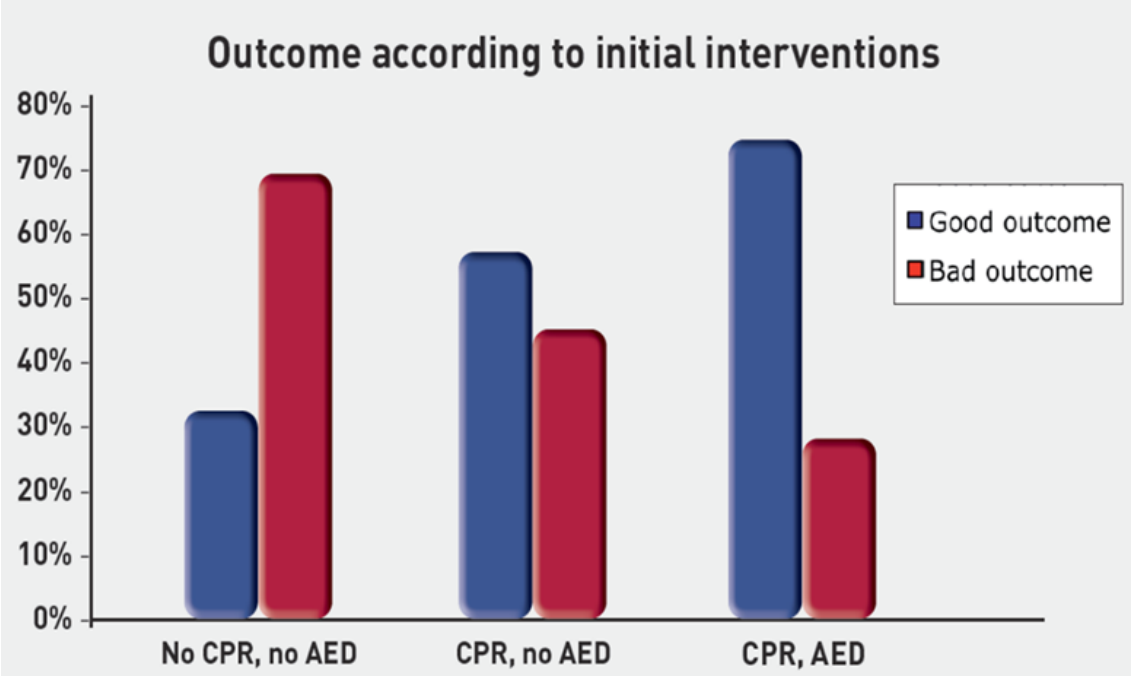


Figure 4: Outcome after cardiac arrest according to initial interventions by bystanders before arrival of the emergency medical services. Adapted from (Stammet, Collas et al. 2012)

Other interventions like dispatcher assisted phone CPR (Rea, Eisenberg et al. 2001, Ghuysen, Collas et al. 2011) may successfully guide lay, untrained bystanders to perform CPR and efficiently reduce the no flow interval until emergency medical service (EMS) personnel arrives at scene and takes over. And this lead to a new “triangle of survival”:

Figure 5: newly suggested “triangle of survival” by the European Resuscitation Council. From (Perkins, Handley et al. 2015)

Also, for the first time, the 2005 chain of survival has a dedicated post resuscitation care element, emphasizing on the importance of the in-hospital care to improve outcome and quality of life.

1.4 The post cardiac arrest care

Unlike what has been done over long periods of time, treatment of cardiac arrest does not end with a successful initial resuscitation “in the field”. Over too many years, physicians were not really attracted by cardiac arrest patients as their outcome was notoriously dismal. Publications in the 1980’s, reported survival rates of about 23% after an out-of-hospital cardiac arrest and even lower good recovery around 15-20% (1986). Because of this poor prognosis, many physicians were not keen on performing “aggressive” and expensive therapies in these patients. Many initially successfully resuscitated patients were admitted to the intensive care units (ICU) to observe how they would finally evolve. If they developed severe cardiogenic shock, very few would have benefitted from immediate percutaneous angiography, as this procedure was judged too invasive, complicated and expensive. There seemed to be no indication to open an occluded coronary artery in a patient with such a high probability of death or neurological sequels. At that time, neurological prognosis was poor, as guidelines for pre hospital resuscitation (as we know now) were not the most efficient, implementation and adherence within the general public was poor. Standardized prognostication methods for outcome prediction did not exist as well. The threshold for withdrawing life supporting therapies was also obviously very low given the pre-cited uncertainties. It all ended up with patients probably being “under-treated” and in a way this vicious circle resulted in overall poor prognosis.

1.4.1 The post cardiac arrest syndrome

One major determinant after cardiac arrest is the post cardiac arrest syndrome (PCAS) which is the result of the ischemic-hypoxic injury to the whole organism after a cardiac arrest. The subsequent reperfusion to all organs that suffered ischemia-hypoxia is the driving force of this syndrome. This “sepsis-like” “post-resuscitation disease” might be considered partially responsible for the high mortality after cardiac arrest in patients that achieve a return of spontaneous circulation (ROSC) (Negovsky 1972, Adrie, Adib-Conquy et al. 2002). It might even be stipulated that one of the cornerstones of the improvement of survival in patients admitted to an ICU after a successful resuscitated cardiac arrest is due to the intensive care. PCAS comprises 4 major determinants which can be classified as 1) the injuries to the brain, 2) the myocardial dysfunction, 3) the systemic ischemia-reperfusion injury and finally 4) the persisting precipitating cause (Nolan, Neumar et al. 2008). The challenges of modern ICU care are embedded in a special challenge: the treatment of the PCAS. As the severity of PACS is highly variable between patients, depending on the severity of the ischemic insult (time to ROSC, quality of CPR, pre arrest status of the patient, …) the treatment to the patient must be tailored to its needs.

After the unsuccessful trial on barbiturates after cardiac arrest in the 1980’s, interventional cardiologists started to show some interest for these patients (1986). Spaulding et al. showed that performing an angiography and percutaneous angioplasty increased the overall survival to around 38% (Spaulding, Joly et al. 1997). Treating one of the precipitating cause of PCAS, was in a way the starting point of modern post-resuscitation care.

2002 was definitely a pivotal year in post-cardiac arrest care with the publication of 2 articles in the same issue of the New England Journal of Medicine showing that induced mild hypothermia dramatically increased survival and improved neurological outcome from some 35% of survivors with no neurological impairment to up to 50-55% (Bernard, Gray et al. 2002, Hypothermia after Cardiac Arrest Study 2002). This triggered the awareness that post-cardiac arrest patients do not necessarily have a futile prognosis and that post-resuscitation care can contribute to significantly lower mortality rates and increase quality of life. With this strong message, the attention of ICU physicians was directed to these patients, as this new, or rather “rediscovered” technique to cool patients, with its potential serious side effects (electrolyte disturbances, malignant cardiac rhythms,…) required a lot of attentiveness. Just

to cite one intervention, more frequent blood gas analysis in order to detect electrolyte disturbances and acid-base disturbances ultimately lead to a closer monitoring of the gas exchanges and consist in a better management of the patient. So probably, not the sole intervention to cool the patient, but also the increased awareness as well as an increased level of care contributed to the improved overall outcome of these patients.

The importance of a well protocolized and systematic approach has also been demonstrated by Sunde et al. in a small but impressive study where they could show that the implementation of a protocol of care resulted in improved outcome compared to “standard” care at the discretion of the treating physician (Sunde, Pytte et al. 2007). The real merit of the “induced hypothermia hype” was probably to have succeeded in redirecting the attention to these patients that formerly had a very poor prognosis. Only by doing so, “therapeutic hypothermia” has probably saved many thousands of lives throughout the world by breaking up the vicious circle of the former “wait and see” strategy.

More recently, the target temperature management after out-of hospital cardiac arrest trial (TTM-trial) has shown that there is no beneficial effect of using a target temperature of 33°C compared to strict 36°C (Nielsen, Wetterslev et al. 2013). This trial might have confused some ICU physicians, but it probably highlights two important messages: the level of the targeted temperature is probably not the most important determinant (as long as the target temperature remains constant, below any febrile temperature and within the studied range of 32-36°C) and by caring intensely for these patients, outcome can be improved. This has been recently integrated in the new post-resuscitation guidelines (Nolan, Soar et al. 2015).

But modern specialized intensive care is well capable of going beyond the therapy of the PCAS and targeted temperature management (TTM), as induced or therapeutic hypothermia should be called today (Nunnally, Jaeschke et al. 2011). Besides percutaneous coronary interventions that should be performed as soon as possible, many other intensive care techniques may apply to patients in severe shock after cardiac arrest. Especially, some more recently advocated or improved techniques can be used. We can cite amongst others the extra corporeal membrane oxygenation (ECMO) or extra-corporeal life support (ECLS) (Sakamoto, Taniguchi et al. 2012). These devices have become miniaturized and portable and can even be implemented in the field before ROSC or be applied in better conditions in the hospitals in patients with refractory cardiac arrest or shock. But these techniques, although being most powerful tools in restoration of an adequate flow of oxygenated blood, have many drawbacks. First of all their start up needs some time, especially in low turnover centers without a permanent stand-by team on site. At least at the moment of implantation, an experienced staff of surgeons, perfusionists, cardiologists and anaesthesiologists are most often needed. Maintenance of the device requires at least trained nursing and medical staff as well as a perfusionist nearby. Many complications inherent to the technique may also be expected, like severe bleeding (as anticoagulation is required), limb or even brain ischemia as well as thrombosis. But this technique can be used as “bridge” therapy awaiting recovery of the heart function (extreme myocardial stunning) or the neurological recovery as well as awaiting a mechanical assist device or a heart transplant. Overall, this technique shows that “extreme” measures can certainly save lots of lives, but it also highlights the need for selection of the patients most suited to benefit from it. There is no point in applying such invasive, time consuming and expensive techniques to patients with a futile neurological prognosis.

Taking care of sudden cardiac arrest patients has dramatically changed over the last decade with the introduction of target temperature management, the more widespread use of percutaneous coronary interventions and phone CPR, but also with in-hospital high level intensive care, which all have in fine largely contributed to the improvement of outcome after cardiac arrest (Rittenberger and Callaway 2013).

1.5 Outcome after cardiac arrest

Emergency care of cardiac arrest victims is of paramount importance as one of the most dreaded complication in survivors is hypoxic-ischemic brain damage. This damage ranges from nearly not perceptible deficits (concentration and attention deficits, anxiety in variable degrees) over severe cognitive impairment with amnesia to vegetative states (Lilja, Nielsen et al. 2015).

Survival of patients admitted to the hospital after cardiac arrest used to be dismal, ranging from well below 30% in the 1980 to somewhere around 38% at the end of the 1990 for those patients admitted to a hospital after ROSC (1986, Spaulding, Joly et al. 1997).

Nowadays, even in the light of many improvements in pre-hospital care as well as in post-cardiac arrest management over the last years, outcome after cardiac arrest remains lower than 10%. Unpublished data from the Luxembourg Resuscitation Council's (LRC) retrospective survey in 2013 within the SAMU ("service d'aide médicale urgente") services of Luxembourg revealed that in all patients suffering from an OHCA, only 22% benefitted from bystander CPR and in the end only 16% of patients were admitted to a hospital.

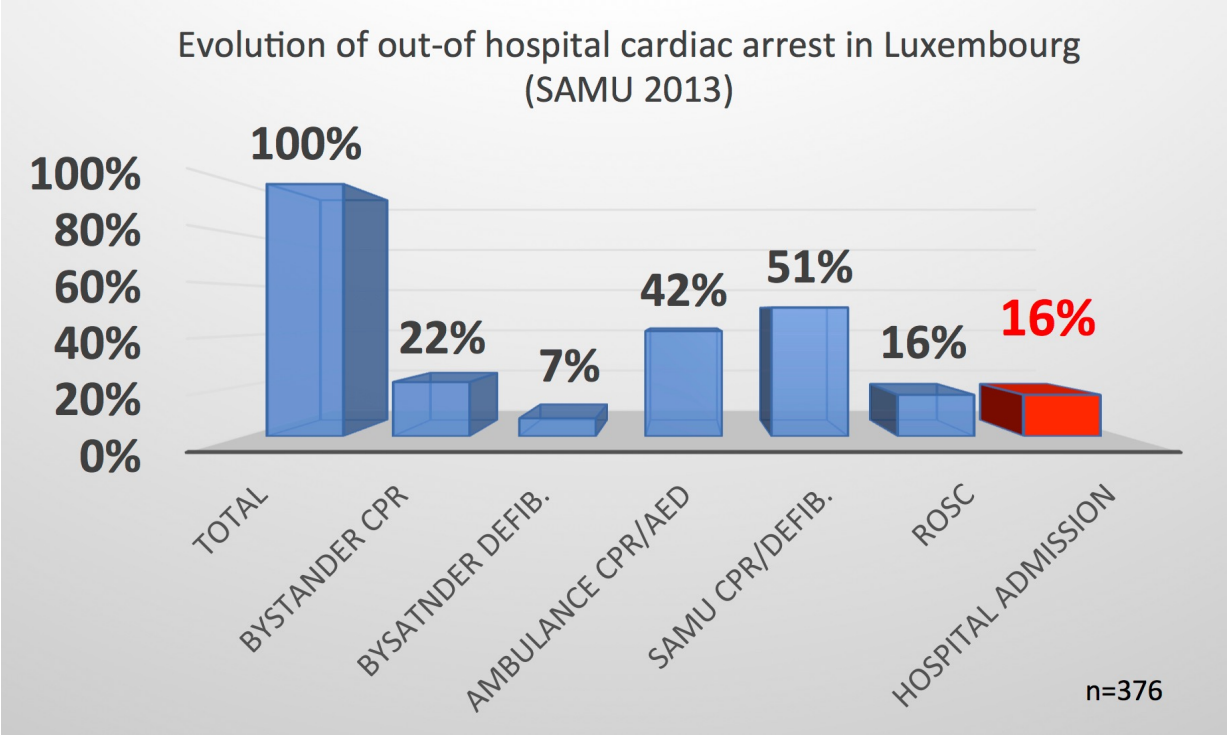


Figure 6: Interventions and early outcome of out-of-hospital cardiac arrest patients in Luxembourg. Query performed at the five SAMU services from Luxembourg in 2013. Source: unpublished data from the Luxembourg Resuscitation Council and P. Stammet.

By extrapolation and applying the actual best practice survival rates (Nielsen, Wetterslev et al. 2013), only 50% of those admitted to the hospital will in the end survive with a good neurological status. Thus in Luxembourg, only some estimated 8% of all OHCA patients will survive in good condition. This dramatically low survival rate is unfortunately encountered in many parts of the western world and highlights the sad impact of sudden cardiac arrest. But there is hope as in some areas, efforts to train the lay population to basic CPR and the use of AEDs show very promising results. In these regions survival rates have been doubled or even tripled due to the implementation of modern resuscitation techniques (Adielsson, Hollenberg et al. 2011, Wissenberg, Lippert et al. 2013). With the exception of these and some other limited areas survival figures are nearly all within the same very low magnitude. Public research and education efforts should be more directed to this field in order to increase the

proportion of patients surviving this critical condition. In this context, the new guidelines focus on improving outcome including different measures like phone CPR, public access AED, teaching of school children, etc.

The existence of severe brain damage after cardiac arrest remains the main matter of concern, as modern and advanced intensive care can substitute nearly every vital organ: lungs and heart can be supported via respirators, vasopressors, inotropes, IABP, assist devices or eventually ECMO and the kidneys can be supported by renal replacement therapies,… but the brain cannot be supported or temporarily “replaced” as no recovery of a severely injured brain is possible!

Most patients that die within the first days after cardiac arrest do so because of refractory heart failure. Those who survive these first 2-3 days and that will nevertheless die, do so because of confirmed or even supposed irreversible, severe brain damage. Owing to the improvements in modern high end intensive care (ECMO, assist devices, …) the majority of deaths after cardiac arrest can be attributed to brain damage (Laver, Farrow et al. 2004). Even more strikingly, most deaths within the first 5 days seem to be due to withdrawal of life supporting therapies (WLST) (Dragancea, Rundgren et al. 2013, Lemiale, Dumas et al. 2013).

1.5.1 Measurement of neurological outcome

Measurement of outcome after cardiac arrest relies on different scales. The most used is the Cerebral Performance Category score which has evolved from the original one described by Jennett (Jennett and Bond 1975, 1986). This five level score allows reliable, reproducible, but somehow subjective and crude assessment of neurological outcome.

Cerebral Performance Categories Scale

CPC Scale

Note: If patient is anesthetized, paralyzed, or intubated, use “as is” clinical condition to calculate scores.
CPC 1. Good cerebral performance: conscious, alert, able to work, might have mild neurologic or psychologic deficit.
CPC 2. Moderate cerebral disability: conscious, sufficient cerebral function for independent activities of daily life. Able to work in sheltered environment.
CPC 3. Severe cerebral disability: conscious, dependent on others for daily support because of impaired brain function. Ranges from ambulatory state to severe dementia or paralysis.
CPC 4. Coma or vegetative state: any degree of coma without the presence of all brain death criteria. Unawareness, even if appears awake (vegetative state) without interaction with environment; may have spontaneous eye opening and sleep/awake cycles. Cerebral unresponsiveness.
CPC 5. Brain death: apnea, areflexia, EEG silence, etc.

Safar P. Resuscitation after Brain Ischemia, in Grenvik A and Safar P Eds: Brain Failure and Resuscitation, Churchill Livingstone, New York, 1981; 155-184.

Table 1: Cerebral performance category scores as suggested by P. Safar.

Other studies report outcome based on the Glasgow Outcome Scale (GOS), which corresponds exactly to the CPC score, but in reverse order. Additionally, the GOS extended (GOSE) has been advocated. This scale has 3 more items, to stratify each degree of disability as low or high, and ranges from 1 to 8 (Jennett, Snoek et al. 1981).

GOS-E 1	Death
GOS-E 2	Vegetative state
GOS-E 3	Severe disability, lower end
GOS-E 4	Severe disability, upper end
GOS-E 5	Mild disability, lower end
GOS-E 6	Mild disability, upper end
GOS-E 7	Good recovery, lower end
GOS-E 8	Good recovery, upper end

Table 2: Glasgow outcome scale-extended. Adapted from (Jennett, Snoek et al. 1981)

Another way of reporting outcome is using the Rankin scale which has been suggested initially in 1957 by Rankin. For the purpose of a study on stroke, this scale has been adapted and this modified Rankin scale (mRs) is now also sometimes used for outcome determination after cardiac arrest (van Swieten, Koudstaal et al. 1988). It ranges from 0 to 6, the lower the value, the better the outcome.

The current Utstein reporting template for cardiac arrest recommends the use of either the CPC or the mRS for outcome assessment at either hospital discharge or at 30 days after the cardiac arrest (Perkins, Jacobs et al. 2015). CPC score 1 and 2 and mRS score from 0-3 are considered good outcome (Becker, Aufderheide et al. 2011). In our work, and in accordance with these recommendations, we constantly used the CPC scale at 6 months for outcome assessment.

More in-depth tools for cognitive outcome and quality of life are under evaluation, but these measurements go beyond the scope of the present work (Cronberg, Lilja et al. 2015, Lilja, Nielsen et al. 2015).

1.6 The burden of cardiac arrest

With some hundred thousand deaths from sudden cardiac death (SCD) in Europe, SCD has a major impact on our societies. Besides the long term burden on the society itself, in the acute phase, one single cardiac arrest binds numerous health care professionals. Starting with the emergency call dispatcher assisting the bystander via phone CPR and thus unable to respond to other emergency calls. First responders, ambulance teams and doctor staffed emergency vehicles rush to the scene and often have to resuscitate under difficult conditions. In the hospitals, emergency physicians care for the patient in the emergency room and prepare for subsequent care. Interventional cardiologists readily perform percutaneous coronary interventions if indicated at every day and night time. Cardiac surgeons and perfusionists implant mechanical assist devices and ECMOs. Finally, ICU physicians and anaesthesiologists care for those patients by implementing target temperature management and best practice intensive care. Thus one single patient, with all uncertainties about neurological outcome, binds huge amounts of health care resources. A reduction in incidence or a less severe post cardiac arrest syndrome due to better quality initial bystander CPR and AED use, could perhaps reduce the need for such resources (Nichol, Huszti et al. 2009). The less sick the patient, the lower the resources needed. Binding of all these resource has a major economic impact (Berdowski, Kuiper et al. 2010).

Finally, a sudden cardiac arrest is by definition, sudden, unexpected and the suffering of the families and relatives is enormous. A loved one is all of a sudden torn out of one's life. Many relatives would appreciate an early "hint" of prognostication to help them to bridge this awful period of waiting and uncertainty about the neurological status of their relative. The period of waiting imposed by TTM and sedation, or during the PCAS shock, is what most relatives experience as extremely stressful. Some relatives might feel hope in desperate cases and others fear of death in some rather good cases. Reliable early prognostication methods could allow the physicians to guide the suffering family in either keep up their hopes of a possible good outcome or rather prepare the relatives to a possible bad outcome.

With the increase of successfully resuscitated patients after cardiac arrest, the potential impact on the society is a dual one; on the one hand, many more lives can be saved and if they recover their previous mental status and place in the society, it is a real win-win situation. On the other hand, if more and more people are resuscitated that in the end have a severe impairment, the society has lost on all aspects.

Amongst the survivors, the proportion of those returning to their previous level of occupation at 6 months is only around 46%, with a maximum of around 65% of survivors returning to some level of work (Cronberg, Lilja et al. 2015, Lilja, Nielsen et al. 2015). This leaves some quite large proportions of patients, mostly of the active population, outside of the money generating circuit. Bearing this in mind, the health care resources needed to be mobilized and the socio-economic impact, SCD constitutes a huge and probably underestimated burden to our society. Proper outcome prediction might allow to select only those patients with a fair chance of neurological recovery and thus limit the burden on the society.

2. PREDICTION OF OUTCOME AFTER CARDIAC ARREST

2.1 Importance of outcome prediction/prognostication after cardiac arrest

The post cardiac arrest shock is part of the PCAS, often dominated by heart failure (myocardial infarction or myocardial stunning) and numerous techniques and support devices exist to support the failing heart, either as a temporary bridge or as a definite therapy. Restoration of blood flow may allow the heart to recover to a certain degree. Other vital organs like the lungs, the kidneys and, to a lesser degree, the liver can also be supported.

But the most vulnerable organ after cardiac arrest is the brain, as it may have suffered from hypoxic-ischemic brain damage during the no or low flow phase. Even after the restauration of a sufficient blood flow and oxygenation, the brain may not recover, but may also experience a worsening of the damage caused by the ischemia-reperfusion injury.

Prognostication of outcome after cardiac arrest is important for therapeutic and follow-up guidance. Healthcare resources, especially those requiring a high level of technicity or are time consuming, should not be allocated to patients with a futile prognosis. Also patients with poor outcome should be efficiently oriented and in a timely manner to nursing facilities or other long term wards in order to keep acute care beds available for those most in need. But, prognostication and especially neurological outcome prediction after CA is very difficult. Not only the correct evaluation of brain function during the ICU stay is difficult enough, also many factors might influence outcome after initial successfully resuscitated cardiac arrest. Comorbidities as well as per-arrest factors play a crucial role and have also to be taken into consideration (Bro-Jeppesen, Kjaergaard et al. 2009).

Even some high impact studies on cardiac arrest had no predefined criteria for continuation or, more importantly, for withdrawal of life supporting therapies. The landmark hypothermia papers (Bernard, Gray et al. 2002, Hypothermia after Cardiac Arrest Study 2002) did neither report WLST nor prognostication methods. In contrast, the recently published TTM-trial (Nielsen, Wetterslev et al. 2013) had a clear protocol for prognostication and eventually WLST. The absence of withdrawal criteria in most of the high impact papers makes conclusions with regard to prognostication very difficult. In order to establish rules for transparent prognostication, the rules of WLST must be clearly defined.

This major issue of lack of reporting of prognostication methods, even worse, supposing of poor prognosis potentially leading to early and unjustified WLST (Perman, Kirkpatrick et al. 2012). Interestingly, the rate and causes for WLST are most often not reported. In this context, the risk of self-fulfilling prophecy is major (Sandroni, Cavallaro et al. 2013, Sandroni, Cavallaro et al. 2013). In the presence of some reputed bad outcome signs, like early myoclonus or some abnormal early performed neurophysiological tests, some physicians might perhaps tend to stop life supporting therapies. Whereas, in recent years it has been shown that delaying prognostication, treating seizures and thus delaying WLST does not only increase the number of survivors, but most importantly does not result in more patients with bad neurological outcome (Sunde, Dunlop et al. 2006, Bro-Jeppesen, Kjaergaard et al. 2009).

A recent survey on prognostication methods after cardiac arrest used in actual clinical practice revealed that there is no consensus and even a fearsome heterogeneity among centers regarding prognostication (Friberg, Cronberg et al. 2015). The absence of clear guidelines on this topic was probably the co-responsible for this undesirable and potentially prejudicial condition in some inexperienced centers. Fortunately, recently these guidelines have been published and with this thesis we try to contribute further to outcome prediction (Sandroni, Cariou et al. 2014, Nolan, Soar et al. 2015).

2.2 Means of prognostication

2.2.1 Demographic and peri-arrest data and clinical neurologic examination

Amongst the many different prognostication tools, clinical peri-arrest parameters must be taken into account. The generally accepted Utstein template sets the standard for reporting those essential parameters (Perkins, Jacobs et al. 2015). Especially, the time from collapse to start of CPR ("no flow" time) and time from start of CPR to ROSC ("low flow" time), are very often indicated as the major pre-hospital indicators of outcome (Bro-Jeppesen, Kjaergaard et al. 2009). Other clinical parameters include age, gender, type of initial rhythm, etc. But besides mitigated results, the sensitivity of those parameters is rather low making them unsuitable for individual outcome prediction.

The most used brainstem reflexes after cardiac arrest are the pupillary light reflex (PLR) and corneal reflex. While fixed pupils on admission are not reliable, the absence of a PLR at 72 hours or later is most often associated with a poor outcome (Bouwes, Binnekade et al. 2012). The prognostic value of the absence of corneal reflexes is even lower.

Motor responses to pain is another parameter studied for outcome prediction. Here too, early assessment of motor response is not able to provide accurate prognostication (Al Thenayan, Savard et al. 2008). The presence of extensor or absent motor response to a painful stimulus is a bad sign, but nevertheless the accuracy of these signs is too low to be of clinical relevance (Rossetti, Oddo et al. 2010). Furthermore, the impact of sedation on clinical examination should not be forgotten as motor responses may be influenced by remainders of sedation and or muscle relaxants (Samaniego, Mlynash et al. 2011).

Myoclonus may originate from anoxic brain damage may be due to seizures or even status epilepticus, but may also be present even in the absence of electroencephalographic signs of seizures. Furthermore, it is sometimes not easy to distinguish between myoclonus, seizures and shivering, all three entities being quite common in patients treated with target temperature management (Seder, Sunde et al. 2015). As such, and mainly because of different definitions and the many confounding factors, myoclonus, without exploratory neurophysiological tests cannot be recommended as clinical outcome predictor whereas only confirmed, early status myoclonus can be (Nolan, Soar et al. 2015).

The major flaw with these parameters is that for a single patient too many confounding factors may exist and limit their use. None of these parameters yields FPR lower than 5% with narrow confidence intervals.

2.2.2 Biomarkers of brain damage

The most known and used biomarkers in the context of prognostication after cardiac are neuron specific enolase (NSE) and protein S100b.

NSE is a 78kDa dimeric glycolytic enzyme involved in glucose metabolism and is mainly found in neuronal and neuroendocrine cells. Its biological half-life is approximately 24 hours. NSE is also found in platelets and red blood cells, cautioning the results when hemolysis is present. NSE has first been described in 1978 by Schmechel et al. (Schmechel, Marangos et al. 1978). Neuronal damage accompanied by blood-brain barrier (BBB) disruption will result in NSE release first into the cerebrospinal fluid (CSF) and ultimately in the blood stream.

NSE has been studied for prognostication in cardiac arrest since 1989 (Roine, Somer et al. 1989), initially in the cerebrospinal fluid and then for practical reasons in the circulating blood. Many studies have evaluated NSE in the pre hypothermia era suggesting its usefulness as outcome predictor (Zandbergen, Hijdra et al. 2006). Since the advent of induced hypothermia and target temperature management, conflicting results have emerged. This has been the

result of studies with small patient sample sizes (Tiainen, Roine et al. 2003, Oksanen, Tiainen et al. 2009), heterogeneous populations (Daubin, Quentin et al. 2011), comparison to historical cohorts (Steffen, Hasper et al. 2010) or different outcome measurements (Cronberg, Rundgren et al. 2011).

Furthermore, NSE measurement is influenced by hemolysis, storage of frozen samples and the assay used for dosing (Mlynash, Buckwalter et al. 2013, Rundgren, Cronberg et al. 2014). All these methodological issued raised the concern of limited reliability of NSE as outcome predictor and called for a multicenter study on this biomarker.

S100 protein is an intracellular calcium-binding protein (dimer) with a molecular weight of 21kDa. It is mainly cleared by the kidneys and its half-life is approximately 2 hours. In human tissues there are at least 4 subtypes that are expressed among which the S100b (found in astroglial and Schwann cells) is the target sub-type used in prognostication. Two subtypes can be found in humans and are measured by actual tests, the dimers S100AB and S100BB. As its molecular weight is lower and its half-life shorter than that of NSE, it is expected that S100 can be found earlier in the blood stream. Furthermore, small amounts of S100 can be found as homodimer or heterodimer in adipocytes, muscle cells and chondrocytes. As circulating amounts of S100 are much lower than NSE, those extra cerebral origins of S100 might have a larger impact on false positive values than for NSE.

Other biomarkers have been used for outcome prediction but their use and utility remain rather sparse.

Creatinine kinase-BB isoenzyme (CK-BB) (Sherman, Tirschwell et al. 2000) has not been investigated any further as it seems to be to less specific and of limited usefulness as outcome predictor. Glial fibrillary acidic protein (GFAP) (Kaneko, Kasaoka et al. 2009, Larsson, Wallin et al. 2014) might be a more promising biomarker but convincing data are still missing. Markers like Tau (Randall, Mortberg et al. 2013) and neurofilament light chain (NFL) (Rana, Schroder et al. 2013), both brain specific biomarkers, are currently under investigation and larger cohorts are required to validate the results from small single center studies.

Biomarkers of inflammatory response and cardiac function are not subject of this thesis and can be reviewed elsewhere (Scolletta, Donadello et al. 2012).

Emerging biomarkers: micro-RNA and long non coding RNAs

“New” and innovative biomarkers are currently under investigation. Amongst them micro ribonucleic acids (RNA) also called miRNA or miR seem to be a new promising category. MiRNAs are small non coding RNAs with about 21 nucleotides. More than 2500 different miRNAs have been identified in humans. Their major role is either the inhibition of gene translation or the destruction of messenger RNA, both pathways leading to a downregulation of target gene expression. MiRNAs start to be extensively described after myocardial infarction or cardiac failure and are now being studied in cardiac arrest populations (Fiedler and Thum 2013). Not only they should be considered as pure biomarkers or biosignatures of the extend of brain damage, but because of their inherent function, mainly as inhibitors of RNA expression and transcription, they might also constitute potential therapeutic targets in the ischemia-reperfusion phase after cardiac arrest (Devaux, Stammet et al. 2015).

2.2.3 Neurophysiology

Electroencephalography (EEG)

Electroencephalography (EEG) in the setting of cardiac arrest is used for detection of seizures and postanoxic status epilecticus (PSE), which have an incidence of 10-40% and are strongly associated with a poor prognosis (Sandroni, Cariou et al. 2014, Nolan, Soar et al. 2015). Two major problems with EEG as outcome predictor arise: pathologic EEG patterns are not

necessarily associated with poor outcome and perhaps even more importantly, there is a large interrater variability in the interpretation of pathologic EEG pattern (Westhall, Rosen et al. 2015). Interestingly, even quite simple and obvious pathologic EEG patterns have quite different definitions and thus interpretation and comparisons of different studies is difficult.

Only highly pathologic pattern, like electroencephalographic status epilepticus have found their way into the guidelines (Wijdicks, Hijdra et al. 2006). More recently, an attempt to standardize the EEG classification has been done rising the hope of a future uniform description of EEG patterns after cardiac arrest (Westhall, Rossetti et al. 2016).

Background reactivity and reactivity to painful stimuli seem to increase the sensitivity of EEG predictive power, but this has not yet been tested in large multicenter trials (Rossetti, Oddo et al. 2010, Thenayan, Savard et al. 2010). Current guidelines do no advocate EEG reactivity as reliable outcome predictor.

Furthermore, it is not known if intermittent EEG recordings are sufficient to detect significant EEG abnormalities as just short periods of more or less 30 minutes are covered.

Continuous (cEEG) and amplitude integrated EEG (aEEG) may partially overcome these weaknesses, but still require specially trained physicians for interpretation of the data (Rundgren, Westhall et al. 2010, Friberg, Westhall et al. 2013).

Because of the absence of a standardized grading system for interpretation of EEGs for outcome prediction, overall EEG yields a low sensitivity and specificity.

Bispectral index

Bispectral index (BIS) is a simplified and automated EEG transformation initially designed to monitor the depth of anaesthesia, that has shown some interest in outcome prognostication (Shibata, Imota et al. 2005, Stammet, Werer et al. 2009). BIS is easy to use as it only requires the placement of a set of electrodes on the forehead of the patient and a check of the signal quality. An internal, publicly unrevealed algorithm uses a Fourier transformation to calculate the value of the BIS. Values range from 0 to 100, with 0 being an isoelectric EEG, values of 40-60 correspond to the normal depth of anaesthesia and values above 80 are indicative of insufficient anaesthesia (awakening). The main advantage of this technique is the absence of qualified personnel to interpret the data. On the other hand, BIS is unable to distinguish the pathologic patterns which frequently occur during cardiac arrest. More recently, other studies have shown interesting and non conflicting results to the prior research, opening the way to further investigate this technique in the context of CA (Seder, Fraser et al. 2010). Especially, the possibility to detect early poor outcome patients is appealing (Seder, Dziodzio et al. 2014).

Somatosensory evoked potentials

SSEPs are evoked potentials on the median nerve stimulation with recordings on the cortical level. They reflect the integrity of the nervous system from the brain to the periphery. SSEPs are not influenced by sedation, unlike EEG, and robust data exists that bilaterally absent N20 peaks bear a very high likelihood of poor prognosis (Bouwes, Binnekade et al. 2009, Nolan, Soar et al. 2015). Therefore, they are another widely used prognostication method. SSEPs are unfortunately not available in every hospital and they need specialized personnel to perform them and trained physicians for interpretation.

2.2.4 Imaging

Amongst imaging techniques, the computer tomography scan (CT scan) is the most widely available and used. Generally a CT scan is performed upon admission, mainly to rule out a neurologic etiology of the cardiac arrest. The main finding is general brain swelling with signs of brain herniation. If, on admission, these signs are present, the prognosis is obviously very poor. But only very few patients show these kinds of signs. Many more patients will present some degree of loss of gray and white matter differentiation. Unfortunately, a universally accepted definition of this dedifferentiation is lacking. The main interest of a CT scan in prognostication after cardiac arrest are the detection of brain swelling and eventually herniation (Sandroni, Cavallaro et al. 2013).

Magnetic resonance imaging (MRI) is another frequently used imaging tool, ideally performed around 5 days after the cardiac arrest. Hypoxia-ischemia induced lesions are mainly represented in diffusion weighted imaging (DWI) and might be quantitatively evaluated using absolute diffusion coefficient. Functional imaging may further enhance the sensitivity of the examination. Definitely, MRI is superior to CT scan after hypoxic brain injury. It may also allow to determine such a damage when other prognostic marker show mitigated results (Sandroni, Cavallaro et al. 2013), but performing an MRI in sedated and/or ventilated ICU patients is time consuming and cumbersome.

2.2.5 Combination of parameters

Efforts have also been made to combine different parameters to improve outcome prediction. One of the first attempts was done by Adrie using mainly pre-hospital and immediately available parameters to calculate the outcome at a very early stage (Adrie, Cariou et al. 2006). But this score never really found its application, as it was rather a general severity score with a calculation of a probability rather than an individual outcome prediction score (Sunde, Kramer-Johansen et al. 2007, Oksanen, Tiainen et al. 2009).

Other groups suggested combining clinical examination, EEG analysis with reactivity tests, SSEPs and NSE (Oddo and Rossetti 2014). In this expert group, clinical examination combined to reactivity testing on the EEG and NSE (using a cut-off of 33ng/ml) revealed the best outcome prediction performance (AUC 0.89 for mortality and 0.88 for outcome), whereas adding SSEPs did not improve the model's performance. These data emerge from one single (EEG) expert center and confirmatory studies as well as the proof of generalizability still lack. Another study comparing CT scan and NSE also concluded to an improved combined effect of both parameters above each one alone (Lee, Jeung et al. 2013). Here too, data came from a single center and expert assistance in the interpretation of CT scan images regarding gray/white matter differentiation was required.

2.3 When to prognosticate? Very early, early or “late”?

Timing of prognostication is of paramount importance. Especially when initiating heavy intensive care, binding many resources, to a patient with multiple organ failure after cardiac arrest, the fear of a poor neurologic outcome is omnipresent. In this particular setting a triage method to help to select patients most likely to benefit from the intensive care would be pivotal. In fact very few prognostic markers fulfil such criteria.

On admission, clinical and demographic data, although associated with outcome are neither specific, nor sensitive enough to reliably base a therapeutic action on them. For instance, patients with prolonged resuscitation measures, having had a bystander witnessed cardiac arrest certainly may have prolonged time to ROSC, but if high quality CPR is continuously provided, they might still experience good neurological outcome despite the prolonged time to ROSC. Mechanical CPR devices also allow transportation of cardiac arrest victims under CPR to the catheterization facility. Although time to ROSC is prolonged in these cases, survival in good condition is still possible (Rubertsson, Lindgren et al. 2014).

Biomarkers do not perform very well too as studies are generally performed on serial biomarker measurements starting at time zero (generally when the patient is admitted to the hospital or ICU) and then harvested every 12-24 hour. Biomarkers have a dynamic evolution of their peak or discriminative values. As such, biomarkers are not the best prognosticators in the very early phase. No biomarker described to date has the ability to reliably predict outcome before 24 hours after CA or ROSC. Even at 24 hours, sensitivity and specificity are quite low, so that the real impact of those markers is certainly later than the first 24 hours.

Finally, neurophysiology might bring an answer. Even though it has been reported that early EEG, especially flat or very low voltage, is not a reliable outcome predictor (Rundgren, Westhall et al. 2010). More recent studies suggest that the processed EEG by a BIS monitoring might perhaps give some indications for the very early “triage” prognostication (Seder, Dziodzio et al. 2014).

Besides the triage or very early prognostication that can be used as guidance for more invasive intensive care treatment, the general prognostication of outcome can only be performed “reliably” at 48 or 72 hours after CA. Many tests are available, but still caution is recommended as not many yield a high specificity and many have low sensitivity.

Once the acute phase of the disease has passed, the prognostication should be performed using a bundle of different tests. Ideally, a combination and a serial performance of clinical and demographic data, biomarkers, neurophysiological tests and perhaps imaging studies should be used (Sandroni, Cariou et al. 2014, Nolan, Soar et al. 2015).

2.4 Problems with prognostication

Initially, many prognostication tools have been studied in “normothermic” patients, but a major confounding factor was added in 2002, when the 2 landmark studies introduced “mild therapeutic hypothermia”. With reducing the core body temperature, metabolism in general and brain metabolism in particular, is slowed down and the exact impact of TTM on prognostication has become unclear. The literature also yielded conflicting results and before 2014 (?!), no adapted guidelines to the targeted temperature existed (Sandroni, Cariou et al. 2014). This has further been highlighted in a recent survey where it could be shown that physicians are facing major uncertainties with regards to prognostication after cardiac arrest (Friberg, Cronberg et al. 2015).

In fact, the seminal hypothermia publications had major impacts, not only by introducing the concept of TH, but perhaps even more importantly, by focusing the attention of the intensive care community to the CA patients. All of a sudden, treatment of those patients formerly easily considered as futile, became challenging and even more important, survival rates of around 50% could be achieved. This lead also to the reconsideration of the formerly accepted criteria for prognostication. In fact, hypothermia does not only slow down the metabolism of the brain and the whole organism, but might also interfere with drug clearance. Furthermore, in order to perform therapeutic hypothermia, patients need to be sedated and even treated with neuromuscular blockade. Sedation might be a powerful confounder when using neurophysiological tests, as sedation per se acts on the brain and alters especially EEG. SSEP are less affected and latest publications seem to indicate that they are also quite unaffected by lowering the temperature (Oddo and Rossetti 2011). Of course, clinical neurological examination might also suffer from delayed drug clearance (liver metabolism slowed down) on an altered brain.

In biomarker studies, the influence of temperature remains also unclear as different studies yielded conflicting results. Most suffer from methodological weaknesses like small sample size, heterogeneous populations, comparisons to historic controls, or are observational studies. The exact impact of the target temperature on NSE, S100 and other biomarkers has not been determined so far.

3. OBJECTIVES OF THIS THESIS

The goal of this thesis is to contribute to an improvement of outcome prediction after cardiac arrest. In particular, the existing and routinely used biomarkers present areas of uncertainty; the cut-off values, the optimal timing of measurement and their evolution over time as well as the potential impact of target temperature remain unclear. We describe a new class of biomarkers that might further improve the accuracy and enlarge the panel of outcome predictors after cardiac arrest. We also studied a simplified electrophysiology method bearing the potential for early and accurate outcome prediction.

In particular, we aimed

- **To refine the role of existing biomarkers NSE and S100 for outcome prediction after cardiac arrest**
- **To study the influence of temperature on the levels of NSE and S100 after cardiac arrest**
- **To test and improve a simplified neurophysiological approach for outcome prediction after cardiac arrest**
- **To study the possibility for early outcome prediction within 24 hours after cardiac arrest**
- **To evaluate the combination of biomarkers and a neuro-physiological measure for outcome prediction after cardiac arrest**
- **To search for novel biomarkers in cardiac arrest**

4. METHODS

4.1 Background data

All demographic data have been collected using the Utstein template in order to have standardized data in accordance with international guidelines (Perkins, Jacobs et al. 2015). This allows us to present data that are comparable to other publications conforming to those guidelines. In all our publications outcome was presented according to the cerebral performance categories. CPC 1 corresponds to no neurological disability, CPC 2 to a minor neurological deficit, CPC 3 to severe neurological impairment with a dependency in everyday life, CPC 4 to coma and CPC 5 corresponds to death. CPC 1 and 2 were always regarded as good outcome whereas CPC 3 to 5 were always regarded as bad outcome.

All studies have been approved either by the national ethics committee on research (CNER, Comité National d'Éthique de Recherche, Luxembourg) or by the respective ethics review boards according to the local legislations in the case of multinational studies.

Papers I and II were predefined sub-studies of the Target Temperature Management trial (TTM-trial) (N° CNER 201007/05, ClinicalTrials.gov number, NCT01020916) (Nielsen, Wetterslev et al. 2013).

The TTM-trial was a multi-center, randomized, parallel-group, assessor-blinded, monitored, and investigator-initiated clinical trial. The study population consisted of 950 adult, unconscious patients with return of spontaneous circulation after out of hospital cardiac arrest included from November 2010 until January 2013. Randomization was performed through a web-based system, assigning patients in a 1:1 ratio to either 33°C or 36°C target temperature management (Nielsen, Wetterslev et al. 2012). Patients older than 18 years of age, comatose (Glasgow Coma Scale < 8) at hospital admission after OHCA with presumed cardiac cause were included. Patients needed to have sustained ROSC for more than 20 minutes. Patients were excluded if randomization was not performed within 240 min after ROSC, if the initial rhythm was unwitnessed asystole, and if the patient remained in refractory shock. TTM was initiated as soon as possible (4 hours foreseen to reach the allocated target temperature), then the target temperature was maintained during 24 hours before a rewarming phase during 8 hours (rewarming at 0.5°C/h maximum). All patients were sedated, intubated and mechanically ventilated until the end of the 36 hours intervention period.

Active treatment was continued in all patients until blinded neurological evaluation had been performed 108 hours after arrest or later.

All data such as initial rhythm, witnessed arrest, bystander performing cardiopulmonary resuscitation (CPR) and time to ROSC were collected according to Utstein template.

The primary endpoint was all-cause mortality 6 months after the end of the trial (July 2013). The main secondary endpoint was poor neurological outcome or death defined as CPC 3 to 5 at hospital discharge and at 6 months.

Papers III, IV and V were based on the NorthPole (Biosignature to predict outcome after hypothermia for patients surviving cardiac arrest) cohort (N° CNER 200803/05). This local, non interventional project aims at discovering new biomarkers after cardiac arrest. All comatose patients admitted to the ICU of the Centre Hospitalier de Luxembourg (CHL) after a cardiac arrest irrespective of the location and initial rhythm were included. All patients were treated by targeted temperature management at 33°C during 24 hours. They were maintained sedated during the 24 hours cooling phase until slow rewarming to 37°C was completed. Then sedation was tapered if possible. Blood drawings took place at 48 hours after CA and at 7 days after CA. BIS monitoring is part of our standard treatment protocol since 2009.

4.2 Statistical analysis

Details of the statistical tests used can be found in detail in the corresponding papers. Missing values were handled using ten-fold multiple imputations in papers I, II and IV. To avoid overfitting, sensitivity and specificity were corrected for optimism using bootstrap internal validation. This technique is used to correct the performances (e.g. AUC, sensitivity, specificity…) of a predictive model for over-learning, which occurs when the same dataset is used to both set up and test the model. To do so, a simple random sampling with replacement is conducted numerous times. The "true" model is generated with the original sample, then the mean of differences between testing and learning set using bootstrap leads to the correction of optimism. This procedure is efficient, since the same number of patients is used for development and for validation. To obtain stable results, the procedure is repeated 100 times.

In papers I, II, IV and V we used reclassification tests to test the ability of a given prognostic marker to improve models already including other independent prognosticators (Pencina, D'Agostino et al. 2008).

A way to determine the possible effect of adding a variable to a model is to compute the Net Reclassification Improvement index (NRI).

The NRI index is a measure evaluating the added predictive value of a new variable of interest, e.g. a biomarker, to a reference model, e.g. a logistic regression, using patients' clinical characteristics.

The NRI is the difference between two components, namely the benefit of adding this new variable for patients having a poor outcome and the benefit for patients having a good outcome.

For patients having a poor (respectively good) outcome, this benefit is evaluated by computing the proportion of patients for which the probability of a poor outcome has increased (respectively decreased) when using the new model as compared to the former one ; to this number is further subtracted the proportion of those patients for which the probability has decreased (respectively increased).

Another measure to qualify the added value of a parameter is the Integrated Discrimination Improvement (IDI). By denoting IS the average "sensitivity of the model over all the possible cut-offs", and IP the average of "one minus specificity", IDI can be defined as the difference between the two models:

$$IDI=(IS\_new-IS\_old\ )-(IP\_new-IP\_old)$$

5. RESULTS

Detailed results of our studies can be found in the appended papers. We want to emphasize that in all our presented studies the overall outcome of patients admitted to the ICU after cardiac arrest was around 50 percent of survival with a good neurological status at 6 months.

Papers I and II were sub-studies of the TTM-trial which did not find any difference between allocated temperature groups regarding death at the end of the trial, neurologic outcome using CPC and mRs as well as no difference in any other secondary or tertiary endpoint (Nielsen, Wetterslev et al. 2013).

Papers III, IV and V report only non-interventional data from the NorthPole cohort.

5.1 Biomarkers

We wanted to investigate the role of existing biomarkers NSE and S100 as outcome predictors after cardiac arrest. Additionally, we aimed to determine the influence of target temperature on the studied biomarkers. Another goal was to try to identify new biomarkers.

5.1.1 Paper I: Neuron specific enolase (NSE)

Design and objectives

This prospective, observational, pre-defined sub-study of the TTM-trial aimed to investigate the prognostic value of NSE in two target temperature populations within a multi-center randomized clinical trial. We investigated the potential influence of target temperature on NSE levels and tried to clarify the role of NSE as outcome predictor.

Patients

686 patients from 29 different centers with out-of-hospital cardiac arrest, randomly allocated to either a target temperature of 33°C or 36°C were included.

Methods

Levels of NSE were assessed in blood samples obtained 24, 48 and 72 hours after return of spontaneous circulation by the electro-chemo-luminescent-immuno-assay (ECLIA) method. The primary outcome was neurological outcome at six months using the cerebral performance category score (CPC).

Results

700 patients were eligible for this sub-study. We analyzed 1823 blood samples from 686 different patients from 29 sites. 51% of the included patients had a good neurological outcome at 6 months.

NSE is predictive of outcome

We could show that median NSE values were significantly higher at each of the 3 time points of blood sampling in the poor vs. the good outcome groups. Median NSE values were 18 versus 35 ng/ml, 15 versus 61 ng/ml, and 12 versus 54 ng/ml for good versus poor outcome at 24, 48, and 72 h respectively ($p < 0.001$) (figure 7). Higher NSE levels were associated with increased mortality (figure 8).

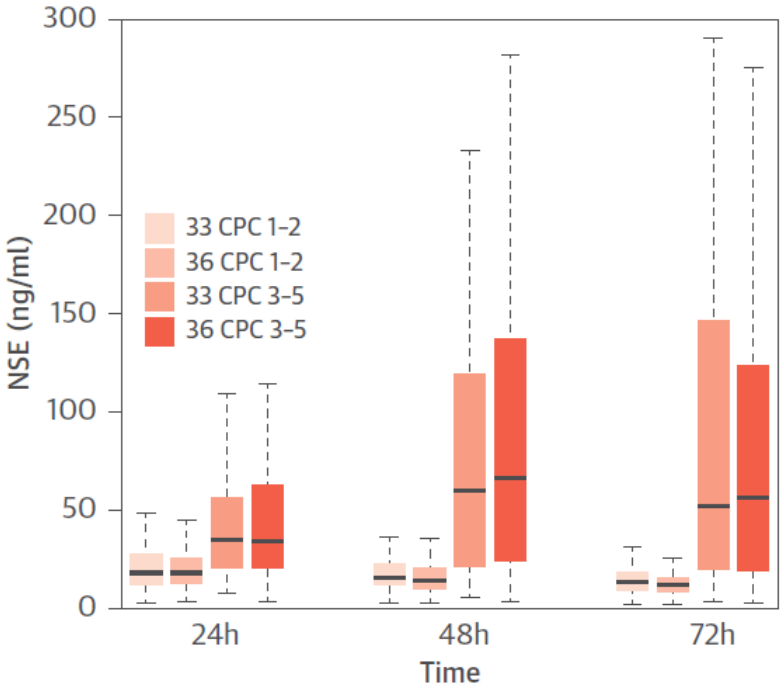


Figure 7: Boxplot representation of median NSE values in each temperature and outcome group. No statistical differences were found between temperature groups. Data are represented as interquartile range with upperand lower fence (q1-1.5(q3-q1) and q3 + 1.5*(q3-q1).*

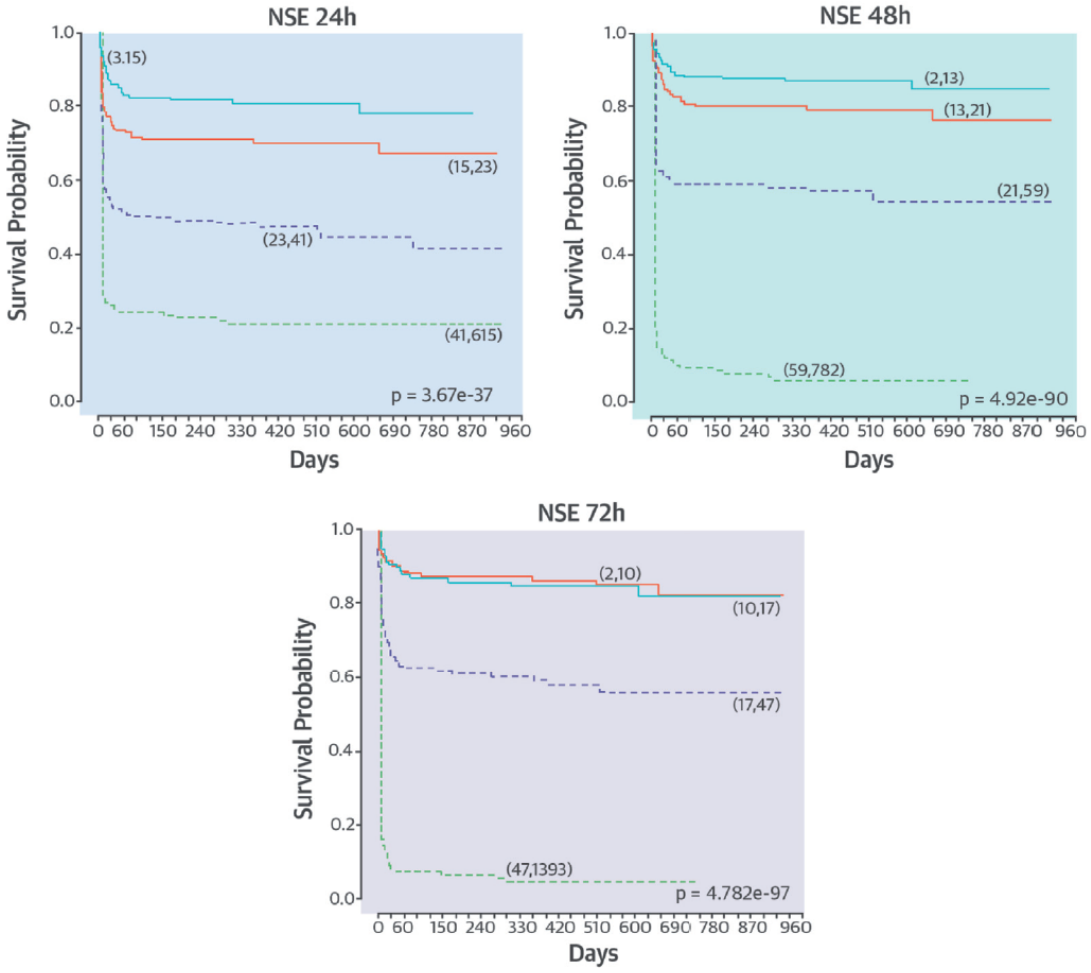
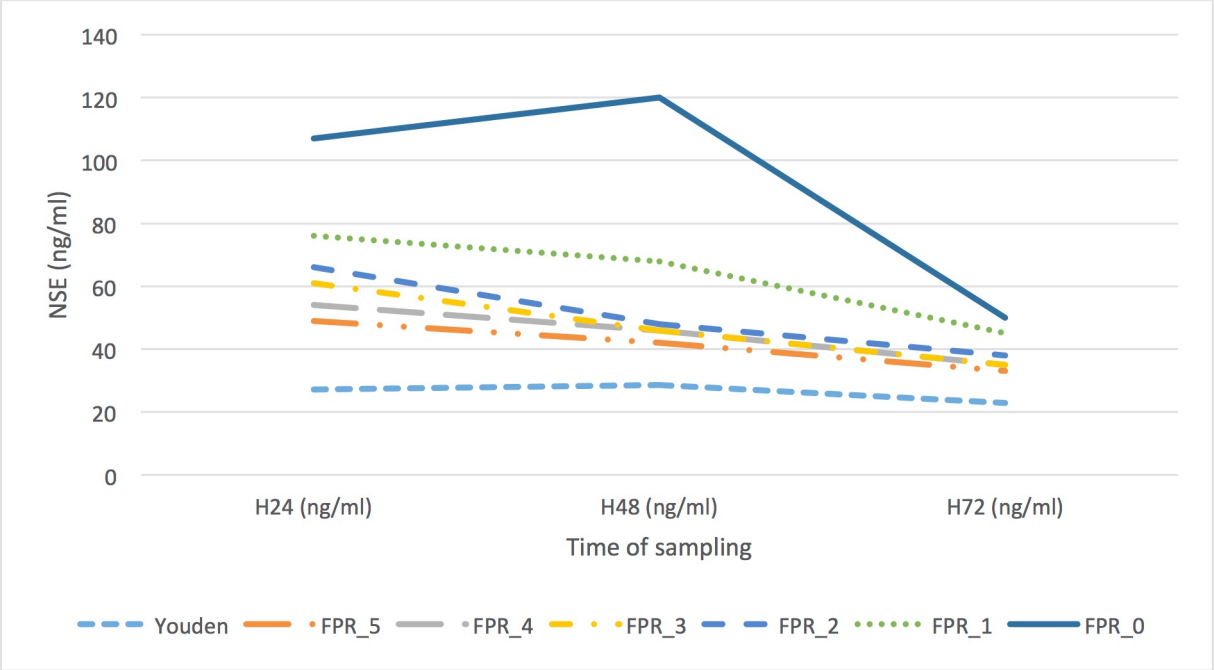


Figure 8: Kaplan-Meier survival curves of NSE values (ng/ml) divided into quartiles at the 3 time points.

An increase of NSE of 6 ng/ml between any 2 time points was associated with a poor outcome (specificity 94% and sensitivity 64% between 24 and 48 h; specificity 93% and sensitivity 39% between 48 and 72 h). A decrease of NSE of 3 to 4 ng/ml in median values between 2 consecutive time points was observed in the good outcome group.

At 24 hours after CA, NSE predicted outcome with an AUC of 0.75, whereas at 48 and 72 hours, the AUC increased to 0.85 and 0.86 respectively. Cut-off values for poor outcome are displayed below according to their respective false positive rates ranging from 0 to 5%.



	H24 (ng/ml)	Sensitivity	Specificity	H48 (ng/ml)	Sensitivity	Specificity	H72 (ng/ml)	Sensitivity	Specificity
Youden	**27**	0,60	0,76	**29**	0,69	0,87	**23**	0,70	0,88
FPR_5	**49**	0,33	0,95	**42**	0,61	0,95	**33**	0,63	0,95
FPR_4	**54**	0,29	0,96	**46**	0,60	0,96	**35**	0,62	0,96
FPR_3	**61**	0,24	0,97	**46**	0,59	0,97	**35**	0,63	0,97
FPR_2	**66**	0,21	0,98	**48**	0,58	0,98	**38**	0,58	0,98
FPR_1	**76**	0,15	0,99	**68**	0,47	0,99	**45**	0,54	0,99
FPR_0	**107**	0,09	1,00	**120**	0,27	1,00	**50**	0,52	1,00

Figure 9: Graphic representation of the calculated NSE cut-offs for poor outcome in our study population according to the false positive rate, ranging from 0% (upper curve) to 5% and Youden index (lower curve). Adapted from (Stammet, Collignon et al. 2015)

Figure 9 illustrates that when using low false positive rates (FPR), cut-off values for NSE tend to become very close. In our cohort, NSE values higher than approximately 50 ng/ml at 48 or 72 hours can be considered strongly predictive of poor outcome (FPR lower than 3 %). At 24 hours, values higher than 80 ng/ml have a FPR lower than 1% to predict poor outcome. Absolute outcome prediction (FPR of zero) needs much higher values because of outliers.

Multivariable analysis, combining serial NSE measurements, target temperature and baseline variables (age, gender, bystander cardiopulmonary resuscitation, first monitored rhythm, time to ROSC, lactate levels on admission, and circulatory shock), identified age, bystander CPR and initial shockable rhythm as independent outcome predictors. Serial NSE was also found to be a strong and independent predictor of poor neurological outcome (specificity 0.88; sensitivity 0.84).

Model with NSE and clinical variables	Effect	Odds ratio	Lower 0.95	Upper 0.95	p-value
Intercept	-6.853	0.001	0.0001	0.001	<0.0001
NSE 24h	**-0.034**	**0.966**	**0.945**	**0.989**	**0.0034**
NSE 48h	**0.040**	**1.041**	**1.012**	**1.070**	**0.0044**
NSE 72h	**0.066**	**1.069**	**1.033**	**1.105**	**0.0001**
Target temperature	0.247	1.280	0.796	2.059	0.3076
Age	**0.094**	**1.098**	**1.073**	**1.124**	**<0.0001**
Gender (male)	-0.307	0.735	0.408	1.326	0.3066
Bystander CPR performed	**-0.601**	**0.548**	**0.323**	**0.930**	**0.0257**
Shockable vs. non-shockable rhythm	**-1.276**	**0.279**	**0.134**	**0.580**	**0.0006**
ROSC after bystander defibrillation	-1.460	0.232	0.038	1.421	0.1142
Time from CA to ROSC	0.010	1.010	0.996	1.023	0.1534
Serum lactate level	0.012	1.012	0.950	1.078	0.7051
Shock on admission	0.332	1.394	0.661	2.939	0.3829

Table 3: multivariable analysis of admission parameters including NSE measured at 3 time points. Independent variables are represented in bold.

Target temperature level does not affect NSE values

We could show that the level of targeted temperature did not significantly affect NSE values nor cut-offs at any time point (figure 7).

Conclusions

Serial and high NSE values are robust and independent predictors of poor neurological outcome after out-of-hospital cardiac arrest. Targeted temperature does not significantly affect the predictive value of NSE.

5.1.2 Paper II: Protein S100 (S100)

Design and objectives

This study was a prospective, observational, pre-defined sub-study of the TTM-trial aiming at investigating the prognostic value of the protein S100 in two target temperature populations within a multi-center randomized clinical trial. We aimed to clarify the role of S100 as outcome predictor including a comparison to NSE and also to investigate the potential influence of target temperature on S100 levels.

Patients

We included 687 patients after out-of-hospital cardiac arrest, originating from 29 different centers, and randomly allocated to either a target temperature of 33°C or 36°C.

Methods

Levels of S100 were assessed in blood samples obtained 24, 48 and 72 hours after return of spontaneous circulation by the ECLIA method. The primary outcome was neurological outcome at six months using the cerebral performance category score (CPC).

Results

700 patients were eligible for this sub-study. We analyzed 1843 blood samples from 687 different patients. 51% of the included patients had a good neurological outcome at 6 months.

S100 is predictive of outcome

We showed that median S100 values were significantly higher at each of the 3 time points of blood sampling in the poor vs. the good outcome groups: 0.19 [IQR 0.10-0.49] versus 0.08 [IQR 0.06-0.11] µg/L, 0.16 [IQR 0.10-0.44] versus 0.07 [IQR 0.06-0.11] µg/L and 0.13 [IQR 0.08-0.26] versus 0.06 [IQR 0.05-0.09] µg/L (all p<0.001) at 24, 48 and 72 hours respectively. Higher S100 levels were also associated with increased mortality.

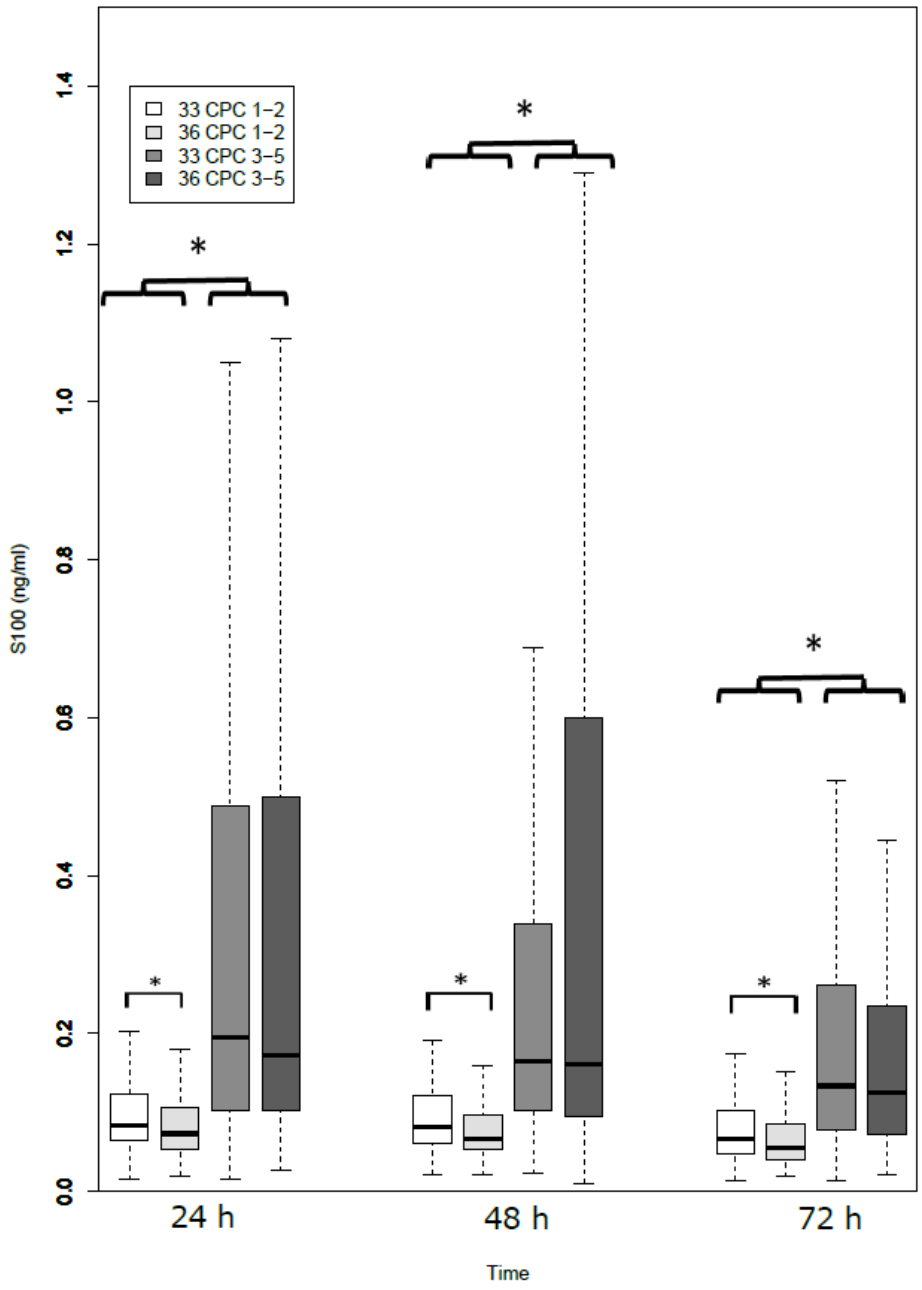
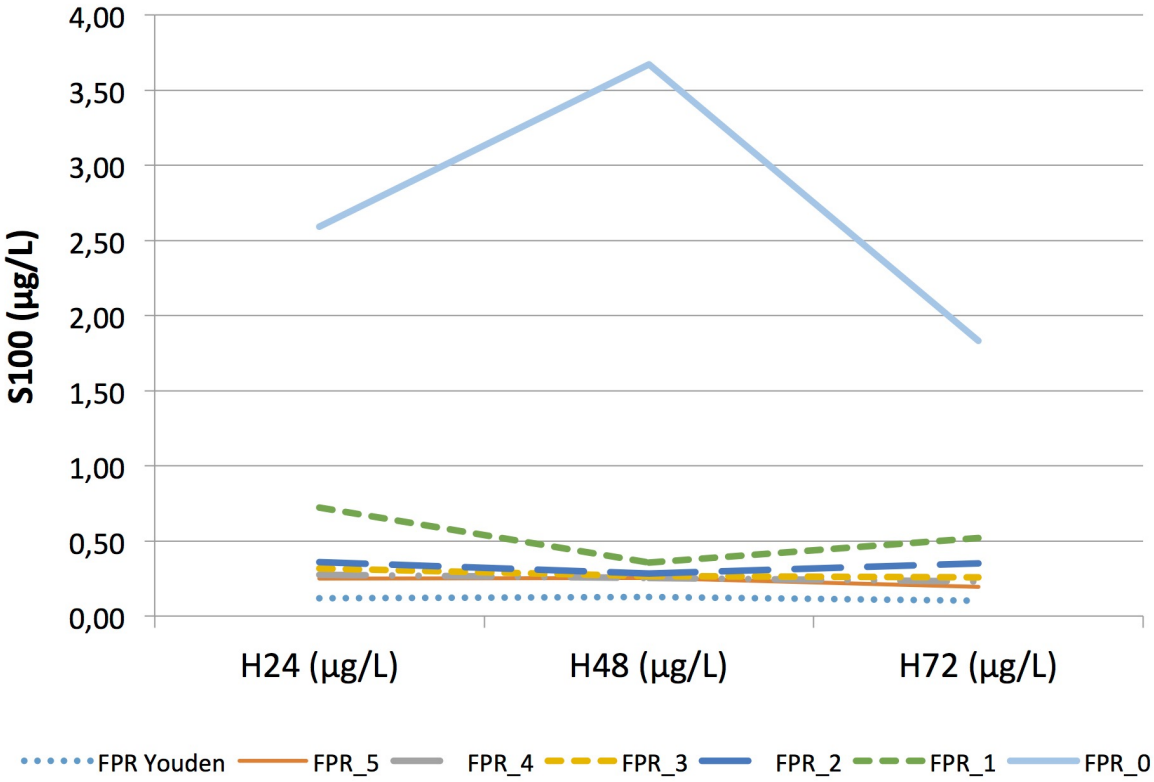


Figure 10: Boxplot representation of median S100 values in each temperature and outcome group. Statistical differences were found between good and poor outcome groups and between temperature groups only in the good outcome groups (p <0.05). Data are represented as interquartile range with upper and lower fence (q1-1.5(q3-q1) and q3 + 1.5*(q3-q1).*

In both outcome groups we observed a significant decrease of S100 levels over time. As such, the timely evolution of S100 could not be used for outcome prediction.

The best performance for outcome prediction was achieved at 24 hours after ROSC, with an AUC of 0.80 (95% CI: 0.77-0.83). At 48 hours and 72 hours, AUCs were lower. There was no significant difference for the AUC between the temperature groups ($p > 0.11$).

S100 cut-off values for poor outcome are displayed in figure 11 according to their respective false positive rates ranging from 0 to 5% and by Youden index.



	H24 (µg/L)	Sensitivity	Specificity	H48 (µg/L)	Sensitivity	Specificity	H72 (µg/L)	Sensitivity	Specificity
Youden	**0,12**	0,68	0,77	**0,13**	0,63	0,82	**0,10**	0,65	0,80
FPR_5	**0,25**	0,41	0,95	**0,25**	0,36	0,95	**0,19**	0,35	0,95
FPR_4	**0,28**	0,40	0,96	**0,25**	0,36	0,96	**0,23**	0,29	0,96
FPR_3	**0,32**	0,35	0,97	**0,27**	0,34	0,97	**0,26**	0,25	0,97
FPR_2	**0,36**	0,32	0,98	**0,28**	0,34	0,98	**0,35**	0,20	0,98
FPR_1	**0,72**	0,22	0,99	**0,36**	0,28	0,99	**0,52**	0,15	0,99
FPR_0	**2,59**	0,10	1,00	**3,67**	0,05	1,00	**1,83**	0,05	1,00

Figure 11: Graphic representation of the calculated S100 cut-offs for poor outcome in our study population according to the false positive rate, ranging from 0% (upper curve) to 5% and Youden index (lower curve).

Multivariable analysis combining serial S100 measurements, target temperature and baseline variables (age, sex, bystander cardiopulmonary resuscitation, first monitored rhythm, time to ROSC, lactate levels on admission, and circulatory shock), revealed that S100 was not an idependent outcome predictor. (table 4)

S100 improved both, the AUC (from 0.80 to 0.84 [95%CI: 0.81-0.87, sensitivity 0.75, specificity 0.81, DeLong test $p<0.001$, likelihood test $p<0.001$]) and the reclassification of patients (NRI (0.53, $p<0.001$) and IDI (0.08, $p<0.001$).

Multivariate analysis (with multiple imputation)			95% CI		
S100 + clinical data	**Effect**	**Odds ratio**	**Lower**	**Upper**	**P-value**
Intercept	-3.670	0.025	0.01	0.11	<0.001
S100 at 24h	1.828	6.221	0.77	50.55	0.09
S100 at 48h	0.873	2.395	0.13	45.81	0.56
S100 at 72h	1.594	4.926	0.21	117.39	0.32
Target temperature	0.085	1.089	0.75	1.59	0.66
Age	**0.062**	**1.064**	**1.05**	**1.08**	**<0.001**
Time CA to ROSC	**0.022**	**1.022**	**1.01**	**1.03**	**<0.001**
Lactate level on admission	-0.001	0.999	0.95	1.05	0.98
Gender	-0.271	0.762	0.47	1.24	0.27
Bystander CPR performed	**-0.527**	**0.590**	**0.39**	**0.90**	**0.02**
VT/VF vs. PEA/asystole	**-1.431**	**0.239**	**0.13**	**0.43**	**<0.001**
ROSC after bystander defibrillation	**-1.560**	**0.210**	**0.05**	**0.88**	**0.03**
Shock on admission	0.160	1.173	0.62	2.21	0.98

Table 4: multivariable analysis of admission parameters including S100 measured at 3 time points. Independent variables are represented in bold.

Multivariate analysis (with multiple imputation)			95% CI		
S100 + NSE+ clinical data	**Effect**	**Odds ratio**	**Lower**	**Upper**	**P-value**
Intercept	-6.480	0.002	0.00	0.01	<0.001
S100 at 24h	1.012	2.751	0.49	15.33	0.25
S100 at 48h	-1.808	0.164	0.00	6.89	0.34
S100 at 72h	2.284	9.820	0.24	401.61	0.23
NSE at 24h	**-0.041**	**0.960**	**0.93**	**0.98**	**<0.001**
NSE at 48h	**0.065**	**1.068**	**1.04**	**1.10**	**<0.001**
NSE at 72h	**0.026**	**1.026**	**1.00**	**1.05**	**0.02**
Target temperature	0.187	1.206	0.76	1.91	0.43
Age	**0.091**	**1.095**	**1.07**	**1.12**	**<0.001**
Time CA to ROSC	0.009	1.010	1.00	1.02	0.17
Lactate level on admission	0.003	1.003	0.94	1.07	0.93
Gender	-0.400	0.671	0.38	1.20	0.18
Bystander CPR performed	**-0.706**	**0.494**	**0.29**	**0.83**	**0.01**
VT/VF vs PEA/asystole	**-1.062**	**0.346**	**0.17**	**0.72**	**<0.001**
ROSC after bystander defibrillation	-0.926	0.396	0.07	2.11	0.28
Shock on admission	0.356	1.428	0.68	2.99	0.34

Table 5: multivariable analysis of admission parameters including S100 and NSE measured at 3 time points. Independent variables are represented in bold.

S100 showed no improvement in the AUC or the reclassification when it was added to the same model including NSE (table 5).

Influence of the targeted temperature level

We could show that target temperature levels influenced the S100 levels only in the good outcome groups at all 3 time-points with statistically significantly higher values in the 33°C group: 0.08 [0.07-0.12] vs 0.07 [0.05-0.10] µg/L (p= 0.004), 0.08 [0.06-0.12] vs 0.07 [0.05-0.10] µg/L (p= 0.002) and 0.07 [0.05-0.10] vs 0.06 [0.04-0.08] µg/L (p= 0.002) at 24, 48 and 72 hours respectively. There was no significant difference in levels of S100 between temperature groups in the poor outcome groups (p> 0.50).

Conclusions

S100 is a predictor of poor outcome after cardiac arrest and targeted temperature does not significantly affect the values. Best outcome prediction is achieved at 24 hours after cardiac arrest. S100 values are not independent outcome predictors and do not add value to a model including NSE.

5.1.3 Paper III: Novel biomarkers: micro-RNAs

Design and objectives

This study was performed as a prospective single center, proof-of-concept study. All patients were admitted to the general ICU of the Centre Hospitalier de Luxembourg after cardiac arrest and being treated with targeted temperature management at 33°C. The aim of this study was to determine whether circulating miRNAs can be used to predict outcome after cardiac arrest. Furthermore, we aimed at comparing these potential new biomarkers to the outcome prediction capacities of NSE and the infection marker procalcitonin (PCT).

Patients

We enrolled 28 successfully resuscitated patients that survived more than 48 hours after cardiac arrest.

Methods

This proof of concept study on the biosignature of miRs after cardiac arrest consisted of 3 steps. First, we pooled the total RNA samples from the good and poor outcome patients to determine miRNA expression profiles by microarrays. Then, cell cultures of neuronal cells were performed to identify the miRNAs originating from neuronal cells. Finally, quantitative polymerase chain reaction (qPCR) was performed on the identified target miRNAs, those expressed by neuronal cells as well as those differentially expressed in the microarrays of the patients. These quantitative results were used to establish the outcome prediction capabilities of miRNAs after cardiac arrest according to the CPC score and in comparison to NSE and PCT.

Results

Expression profiles of circulating micro-RNAs

On the pooled plasma of the 28 patients, a panel of 695 miRNAs was tested and 115 miRNAs were consistently detected. Amongst those, miR-122 was differentially expressed between groups.

Expression of micro-RNAs in neuronal cells

A cell culture was performed on neuroblastoma cells until complete cell differentiation. On conditioned growth medium, miR-21/-122/-150*/-451 were reliably detected. This step served as a confirmatory test that the presence of those miRNAs in the blood stream might find its origin in neuronal cells.

Prediction of outcome

The outcome prediction capacity of miR-122 and miR-21 was compared to NSE and PCT, both also measured at 48 hours after ROSC. For miR-122 we determined an AUC of 0.73 (95% confidence interval, 0.54–0.93) and for miR-21 an AUC of 0.77 (95% confidence interval, 0.58–0.95) to predict neurological outcome after CA. Combining both miRNAs did not improve prediction accuracy.

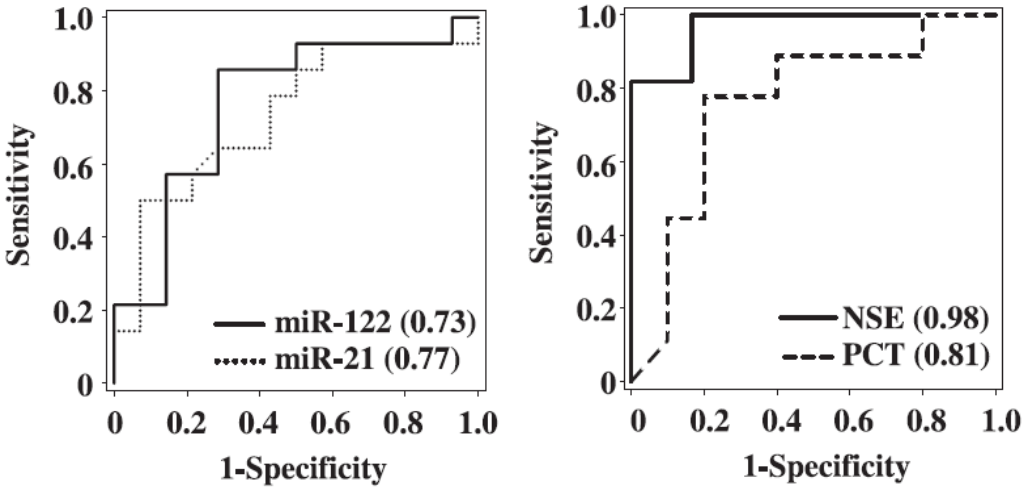


Figure 12: Prediction analyses. Areas under the receiver operating characteristic curve for circulating levels of miR-122 and miR-21 determined by quantitative polymerase chain reaction as well as NSE and PCT. Displayed are receiver operating characteristic curves showing the value of biomarkers to predict neurological outcome (CPC 1–2 vs. CPC 3–5).

Prediction of mortality

Patients within the highest third of miR-122, miR-21 or NSE values had the highest mortality rate. The performance of NSE to predict mortality was more significant ($p < 0.001$) than for miR-122 and miR-21 ($p = 0.02$ for both). PCT did not have a significant predictive value for mortality. Although, in our study group, miR-122 and miR-21 were significant predictors of mortality they did not outperform NSE.

No significant correlation between miRNAs and any risk factor could be detected. MiR-122 levels were not different between patients with acute myocardial infarction (AMI) and patients without AMI. MiR-21 was lower in patients with AMI compared to patients without AMI. MiRNA levels did not correlate with the markers of myocardial injury creatine kinase (CK-MB).

Conclusions

This study was the first to describe a new class of biomarkers, miRNAs, for outcome and survival after cardiac arrest. Although not being as potent as NSE in this pilot study, miRNA can predict outcome after cardiac arrest. The clinical and physiological relevance of miRNAs need to be further explored in upcoming studies.

5.2 Neurophysiology

5.2.1 Paper IV: Bispectral index (BIS)

We aimed to test and improve the simplified electrophysiology approach with bispectral index (BIS) to predict outcome after cardiac arrest.

Design and objectives

This prospective, observational single center study in adult comatose patients treated by target temperature management at 33°C after cardiac arrest, aimed to determine the use of Bispectral index (BIS), measured during the first 24 hours after ICU admission, as early outcome predictor.

Patients

96 patients after cardiac arrest admitted to the ICU of the Centre Hospitalier de Luxembourg were included.

Methods

All causes of cardiac arrest were included. Patients benefited from target temperature management at 33°C with mandatory sedation and neuromuscular blockade according to our local protocol. This sedation regimen allowed suitable sedation with minimal electromyogram (EMG) artefacts on the electroencephalogram (EEG) signal. After 24h, patients were rewarmed to 36°C at a maximum rate of 0.5°C/h and sedation was tapered. CPC score at 6 months was the primary endpoint. Only patients with complete datasets and high quality EEG signal on BIS recordings were included. Time course of BIS was analyzed as well as mean BIS was calculated over the first 24 hours after ICU admission, first using data from every half hour and then also every minute. We determined cut-off values for poor outcome.

Results

96 patients admitted after cardiac arrest were included in the analysis of this single center study. 48 % of the patients had a good neurological outcome at 6 months.

We could demonstrate that initial mean BIS values were higher in the good outcome patients as compared to the poor outcome patients.

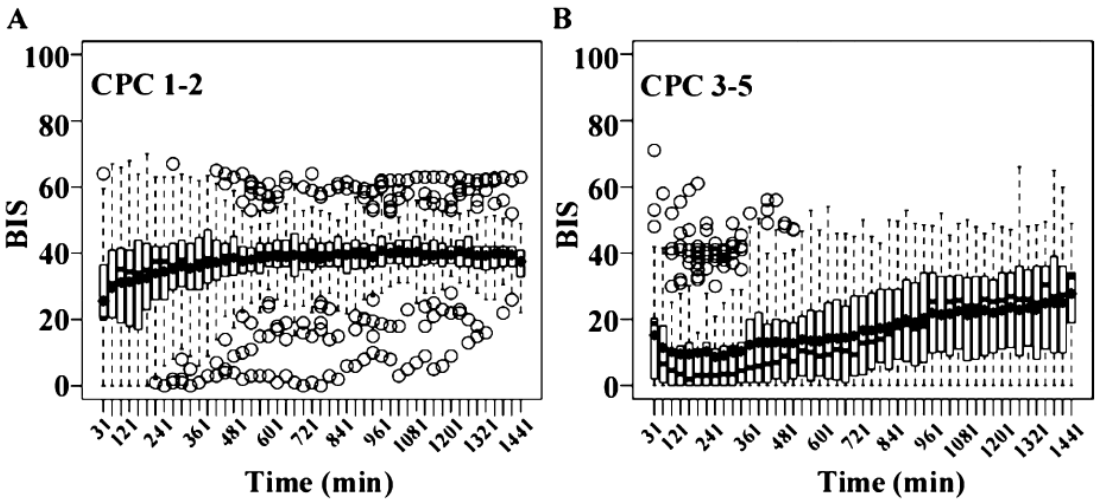


Figure 13: Time-course over 24 hours of BIS values according to patient outcome. Displayed are boxplot of BIS values at each time point in good outcome (A) and poor outcome (B) patients. Black dots represent the mean, length of the squares are marked out by the first and third quartile, dotted lines are drawn from the lower to the upper fence (q1-1.5(q3-q1) and q3 + 1.5*(q3-q1)) and white circles represent outliers.*

Over the first 24 hours after ICU admission, mean BIS values were significantly higher in the good outcome group (38 ± 9) as compared to the poor outcome group (17 ± 12) ($p < 0.001$).

Outcome prediction and cut-offs

The optimal specificity (89%, [95% CI: 80%,98%]) and sensitivity (86%, [95% CI: 76%,96%]) for 6-months neurological outcome prediction was obtained from the mean over the first 12.5 hours using a cut-off BIS value of 23 (AUC: 0.88).

Absolute outcome prediction, with a false positive of zero, in our cohort was achieved with a mean BIS of 2.4 calculated over the first 6.5 hours after admission to the ICU (specificity 100%, [95% CI: 92%,100%], sensitivity 26%, [95% CI: 14%,38%]). All patients with a mean BIS below 2.4 over the first 271 minutes evolved to CPC 3-5.

Analysis and calculation of the mean BIS value using minute by minute values did not improve accuracy of outcome prediction over a mean calculated using every 30 minutes values.

Multivariable analyses

In a multivariable analysis of clinical parameters, including: age, gender, SAPS II, time to ROSC, initial rhythm, associated factors and medical history, only age and time to ROSC were significant predictors of outcome ($p = 0.002$ and $p = 0.001$, respectively). After adding mean BIS value over the first 12.5 hours to this model, only age and mean BIS remained significant outcome predictors. Continuous NRI (1.28, $p < 0.001$) and IDI (0.31, $p < 0.001$) showed that mean BIS significantly improved patients classification with regards to the clinical model.

Without mean BIS				
	estimate	**CI 2.5 %**	**CI 97.5 %**	**p-value**
(Intercept)	5.7244	2.6804	8.7683	2.28E-04
Age	**-0.0605**	**-0.0990**	**-0.0219**	**0.002**
time to ROSC	**-0.0816**	**-0.1277**	**-0.0354**	**0.001**
With mean BIS				
(Intercept)	1.3122	-2.236	4.861	4.69E-01
Age	**-0.0497**	**-0.094**	**-0.005**	**0.029**
time to ROSC	-0.0437	-0.098	0.011	0.116
mean BIS at 12.5h	**0.114844844**	**0.068**	**0.162**	**1.70E-06**

Table 6: multivariable analysis without and with BIS;
only the independent variables detected by the model are shown.

Conclusions

BIS is an easy to use simplified electrophysiology method, able to reliably predict poor outcome after cardiac arrest within the first 12.5 hours after ICU admission. A standardized sedation and neuromuscular blockade protocol is mandatory to reduce EMG artefacts.

5.3 Paper V: Combination of biomarkers and neurophysiology (BIS-S100-NSE)

Considering the previously reported data on biomarkers and BIS, we aimed to answer the question if there is an added value in combining different prognostic tests to improve outcome prediction.

Design and objectives

This prospective, observational, single center study was designed to evaluate a multimodal approach, combining biomarkers (NSE, S100) and a neurophysiologic test (BIS) for outcome prediction after cardiac arrest.

Patients

Consecutive patients admitted to the ICU of the Centre Hospitalier de Luxembourg after cardiac arrest and treated with target temperature management at 33°C were included in this study.

Methods and statistics

Serum levels of neuron-specific enolase (NSE) and neuron-enriched protein S100 (S100) were measured 48 hours after CA. Bispectral index (BIS) was continuously monitored during the first 48 hours after cardiac arrest and the lowest BIS value within this timeframe was used for outcome prediction. The primary endpoint was neurological outcome, as defined by the cerebral performance category (CPC) at 6 months. The secondary endpoint was survival.

Results

75 patients successfully resuscitated from cardiac arrest were enrolled in this study. 55% had a good neurological outcome and 45% a poor outcome at 6 months.

Biomarkers and BIS and outcome

Serum levels of NSE and S100 were significantly higher in patients with poor outcome compared to those with good outcomes. We could also show that serum levels of NSE and S100 were highly correlated (r: 0.61; $p < 0.001$). BIS was higher in patients with good outcome compared to patients with poor outcome (10-fold; $p < 0.001$). The median time until the lowest BIS value was 5 h (range: 4 to 14.5 h) after CA.

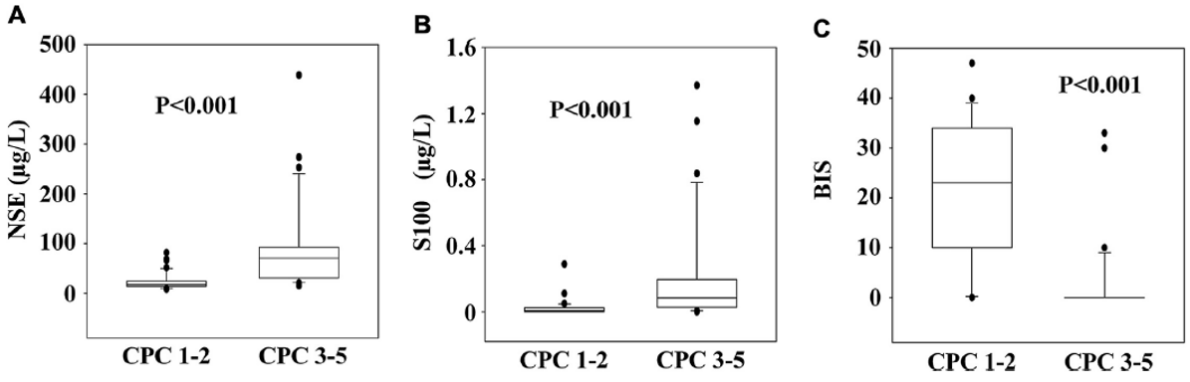


Figure 14: Serum levels of NSE (A) and S100 (B) determined 48 hours after cardiac arrest according to outcome. The lowest BIS value over 48 hours is presented (C).

Outcome prediction and cut-offs

All three outcome predictors, S100, NSE, and BIS, taken separately had significant predictive values, with AUCs above 0.80.

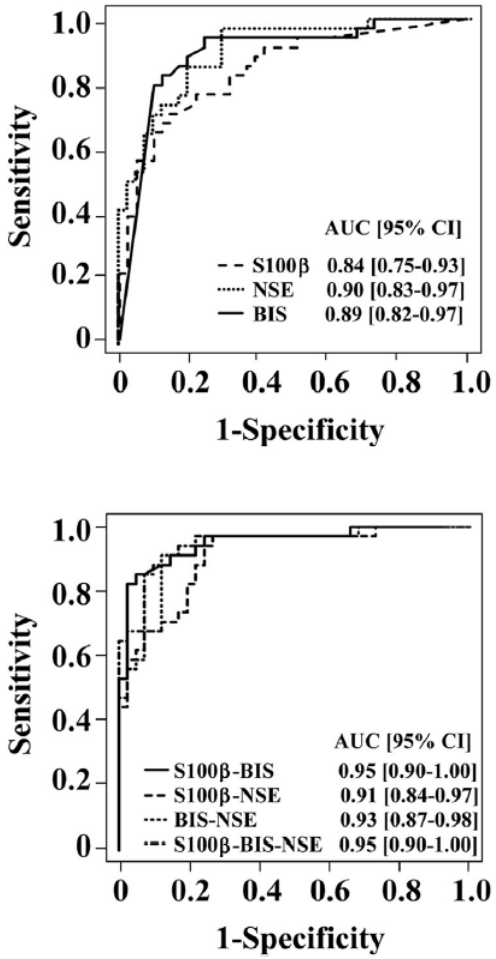


Figure 15: Receiver-operating characteristic (ROC) curves for single markers: S100, NSE and BIS monitoring for outcome prediction at 6 months (upper graph). ROC curves for combinations of markers (lower graph). The areas under the ROC curve (AUC) are indicated.

Combined determination of S100 and BIS had an incremental predictive value, with an AUC of 0.95. Combination of S100 and NSE, or BIS and NSE, had slightly lower predictive values (0.91 and 0.93 respectively). Adding NSE to S100 and BIS did not further improve the prediction accuracy. Analysis of deviance confirmed that the addition of S100 to the model with BIS improved the prediction ($p < 0.001$). However, the addition of NSE to the model with S100 and BIS did not improve prediction ($p = 0.10$).

S100 significantly improved the discrimination based on lowest BIS value (IDI = 0.13; $p < 0.001$). Also, BIS improved the discrimination of S100 with an IDI of 0.32 ($p < 0.001$). NSE failed to improve the discrimination of patients misclassified by a model with BIS and S100. BIS also improved the classification of NSE alone (IDI = 0.14; $p < 0.001$). A model with BIS and S100 also improved the classification of NSE alone (IDI = 0.20; $p < 0.001$).

The cut-off for the lowest BIS value predicting poor outcome was 5.5, providing a sensitivity of 85%, a specificity of 83%, and a false positive rate of 17%. Patients with a S100 serum level above 0.03 mg/l had a 3.4-fold higher risk of poor neurological outcome (95% CI: 1.88 to 6.34; $p < 0.0001$). Patients with a lowest BIS below 5.5 had a 6.1-fold higher risk of poor neurological outcome (95% CI: 2.66 to 10.07; $p < 0.0001$). Patients with both, S100 serum levels above 0.03 mg/l and a BIS value below 5.5, had a 3.6-fold higher risk of poor neurological outcome (95% CI: 2.28 to 5.71; $p < 0.0001$).

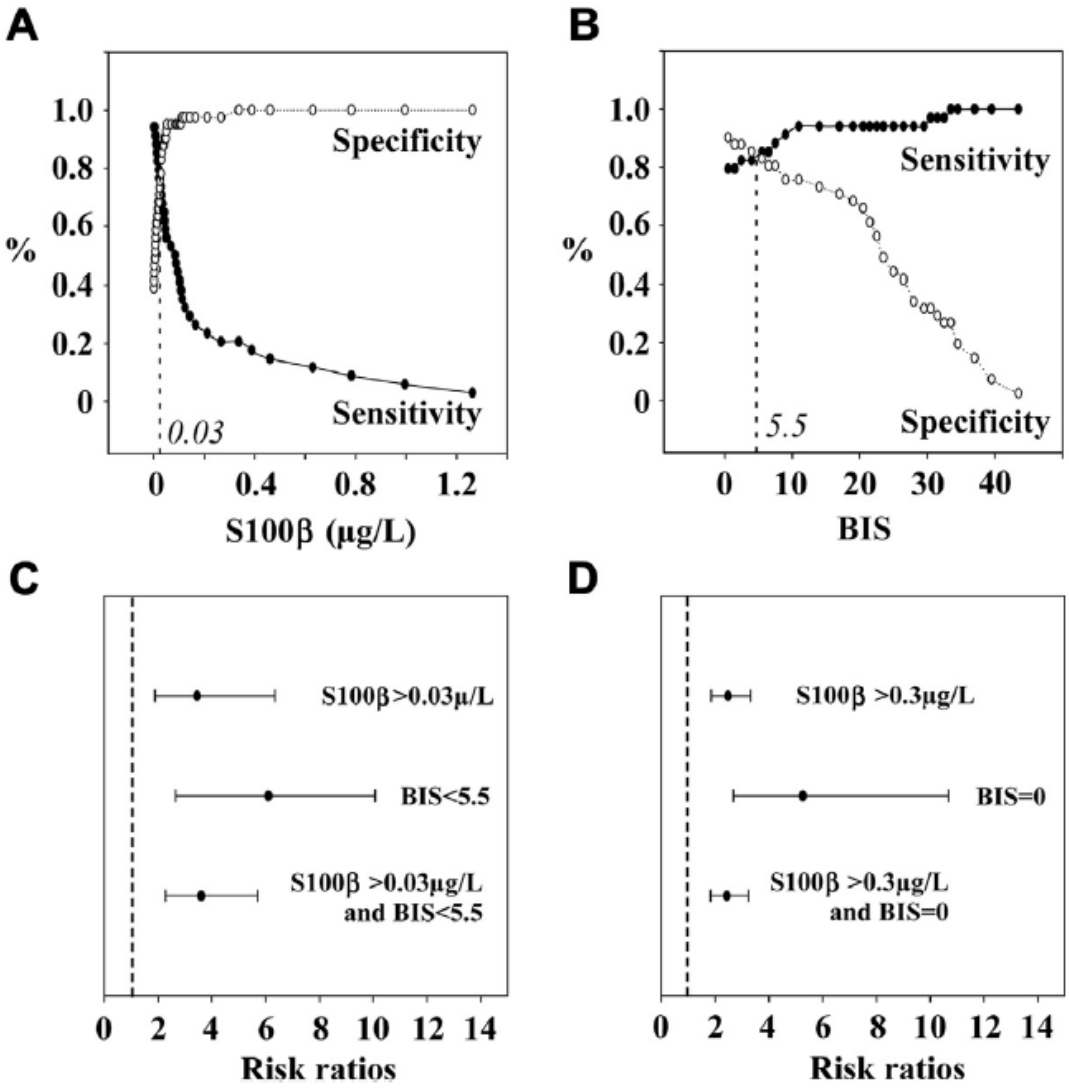


Figure 16: A and B: Cut-offs, determined by plotting sensitivity versus specificity values, are indicated by dotted lines and italicized values. C and D: Risk ratios of poor neurological outcome (CPC 3–5) according to cutoff values for S100b and BIS. The dotted vertical line indicates a risk ratio of 1. Risk ratios ± 95% CI are presented.

In multivariable analysis, we found an added value of combining S100 and BIS over traditional prognostic parameters like: age; sex; SAPS II; time to ROSC; presenting rhythm (asystole/pulseless electric activity vs. ventricular fibrillation/ventricular tachycardia); and associated factors such as cardiogenic shock, acute myocardial infarction, EEG status epilepticus, and seizures. These analyses showed that SAPS II ($p = 0.04$), presenting rhythm ($p = 0.01$), S100 ($p = 0.01$), and BIS ($p = 0.01$) were independent predictors of neurological outcome.

Conclusions

The combination of a biomarker and a simplified electrophysiology method (BIS) is able to more reliably predict poor outcome after cardiac arrest than the biomarker or BIS alone.

6. DISCUSSION

With this doctoral thesis project, we aimed to improve outcome prediction of patients admitted after cardiac arrest to an intensive care unit. We have studied different prognostication tools, NSE, protein S100 and bispectral index, in patients treated with targeted temperature management. We confirmed and refined the utility of the routinely used biomarkers NSE and S100 in a large multicenter trial. We could also show that the influence of temperature was not significant.

We were the first to describe that miRNAs may serve as biosignatures in patients resuscitated from cardiac arrest.

We used an innovative approach by applying BIS to cardiac arrest patients and could demonstrate its use, either alone or in combination with biomarkers to reliably predict outcome.

Even in our purely observational data, we constantly report good neurological survival rates of patients admitted to the ICU after CA of around 50%. This highlights that even outside the narrow borders of clinical trials, good outcome can be achieved by providing attentive and devoted care to this most vulnerable patient population.

Biomarkers

Amongst the routinely used biomarkers for prognostication after cardiac arrest, NSE and S100 are the most studied and used in everyday clinical practice. Both tests are readily available as routine biochemical tests in most hospital laboratories.

Our studies confirmed that both biomarkers are very well correlated with outcome after cardiac arrest. Higher values are invariably associated with poor outcome as demonstrated by the Kaplan-Meier curves. On the other hand, lower values, do not necessarily correlate to good outcome as there might be many confounders as some patients with “intact” brain function may die from other causes, like sepsis, heart or multi organ failure. We could also show that NSE is the more robust outcome predictor compared to S100. NSE has consistently higher AUC for outcome prediction and the overall correlation to outcome is better than for S100.

After cardiac arrest, brain biomarkers are released into the blood stream after neuronal or cerebral hypoxic ischemic insult. They reflect the severity of the neuronal damage, and possible blood-brain barrier damage. As such they can be considered as surrogate markers of the severity of the brain damage, although they are obviously not able to indicate which areas or even functions of the brain are the most affected. Any brain protective measure should thus have a measurable influence on the level of biomarkers and have a clinical correlation. With our work we were unable to address the question whether sedation by itself has a brain protective virtue as all our patients had been sedated in order to properly perform targeted temperature management. It is nevertheless admitted that sedation does not *per se* affect biomarkers, making them ideal candidates in TTM patients where sedation is mandatory. Unlike EEG which can be prone to artefacts either by too deep sedation or EMG noise in non-paralyzed patients, biomarkers can be safely used in these circumstances (Samaniego, Mlynash et al. 2011).

Despite the robustness of the studied biomarkers, we still are reluctant in promoting fixed and immutable cut-off values for outcome prediction. In fact, biomarkers are just single measurements at certain time-points, whereas potential brain damage is an ongoing, dynamic process (Sandroni, Cavallaro et al. 2013). Thus only relying on one (or even a few) measurements of one single parameter bears potential risks of giving an incomplete view of the brain damage. Additionally, an absolute cut-off value should not be used as the

interpretation in an individual patient may be difficult. Rather than limiting to a single value, there is evidence that high and increasing NSE levels are more accurate predictors than single absolute cut-offs alone (Storm, Nee et al. 2012, Huntgeburth, Adler et al. 2013). This is particularly true for NSE. As we highlighted, a specificity of 100% (false positive rate of zero) will probably not hold true in clinical medicine. This is regularly demonstrated in various studies reporting good survivors with very high NSE values (Zellner, Gartner et al. 2013). We introduced a new concept for defining cut-offs: instead of fixing an absolute cut-off value we report a range, taking into consideration the false positive rates. When looking at the different cut-off values, probably the best compromise lies in a low FPR of around a few percents. This approach provides a range or an order of magnitude, with cut-offs very close each to the other, with a high probability of poor outcome. Our work served as reference for serial NSE measurements in the recent 2015 ERC guidelines on prognostication (Nolan, Soar et al. 2015). In 2006, the American Academy of Neurology guidelines (Wijdicks, Hijdra et al. 2006) suggested a "historical" cut-off for NSE, based on a single multicenter trial (Zandbergen, Hijdra et al. 2006). It should be emphasized that there is at least one serious methodological issue in this paper as the definition of FPR was false positive/ true positive instead of false positive/(false positive + true positive), which lead to a lower FPR value and an overestimation of the accuracy of the test. In our data, this historical cut-off of 33 ng/ml yielded only a specificity of 0.91 and a sensitivity of 0.65. With a false positive rate of 9%, this cut-off is far too low to be acceptable in terms of reliable prognostication as the suggested FPR should be 5% or lower (Sandroni, Cariou et al. 2014). The magnitude of our cut-offs is in line with those reported in other more recent studies (Wennervirta, Ermes et al. 2009, Bouwes, Binnekade et al. 2012, Storm, Nee et al. 2012).

A pitfall in using NSE is hemolysis that increases NSE levels. The risk of hemolysis is particularly elevated in post-cardiac arrest patients as they may experience hemolysis from the resuscitation itself or the medical care using IABP or ECMO. In our study we carefully discarded all samples with hemolysis, but the risk of extra cerebral origins of NSE still persists even in the absence of detectable hemolysis. This might be due to the fact that free hemoglobin in the bloodstream, originating from the erythrocytes (which also contain NSE), has a much shorter half-life than NSE.

As NSE has initially been described as tumor marker for small cell lung cancer. Although this is a potential confounder, reports of patients with elevated NSE values after cardiac arrest in whom this lead to the diagnosis of a tumor are rare. In most cases, anamnesis would reveal an already existing tumor (Fugate, Wijdicks et al. 2010). Furthermore, a constantly elevated NSE value might be indicative of a possible tumor and should thus be set in this context. This potential confounder also emphasizes that no biomarker should be used as sole outcome predictor.

Besides the potential risk of dosing errors, the dosing method or even the laboratory and the storage of samples may be responsible for yielding different results (Mlynash, Buckwalter et al. 2013, Rundgren, Cronberg et al. 2014).

Although S100 is a good outcome predictor, it is clearly outperformed by NSE. Furthermore the suggested cut-offs with low FPR are very close each to the other and only very high S100 values are actually discriminative, but with a sensitivity limiting its clinical utility. Given the inherent precision of the dosing method, with observed errors between 2.6 and 3.6%, the clinical utility of these cut-offs must be questioned further. Like NSE, S100 may also originate from extra-cerebral sources like chondrocytes, adipocytes, melanocytes and even Langerhans cells (Olsson, Zetterberg et al. 2011). Because of the very low S100 values encountered after cardiac arrest, every part of S100 not coming from the brain might potentially skew the results. On the other hand, as S100 originates mainly from the astroglial cells, as compared to neurons and neuroendocrine cells for NSE, there might be a potential benefit in a combined neuronal biomarker approach.

We consider that our results are robust as the population in the well designed TTM-trial accounted for more than twice than in the largest trials on these biomarkers (Zandbergen, Hijdra et al. 2006, Bouwes, Binnekade et al. 2012).

In our studies, all the biomarkers were measured a posteriori, ruling out the risk of a self-fulfilling prophecy. We used a central biobank for storage of the samples and all analyses have been performed at a single core laboratory. Furthermore, we used a blinded approach, where neither the biomarker data were available to the treating physicians, nor to the outcome assessors at 6 months.

Influence of temperature

The concept of therapeutic hypothermia for out-of-hospital cardiac arrest was introduced on a global scale after the 2002 landmark trials (Bernard, Gray et al. 2002, Hypothermia after Cardiac Arrest Study 2002). This intensive care strategy certainly encounters for many saved lives after OHCA over the last decade. Albeit, this concept has been challenged especially by Nielsen et al., who questioned the scientific evidence for incorporating therapeutic hypothermia in the 2005 clinical guidelines on post cardiac arrest care (Nielsen, Friberg et al. 2011). This critique culminated in the TTM-trial, introducing the targeted temperature management concept (versus the therapeutic hypothermia concept), which studied 2 sub-febrile target temperatures. Applying a strict TTM protocol at either 33°C or 36°C after cardiac arrest resulted in no difference in survival. Furthermore, no difference on any other studied parameters in the sub-studies and the post hoc analysis published so far could be found (Bro-Jeppesen, Kjaergaard et al. 2015, Cronberg, Lilja et al. 2015, Dragancea, Horn et al. 2015, Kjaergaard, Nielsen et al. 2015, Lilja, Nielsen et al. 2015). Our biomarker papers I and II were pre-defined sub-studies of the TTM trial and allowed us to study the potential impact of TTM on biomarkers. Although, therapeutic hypothermia has shown various neuroprotective effects mainly in laboratory experiments, there was no clinical impact in the TTM trial (Sterz, Safar et al. 1991). None of our 2 studied biomarkers was significantly affected by the level of target temperature. Although, we found significantly lower S100 values in the 36°C arm, this only held true for those with good outcome, thus values well below any significant cut-off point. We have no explanation for this, except that median values are so low with a substantial overlap in interquartile ranges and that inherent laboratory imprecisions or some outliers may just reflect a statistical finding. Definitely, with the biomarkers we studied, we could not identify any clinical relevant impact of temperature. If we consider that biomarkers are surrogate markers of brain injury, we must conclude that TTM at 33°C or 36°C does indeed not seem to have a neuroprotective effect. We might speculate that the way TTM is performed nowadays, regarding the speed of cooling, the level of target temperature, the duration of TTM as well as the speed of rewarming, does not have any impact, neither on clinical outcome endpoints, nor on biochemical markers of brain damage.

Another issue might be the impact of lower temperatures on the metabolism of sedatives. It has been shown that lower temperatures influence the clearance of certain drugs and that especially neurological clinical examination might be affected (Samaniego, Mlynash et al. 2011, Bjelland, Klepstad et al. 2013). The effect of sedation is further enhanced in TTM patients as sedation is mandatory to be able to perform TTM compared to “normothermic” patients where sedation would perhaps be less important and tapered earlier. As brain biomarkers are unaltered by sedation this issue has no relevance.

Furthermore, temperature does not seem to have a major impact on EEG (Rossetti, Carrera et al. 2012). As an EEG derivate, this could also be supposed for BIS given the fact that other EEG derived analyses have been successfully investigated in these patients (Rundgren, Westhall et al. 2010). No data on the influence of mild hypothermia (32-34°C) on BIS and only scarce data on deep hypothermia exist, suggesting a decrease of 1.8 per degree in cardio-thoracic surgery with extracorporeal circulation and deep hypothermia around 20°C (Ziegeler,

Buchinger et al. 2010). All published work on BIS after cardiac arrest has been done in TTM patients treated at 33°C (Stammet, Werer et al. 2009, Seder, Fraser et al. 2010, Seder, Dziodzio et al. 2014). If there is an impact of temperature it is likely to have affected all patients in the same way. It should also be highlighted that mild hypothermia does not affect SSEPs neither, making them also a recognized prognostication tool in TTM patients (Rothstein 2014, Nolan, Soar et al. 2015).

Bispectral index

Bispectral index (BIS) has originally been developed as a tool to measure the depth of anaesthesia in the operating theatre. We have been the first to describe its use as an outcome predictor after cardiac arrest in a small single center study (Stammet, Werer et al. 2009). Based on this experience, we have refined our work and could further strengthen the utility of BIS as outcome predictor. By calculating the mean BIS value over the first 24 hours after admission to an ICU, we could increase the accuracy of outcome prediction. Instead of relying on a single measurement, like in our first publication, or an analysis over a shorter period of time (Seder, Fraser et al. 2010, Riker, Stone et al. 2013), we used a longer period of measurement and thus reduced potential errors of a single time point measurement. On the other hand, introducing multiple measurements bears the risk of including new errors. Per protocol, we tried to avoid these, by only taking into account high quality data (high signal quality index and low EMG noise) and only using every 30 minutes data. We could show that when calculating the mean on an every minute basis, the accuracy of our model did not increase, which may be indicative that too many data add too much noise to the calculations without adding any value. Our results are completely supportive and in line with those published by others and highlight the potential utility of BIS as outcome predictor (Seder, Fraser et al. 2010, Burjek, Wagner et al. 2014, Seder, Dziodzio et al. 2014). Regardless the way of looking at the results, the lowest observed BIS value or the lowest mean, low BIS values are constantly associated with poor outcome. BIS is an easy to use tool and a widespread use could be achieved easily without the need of specially trained personnel. One limitation is certainly the mandatory use of a neuromuscular blocking (NMB) agent in order to suppress any muscular activity (EMG noise). The necessity of NMB has also been demonstrated in the study by Seder et al. where BIS in non-paralyzed patients had no prognostic value at all (AUC 0.44) (Seder, Fraser et al. 2010). Besides the inherent risks of any neuromuscular block (mainly ICU acquired polyneuropathy and the risk of pneumonia), this drug also masks the early onset myoclonus which is of poor prognosis (Bouwes, van Poppelen et al. 2012). If early myoclonus occurs, it is generally already present on ICU admission, before start of the NMB infusion and thus treatment or specific neuromonitoring can be initiated already at that time. Furthermore, our protocol only advocates a 24 hours neuromuscular blockade, which should only have minimal influence on potential side effects. We must also acknowledge that our study was a single center study with a limited number of patients.

Standard EEG, continuous EEG or amplitude integrated EEG may certainly provide more detailed information, such as epileptic activity, burst suppression or other malignant patterns. Two major drawbacks nevertheless hamper the generalizability of these techniques. First, in any case, you need specially trained physicians to make the interpretation of data and secondly, there is not yet a generally accepted definition of malignant EEG patterns. Furthermore, it has been shown that there is a significant interrater variability in the interpretation of EEG data even in expert hands (Westhall, Rosen et al. 2015). Although, recent efforts by the TTM trial group have resulted in the publication of a new and simplified classification aiming at reducing the interrater variability, this concept needs to be verified in upcoming prospective studies (Westhall, Rossetti et al. 2016). Because of this interrater variability and the non-standardized definitions of the different malignant patterns, the interpretation of EEG data remains problematic. Only status epilepticus and unreactive burst suppression are part of the guidelines (Nolan, Soar et al. 2015).

Somatosensory evoked potentials (SSEP) are apparently unaffected by TTM, unlike auditory evoked potentials (Tooley, Greenslade et al. 1996, Rothstein 2014). According to a recent review, bilateral absence of N20 peaks is one of the most reliable outcome predictor after cardiac arrest with a FPR of zero and a very low 95% confidence interval (0-2%) (Sandroni, Cavallaro et al. 2013). In our study, the cut-off for BIS with a FPR of zero had a slightly higher, but still low 95% confidence interval (0-8%). We acknowledge, that SSEP have been validated in numerous studies whereas, our study on BIS is monocentric. Like BIS, SSEP can be performed during the hypothermia phase and can thus also be considered as early outcome predictor. Unlike BIS, SSEPs need qualified personnel to perform the test and to make the interpretation. Because of these constraints, SSEPs are mostly not feasible off hours and weekends as they may also not be available in every hospital. BIS has the advantage of providing early information and being easily available in every hospital without the need of specially trained personnel.

Regarding the effect of sedation, we believe that its impact is not a major issue as all bis studies, like all the different EEG studies, have been performed in sedated patients. Unlike Burjek and coworkers, we and others, did not adapt sedation to the BIS value, but rather applied a defined sedation protocol and studied the evolution of BIS over time (Burjek, Wagner et al. 2014, Seder, Dziodzio et al. 2014). We cannot rule out a possible confounder with regards to sedation as our BIS values were not blinded to the treating personnel.

Timing of prognostication and time course of outcome predictors

In prognostication after cardiac arrest, timing is of paramount importance. As very early predictors are being investigated, it might be tempting to prognosticate very early but great caution must be applied. Indeed, the TTM trial had a strict protocol on prognostication and withdrawal of life supporting therapies, which was not the case in previous studies. Prognostication was done only 108 hours after ROSC by a blinded physician and WLST was possible thereafter only in the presence of findings of severe brain damage. Earlier WLST was only possible if brain death was established or in the presence of early status myoclonus and bilaterally absent N20 peaks or for documented and obvious ethical reasons (Nielsen, Wetterslev et al. 2012). This strict and protocolized approach avoided a self-fulfilling prophecy and allowed for the clearance of sedation.

The recently published 2015 guidelines on post resuscitation care insist on a rigorous approach for prognostication which should extend from at least 72 hours until even 5 days after ROSC to allow for potential confounding factors to be cleared as much as possible (Nolan, Soar et al. 2015). NSE showed a constant increase over time in poor outcome patients. High and rising values over time were strongly associated with poor outcome. This dynamic approach of serial measurements adds accuracy to the prediction of outcome by NSE (Einav, Kaufman et al. 2012, Storm, Nee et al. 2012, Huntgeburth, Adler et al. 2013). In patients with good outcome, we noticed a decrease over time, although it was not correlated to outcome. On the other hand, S100 values peaked at 24 hours and then decreased constantly, regardless of outcome. The highest AUC for outcome prediction with S100 was found at 24 hours. Interestingly, an older study could not determine an earlier peak of S100 (Bottiger, Mobes et al. 2001). Therefore, the dynamics of S100 release had no prognostic value. The actual guidelines include NSE as only biomarker with enough evidence to be included in the decision algorithm. We think that it should be highlighted that biomarkers and other parameters should already be measured and recorded at an early stage after cardiac arrest. Even though, no decision should be made on solely those early parameters, they must be taken into account at a later stage, when normothermia has been restored and sedation tapered. We could show that NSE had its highest sensitivity and specificity for outcome prediction at 48 and 72 hours after ROSC.

Interestingly, BIS was a very early marker of poor outcome who also showed a timely evolution. Patients with a good outcome presented with rather high levels of BIS (around 20)

and maintained these levels throughout the first 24 hours of their ICU stay (figure 13A). Patients with poor outcome generally had low or very low levels of BIS and only showed a slow increase over the first 24 hours without even reaching the initial BIS levels of the good outcome patients (figure 13B). In Paper V, the lowest BIS value could already be measured at 5 hours after ICU admission, whereas in paper IV, mean BIS values lower than 2.4 during the first 6.5 hours after ICU admission were predictive of a certain poor outcome. These findings confirm our earlier results as well as those found by others (Seder, Fraser et al. 2010, Riker, Stone et al. 2013). In all BIS studies, BIS was not used to terminate intensive care prematurely, but because of the absence of blinding of the physicians, it cannot be ruled out that there might be a confounding factor. Furthermore, these studies have all been performed in single centers.

Some suggested a “triage” parameter to allocate maximal healthcare to those with supposed “milder brain injury” after cardiac arrest (Seder, Dziodzio et al. 2014). Although we agree that today prognostication must be delayed later than 72 hours after CA, we think that given the evidence from our work, there might be a place for at least “triage tools” to direct the intense high end treatment to individuals prone to a low risk of poor neurological outcome (Nielsen 2012). Based on the consistent data in different studies on BIS, both regarding the timing and the low BIS values predictive of poor outcome, BIS might indeed open the way for very early triage of cardiac arrest patients. This would allow to tailor the treatment to the potential severity of the brain damage of the patients. With its ease of use, the immediate availability of the values and with its early prognostic value, BIS has the potential to become the most suitable tool in this context. In the case of sustained high BIS values in a patient with severe shock or cardiac failure for instance, every effort should be made to overcome the acute condition as the risk of severe brain damage can be considered quite low. Except in obvious futile cases, like those defined in the TTM-trial protocol, such an early approach should never be used to WLST but rather encourage physician to apply standard, recommended intensive care until guideline supported prognostication can be performed.

Combination of different outcome predictors

The analysis of single outcome predictors is mandatory to study their individual prognostic value. In paper V, we combined 2 biomarkers, NSE and S100, with our previously studied neurophysiological parameter, BIS. Our innovative approach has also been acknowledged in the 2015 guidelines on post-resuscitation care (Nolan, Soar et al. 2015). We could show, that by combining a blood biomarker to an electrophysiological tool, it was possible to increase the outcome prediction accuracy. Such a combined approach has the potential to accurately explore at the same time, the actual physical brain damage by a biomarker, and the brain function by BIS. As such, it is possible to reduce the risk of possible errors and to reclassify the patients that may have been misclassified if one of both techniques were used alone. The combination approach also allows to take into account the different optimal timings of the markers used. We must acknowledge that in our study, we only used the 48 hours samples because of availability of the samples. Given the kinetics of S100, a sample at 24 hours might have increased the accuracy further. The combination of different biomarkers measured at their optimal time point, 24 hours for S100 and 48 hours or 72 hours for NSE and determination of the lowest (mean) BIS values, might also have improved the model’s prediction capacities further. Another issue is sedation as it may as well interfere with electrophysiology of the brain, by altering especially the EEG, as well as with clinical examination. We could show, that with our sedation protocol including NMB, BIS still offers reliable outcome prediction capacities, although we cannot definitely rule out an effect of sedation on BIS. On the other hand, biomarkers are unaltered by sedation and thus offer a perfect counterpart to the BIS. Their major drawback is that they cannot be measured continuously but only intermittently at fixed time points. We could also show that a combination of 2 biomarkers, measured at the same time point, does not necessarily add power to the model. Adding NSE to the model including S100 and BIS did not further

improve the accuracy. Similar findings have also been reported by Einav and coworkers (Einav, Kaufman et al. 2012).

When looking at our multivariable analysis results, some parameters turn out to be consistently associated with outcome. Aside our reported study parameters, age, initial rhythm, time to ROSC and bystander CPR are constantly and independently associated with outcome. But one major issue is the real clinical impact of statistically improved models. It is unclear if an increase of a few percents of AUC or specificity/sensitivity really have an impact in every day patient care. For instance, if gender turns out to be an independent risk predictor in a model, this clearly does not influence clinical decisions as it is barely imaginable that simply because of your gender, WLST will be performed. Any future combined approach of outcome predictors should take into consideration the optimal timing, the timely evolution and the target or mechanism of action of each prognosticator in order to get the most clinically relevant items out of such a combination.

New biomarkers

Existing "classical" biomarker are mostly peptides and enzymes that can readily be measured in routine by the hospital biochemistry laboratory. As proteins, they are located at the end of the translational process from DNA to proteins. Amongst the regulatory mechanisms, micro RNAs constitute a new field of research as they have only been discovered in 2001 (Lagos-Quintana, Rauhut et al. 2001, Lau, Lim et al. 2001, Lee and Ambros 2001). MiRNAs, small non coding RNAs with about 21 nucleotides, don't have a proper coding function but regulate gene expression by two mechanisms, RNA degradation and repression of gene translation, both resulting in inhibition of the translational process.

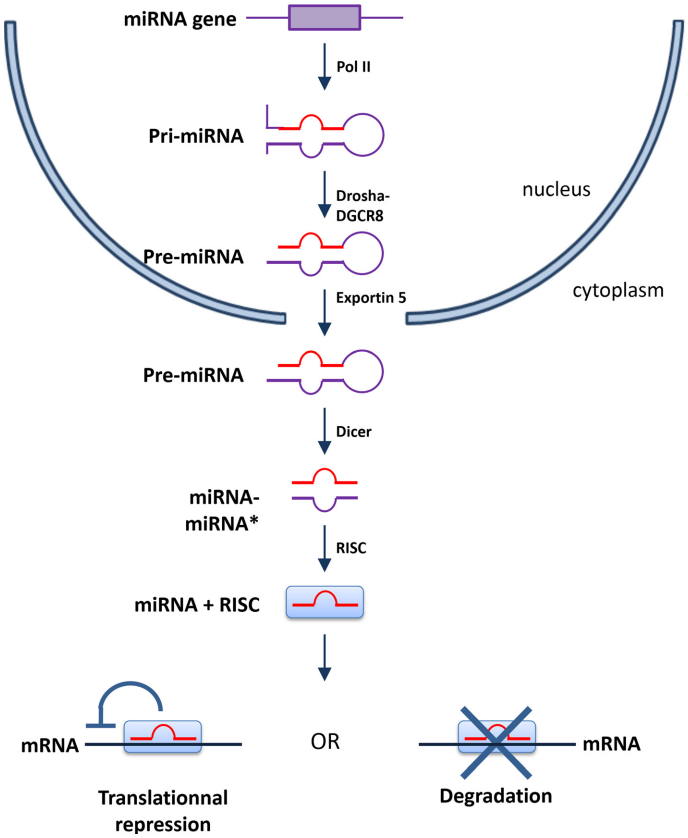


Figure 17: schematic representation of miRNA synthesis and action. MiRNAs are synthesized in the nucleus as primary miRNA and then cleaved to form precursor miRNA which are exported to the cytoplasm. After another cleavage by a complex called DICER, the mature miRNA binds to its target, the messenger RNA (mRNA). MiRNA either induce mRNA destruction or a translational blockade. Pol II: polymerase II; RISC: RNA-induced silencing complex. From (Devaux, Stammet et al. 2015), adapted from (Goretti, Wagner et al. 2013).

In this context, we launched our NorthPole protocol back in 2008. The main goal of this project was to identify novel biosignatures after cardiac arrest in patients treated with therapeutic hypothermia. With paper III, we were able to perform a princeps description of a biosignature after CA: miR-122 and miR-21 could be identified as markers for poor outcome after cardiac arrest. Both miRNAs, although being associated with outcome, were still modest outcome predictors compared to the routine biomarkers NSE and S100. Another study performed on 65 patients could not confirm our results, but identified miR-124 as robust outcome predictor with an AUC of 0.87 (95 CI = 0.79 - 0.96) and 0.89 (95% CI = 0.80 - 0.97) at 24 and 48 hours, respectively (Gilje, Gidlof et al. 2014). The absence of correlation between these 2 studies might emerge from the low overall sample size, the associated comorbidities of the patients, different laboratory approaches because of a lack of standardization of laboratory proceedings and the unclear origin of the miRNA. Although, we could show that miR-21 and miR-122 were present in vitro in neuronal cell cultures, it appears that miR-122 is more liver specific and could not be identified as differentially expressed between good and poor outcome groups in the latter publication.

As miRNAs precede the translation from RNA to proteins, they might theoretically be earlier biomarkers than the proteins themselves. Furthermore, they may form a future potential therapeutic target with regards to gene regulation by using “antagomirs” or by the insertion of miRNAs in artificial exosomes crossing even the blood brain barrier (Devaux, Stammet et al. 2015). Recent experimental studies report success in extending neuroprotection of insulin-like growth factor after stroke in middle age female rats after injection of “antagomirs” (Selvamani, Sathyan et al. 2012). Another report could demonstrate the benefit of locking mir-130a to reduce the cerebral infarct volume in a transient ischemic attack in rats (Sepramaniam, Ying et al. 2012). More recently, in a mouse model of stroke, it could be shown that intravenous injection of an anti-miRNA can have a positive effect on recovery (Caballero-Garrido, Pena-Philippides et al. 2015). Before these strategies can be implemented in clinical practice, many open questions have to solved as many miRNAs are not fully explored; their molecular targets, their origins and their functions are still under investigation. Furthermore, new miRNAs are regularly discovered.

We must acknowledge, that our miRNAs study was a proof of concept study and with only 28 patients the sample size was very small. Given the fact that we performed an *a posteriori* selection of the patients regarding outcome (CPC1 vs CPC5 exclusively) and because of the availability of the different blood samples, a selection bias may not be ruled out. Also, we only performed testing at 48 hours after ROSC because of the availability of the samples in our cohort. The biospecimens originating from the TTM trial provide an optimal platform for ongoing research in a larger population and with samples available at 24, 48 and 72 hours after ROSC. As such, our work must be considered as a preliminary step towards a potential new field of biomarkers with potential therapeutic implications.

7. CONCLUSIONS AND PERSPECTIVES

This thesis aimed at improving the prediction tools for neurological outcome after cardiac arrest. Different parameters have been studied and we could refine the role of NSE and S100. Both biomarker are unaffected by the level of target temperature management. NSE outperformed S100 in terms of accuracy of neurologic prediction. For both biomarkers, we advocate an order of magnitude rather than a fixed cut-off value. For NSE, serial measurements over the first 72 hours after ROSC are an additional tool to improve the power of outcome prediction. For S100, the most discriminative time point is 24 hours after ROSC and timely evolution has no prognostic value.

With the description and identification of micro RNAs in our proof of concept study, we were able to show that these molecules constitute a new category of biomarkers after cardiac arrest. As research within this particular field is only in the beginning, many more discoveries are to be expected in the future. Additional data on miRNAs from our group are currently under review for publication. Besides the studied micro RNAs, more recently long non coding RNA and circular RNA were described and have also been shown to have a role in heart failure (Kumarswamy, Bauters et al. 2014, Devaux, Zangrando et al. 2015). Both types of this new “non coding RNAs” family, comprising miRNA, lncRNA and circRNA, are already part of an ongoing follow-up project on the TTM trial biobank material.

BIS has the potential to become a very early outcome predictor as it consistently yields accurate outcome prediction within the first hours after admission to the ICU. As all reported studies have been performed only in single centers, there is a need for incorporating BIS into a larger multicenter trial. Although this approach is tempting for prognostication, we suggest, at best, to use it to select patients with the lowest risk of brain damage for allocation of maximal therapy and keeping standard intensive care for those with supposed severer brain damage.

It is also noteworthy that current prognostication tools have been studied to predict poor outcome. A future application, especially with regard to early triage of patients would be the development of a tool to predict good outcome, or minimal brain damage. Here too, BIS, as a functional parameter with early prediction capacity might be an interesting parameter. A recent study on amplitude integrated EEG could detect good outcome based on the time to normal trace <24 hours (Oh, Park et al. 2015). This study found impressive AUC (0.97, 95%CI 0.92-0.99) and sensitivity (0.95, 95%CI 84.9–98.9) and specificity (0.91, 95% CI 81.7–96.2) values, but the confidence intervals remained large.

Future research on prediction parameters should distinguish between triage methods, allowing to tailor the individual intensive care, and prognostic parameters, to be used at a later stage and with more robust background to allow WLST. In agreement with the current guidelines, we acknowledge that existing outcome predictors at 24 hours after ROSC are not robust enough in terms of specificity/sensitivity to allow WLST or even downgrading of intensive care. At best they might be used no to upgrade care to the highest level of complexity or cost in patients with supposed poor outcome.

A multimodal predictor approach within a single model is prone to improve the prediction accuracy. By combining different parameters, like brain biomarkers of different origins, but perhaps also inflammatory or cardiac markers, peri-arrest parameters (Utstein data), neurological findings, neurophysiology tests (BIS, malignant patterns on EEG, SSEP) and perhaps imaging findings, it might become possible to determine a score in the future with a very low false positive rate and small confidence intervals. To achieve this goal, the model will have most likely to incorporate data collected starting from ICU admission up to 72 hours after ROSC. Based on some large trials, like the TTM trial, a preliminary work could be launched to serve as hypothesis to test in future trials. As we showed in paper V, it must be born in mind that a model combining single well performing outcome parameters does not

per se increase the performance of the model (Einav, Kaufman et al. 2012). Therefore, great caution and skill is required to construct such a model that will also become applicable and useful in every day clinical practice.

When considering prognostication with an inherent implication regarding the treatment strategy for a patient, ethical issues must also be taken into consideration. Although prognostication is a necessary step in the modern care of post cardiac arrest patients, greatest care must be taken not to prematurely condemn patients. As such prognostication tools must be as robust as possible with highest specificity and sensitivity, lowest false positive rate, and small confidence intervals. Obviously no such test, or even battery of tests exists to date. In the end the clinical decision to start, to increase care or to limit and eventually withdraw care is up to the treating physician. These decisions must always be taken with the complete knowledge of the individual clinical case. Evidently, if there is a doubt about one or more prognostic evaluations, the doubt should benefit the patient. There are cases in the literature describing patients with good outcome despite pejorative clinical or paraclinical findings, especially after the introduction of TTM at 33°C (Sunde, Dunlop et al. 2006). Therefore, the current guidelines wisely indicate that prognostication should be a dynamic process from the event up until at least 5 days after ICU admission, carefully considering any possible confounding factors.

Prognostication after cardiac arrest is a major area of research and interest as the patients resuscitated from cardiac arrest account amongst the most severely ill. They bind a lot of healthcare resources and during the first days after admission their outcome is unclear. With this doctoral thesis, we aimed at contributing to the refinement of some prognostic tools.

To translate our findings into clinical practice, we suggest an approach conforming to the most recent guidelines. Post cardiac arrest care builds on target temperature management. As we were unable to detect a difference in the levels of biomarkers between the 2 temperature groups, we can suppose that the level of TTM does not have any measurable effect on the brain. These findings are supported by the absence of temperature effect in any other analysis of the TTM trial. As such, and for the ease of realization, TTM at 36°C can be safely applied.

Given the early outcome prediction potential of the easy to use BIS, this neuromonitoring should be applied to every patient after cardiac arrest. Its values might be used to guide therapy immediately after ICU admission, but should not be used for outcome prediction at this stage. The use of BIS implicates that a rigorous sedation protocol is used, including neuromuscular block. When it comes to prognostication, it should be performed according to the guidelines at a later stage, when the patient is normothermic and confounding factors are cleared as much as possible. To date, NSE is the outstanding biomarker. To avoid any measurement errors and to improve accuracy of prediction, it should be measured serially at 24, 48 and 72 hours after cardiac arrest. Clinical features, like initial rhythm or absent pupillary light reflex after 24 hours, and other outcome predictors, like SSEPs, must be taken in consideration according to the guidelines. The combination of BIS and NSE (or S100) is a very strong predictor of poor outcome that can advantageously complement the advocated multimodal outcome prediction algorithm.

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8. APPENDIX I: SCIENTIFIC PUBLICATIONS

8.1 Paper I:

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Neuron-Specific Enolase as a Predictor of Death or Poor Neurological Outcome After Out-of-Hospital Cardiac Arrest and Targeted Temperature Management at 33°C and 36°C

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ABSTRACT

BACKGROUND Neuron-specific enolase (NSE) is a widely-used biomarker for prognostication of neurological outcome after cardiac arrest, but the relevance of recommended cutoff values has been questioned due to the lack of a standardized methodology and uncertainties over the influence of temperature management.

OBJECTIVES This study investigated the role of NSE as a prognostic marker of outcome after out-of-hospital cardiac arrest (OHCA) in a contemporary setting.

METHODS A total of 686 patients hospitalized after OHCA were randomized to targeted temperature management at either 33°C or 36°C. NSE levels were assessed in blood samples obtained 24, 48, and 72 h after return of spontaneous circulation. The primary outcome was neurological outcome at 6 months using the cerebral performance category score.

RESULTS NSE was a robust predictor of neurological outcome in a baseline variable-adjusted model, and target temperature did not significantly affect NSE values. Median NSE values were 18 ng/ml versus 35 ng/ml, 15 ng/ml versus 61 ng/ml, and 12 ng/ml versus 54 ng/ml for good versus poor outcome at 24, 48, and 72 h, respectively ($p < 0.001$). At 48 and 72 h, NSE predicted neurological outcome with areas under the receiver-operating curve of 0.85 and 0.86, respectively. High NSE cutoff values with false positive rates ≤5% and tight 95% confidence intervals were able to reliably predict outcome.

CONCLUSIONS High, serial NSE values are strong predictors of poor outcome after OHCA. Targeted temperature management at 33°C or 36°C does not significantly affect NSE levels. (Target Temperature Management After Cardiac Arrest [TTM]; NCT01020916) (J Am Coll Cardiol 2015;65:2104–14) © 2015 by the American College of Cardiology Foundation.

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Comatose patients admitted to an intensive care unit (ICU) after an out-of-hospital cardiac arrest (OHCA) have a mortality rate of around 50%. In the majority of cases, initial ICU mortality is driven by hemodynamic failure, whereas later morbidity and mortality are due to brain damage (1). A large proportion of patients die of withdrawal of life-sustaining therapies because of presumed poor prognosis (2,3). Thus, adequate prognostication tools for neurological outcome prediction are crucial for therapeutic guidance in this severely ill population.

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Biomarkers of brain damage, particularly neuron-specific enolase (NSE), have been widely studied as markers for outcome prognostication (4,5). The protein NSE is a 78kDa glycolytic enzyme involved in glucose metabolism and is mainly found in neuronal and neuroendocrine cells. Its half-life is approximately 24 h. Previous studies on patients not treated with hypothermia after cardiac arrest suggested a cutoff level of 33 ng/ml at 48 h to be predictive of death and poor neurological function (6); the American Academy of Neurology subsequently adopted this cutoff into prognostication guidelines (7). With the implementation of induced hypothermia and its assumed neuroprotective effect, the validity of this cutoff has been questioned. Subsequent studies yielded conflicting results, probably due to methodological issues and the lack of standardization of dosing methods (8). Consequently, current guidelines do not advocate NSE for outcome prediction (9), and a recent advisory statement suggests a cautious use of "high NSE levels" within a multimodal prognostication algorithm (10).

In this context of uncertainty, the TTM trial (Target Temperature Management After Out-of-Hospital Cardiac Arrest) (11), a multicenter clinical trial that included 950 patients randomized to targeted temperature management of 33°C or 36°C, provided a platform to investigate the role of NSE as a prognostic marker of outcome after OHCA in a contemporary setting.

ABBREVIATIONS AND ACRONYMS

CPC = cerebral performance category

FPR = false positive rate

ICU = intensive care unit

IDI = integrated discrimination improvement

NRI = net reclassification index

NSE = neuron-specific enolase

OHCA = out-of-hospital cardiac arrest

ROSC = return of spontaneous circulation

METHODS

All patients included in this study were part of the TTM trial (November 2010 to July 2013) comparing 2 temperature regimens in unconscious adult patients admitted to an ICU after an OHCA of a presumed cardiac cause. The TTM trial design, the statistical analysis plan, and the main results have been published previously (11–13). The randomization was stratified by site and performed centrally with adequate allocation concealment and sequence generation. A target temperature of 33°C or 36°C was initiated in each group according to allocation. At 28 h after start of the intervention, rewarming to 37°C was commenced at a maximum speed of 0.5°C/h. This pre-defined substudy of the TTM trial on NSE was approved by the steering committee before starting NSE analysis.

The TTM trial protocol was approved by ethical committees in each participating country, and informed consent was waived or obtained from all participants or relatives according to national legislations, in line with the Helsinki declaration (14).

Serum blood samples were taken from the patients at 24, 48, and 72 h after return of spontaneous circulation (ROSC). All samples were pre-analytically processed at the different sites, aliquoted, and frozen to −80°C before shipment to the Integrated BioBank of Luxembourg before analysis. NSE values were not available to the treating physicians during the trial.

NSE analyses were performed 6 months after trial completion at the clinical biology laboratory of the Centre Hospitalier de Luxembourg. All serum samples

Luxembourg, Luxembourg; and the §§§Department of Anesthesia and Intensive Care, Helsingborg Hospital, Helsingborg, Sweden.
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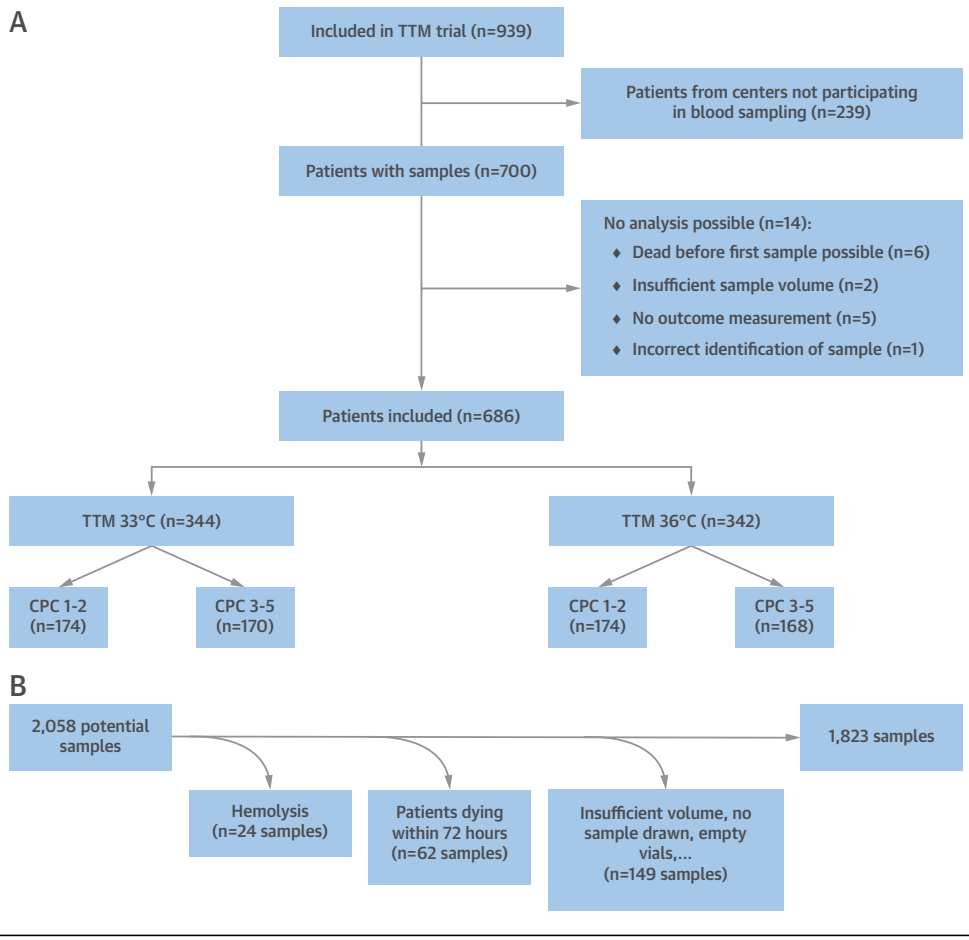
FIGURE 1 Study Flow Charts



(A) This flow chart illustrates the number of patients enrolled in the TTM trial and included in this substudy; **(B)** the accompanying chart provides reasons for eliminating serum samples from analysis. CPC = cerebral performance category; TTM = target temperature management.

were tested for hemolysis using the Roche hemolysis index with measurements at 600 and 570 nm. Because of measurement interference, all samples with a positive hemolysis index (≥500 mg/l of hemoglobin) were discarded.

Determination of NSE was performed using a COBAS e601 line with an Electro-Chemi-Luminescent-Immuno-Assay (ECLIA) kit (Roche Diagnostics, Rotkreuz, Switzerland). The measuring range extended from 0.05 to 370 ng/ml. Samples with values above the measuring range had to be diluted accordingly. Functional sensitivity was at 0.25 ng/ml, and expected normal values were <17.0 ng/ml. In our laboratory, between-run precision at concentrations of 10.5 and 83.3 ng/ml was 6.8% and 5.7%, respectively.

Neurological prognostication as well as withdrawal of life-supporting therapies were standardized and reported according to the trial protocol (12,13,15).

We aimed to investigate NSE as a predictor of death and cerebral performance after OHCA in 2 targeted temperature groups as well as in a pooled sample. We studied the influence of the targeted temperature, evolution over time, predictive power of NSE, and cutoff values, including a multivariable analysis. We defined high NSE cutoff values as having a false positive rate of ≤5%.

TABLE 1 Main Demographic and Utstein Data

	33°C Group (n = 344)	36°C Group (n = 342)	p Value*
Age, yrs	64 ± 12	63 ± 13	0.53
Male	292 (83)	273 (79)	0.18
Bystander CPR performed	255 (72)	249 (72)	0.91
First monitored rhythm			
VF or nonperfusing VT	273 (79)	272 (80)	
Asystole or PEA	67 (19)	64 (19)	
ROSC after bystander defibrillation	6 (2)	3 (1)	0.68†
Time from CA to ROSC, min	30 ± 22	31 ± 24	0.85
Initial serum lactate levels, mmol/l	7 ± 4	7 ± 4	0.93
Circulatory shock on admission	45 (13)	43 (12)	0.97

Values are mean ± SD or n (%). *Wilcoxon rank sum test for continuous variables and chi-square test for categorical variables. †Fisher exact test.

CA = cardiac arrest; CPR = cardiopulmonary resuscitation; PEA = pulseless electric activity; ROSC = return of spontaneous circulation; VF = ventricular fibrillation; VT = ventricular tachycardia.

The primary outcome in this study was neurological function at 6 months, dichotomized to good or poor outcome according to the Cerebral Performance Category (CPC) scale (16). The CPC score classifies patients into 5 categories: CPC 1 (no neurological disability); CPC 2 (minor neurological deficit); CPC 3 (severe neurological impairment, dependent in everyday life); CPC 4 (coma); and CPC 5 (brain death). Secondary outcomes were an assessment of disability according to modified Rankin scale (mRS) at 6 months and all-cause mortality at the end of the trial. Scores on the mRS range from 0 to 6, with 0 representing no symptoms, 1 no clinically significant disability, 2 slight disability, 3 moderate disability, 4 moderately severe disability, 5 severe disability, and 6 death.

CPC scores 1 or 2 and mRS 0 to 3 were considered a good outcome, whereas CPC 3 to 5 and mRS 4 to 6 were considered a poor outcome.

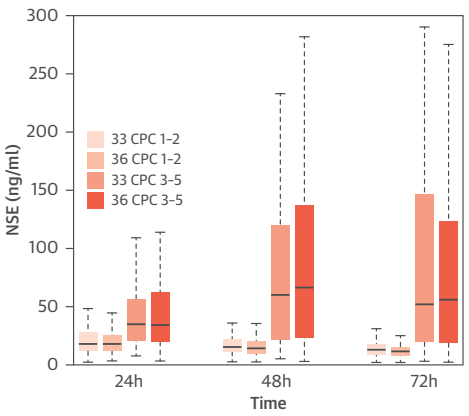
STATISTICAL ANALYSIS. Comparisons of patients' clinical characteristics between temperature groups were performed using the Wilcoxon rank sum test for continuous variables and chi-square or Fisher exact test for categorical variables. Medians with interquartile range (IQR) and mean ± SD are presented.

Changes of NSE concentrations over time were tested for significance using the Wilcoxon signed rank test. Comparison of NSE distribution between CPC groups was performed with the Wilcoxon rank sum test.

At each time point, receiver-operating characteristic curves were plotted and corresponding areas under the curve (AUCs) were determined to evaluate the predictive power of NSE on CPC. Cutoffs were provided as a compromise between sensitivity and specificity by maximizing the Youden index, as defined by sensitivity + specificity - 100%, and by providing 95% to 100% specificity. The same analyses were then performed on NSE change from 24 to 48 h and from 48 to 72 h. All sensitivity and specificity values were corrected for optimism using bootstrap internal validation (100-fold) to avoid overfitting (17). When possible, the normal approximation was used to obtain confidence intervals (CIs); otherwise, the Wilson formula was used (18).

Finally, NSE at 24, 48, and 72 h were added to a clinical multivariable logistic model containing temperature allocation, age, sex, bystander cardiopulmonary resuscitation, first monitored rhythm, time from cardiac arrest to ROSC, lactate levels, and circulatory shock on admission. The relationship between NSE and CPC was supposed to be linear; Pearson residuals were plotted and did not reveal any strong pattern. Restricted cubic splines were also used to model the nonlinear relationship

FIGURE 2 NSE Time Course



In these boxplots of neuron-specific enolase (NSE) over the first 72 h after return of spontaneous circulation (ROSC), data are represented as median, quartile 1, quartile 3, and lower fence (i.e., lowest value above quartile 1 − 1.5 [quartile 3 - quartile 1]) and upper fence (i.e., greater value below quartile 3 + 1.5 [quartile 3 - quartile 1]). No statistical differences were found in NSE values between temperature groups (at all 3 time points, lowest p values were 0.46 in the poor outcome group and 0.09 in the good outcome group). CPC = cerebral performance category.

TABLE 2 NSE Cutoff Values

		Cutoff (ng/ml)	Sensitivity	95% CI	Specificity	95% CI
Pooled						
24 h	NSE Youden	27	0.60	0.55–0.65	0.76	0.71–0.80
	NSE 5	49	0.33	0.28–0.38	0.95	0.93–0.97
	NSE 4	54	0.29	0.24–0.34	0.96	0.94–0.98
	NSE 3	61	0.24	0.19–0.28	0.97	0.95–0.99
	NSE 2	66	0.21	0.16–0.25	0.98	0.97–1.00
	NSE 1	76	0.15	0.11–0.19	0.99	0.97–1.00
	NSE 0	107	0.09	0.06–0.12	1.00	0.99–1.00
48 h	NSE Youden	29	0.69	0.64–0.75	0.87	0.83–0.91
	NSE 5	42	0.61	0.55–0.67	0.95	0.92–0.97
	NSE 4	46	0.60	0.55–0.66	0.96	0.94–0.98
	NSE 3	46	0.59	0.53–0.64	0.97	0.95–0.99
	NSE 2	48	0.58	0.52–0.64	0.98	0.96–0.99
	NSE 1	68	0.47	0.42–0.53	0.99	0.97–0.97
	NSE 0	120	0.27	0.22–0.32	1.00	0.99–1.00
72 h	NSE Youden	23	0.70	0.64–0.76	0.88	0.85–0.92
	NSE 5	33	0.63	0.57–0.69	0.95	0.92–0.97
	NSE 4	35	0.62	0.56–0.68	0.96	0.93–0.98
	NSE 3	35	0.63	0.57–0.68	0.97	0.95–0.99
	NSE 2	38	0.58	0.52–0.64	0.98	0.96–0.99
	NSE 1	45	0.54	0.48–0.60	0.99	0.97–1.00
	NSE 0	50	0.52	0.46–0.58	1.00	0.99–1.00
33°C						
24 h	NSE Youden	27	0.60	0.52–0.67	0.73	0.66–0.79
	NSE 5	49	0.31	0.24–0.39	0.95	0.91–0.98
	NSE 4	54	0.27	0.20–0.34	0.96	0.93–0.99
	NSE 3	61	0.22	0.15–0.28	0.97	0.94–0.99
	NSE 2	68	0.19	0.13–0.25	0.98	0.95–1.00
	NSE 1	96	0.11	0.06–0.16	0.99	0.96–1.00
	NSE 0	103	0.11	0.06–0.16	1.00	0.97–1.00
48 h	NSE Youden	33	0.64	0.56–0.71	0.90	0.86–0.95
	NSE 5	46	0.58	0.50–0.65	0.95	0.92–0.98
	NSE 4	46	0.58	0.50–0.66	0.96	0.93–0.99
	NSE 3	48	0.57	0.49–0.65	0.97	0.93–0.99
	NSE 2	48	0.56	0.48–0.64	0.98	0.95–1.00
	NSE 1	52	0.54	0.46–0.62	0.99	0.96–1.00
	NSE 0	120	0.25	0.18–0.32	1.00	0.97–1.00
72 h	NSE Youden	26	0.69	0.62–0.77	0.91	0.86–0.95
	NSE 5	33	0.65	0.57–0.73	0.95	0.91–0.98
	NSE 4	35	0.63	0.55–0.71	0.96	0.93–0.99
	NSE 3	35	0.63	0.55–0.71	0.97	0.93–0.99
	NSE 2	41	0.58	0.50–0.66	0.98	0.96–1.00
	NSE 1	45	0.53	0.45–0.61	0.99	0.96–1.00
	NSE 0	48	0.54	0.45–0.62	1.00	0.97–1.00

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between NSE and CPC, but the findings were not markedly different (data not shown). The additional predictive power brought by NSE to these markers was evaluated by computing the continuous net reclassification index (NRI) and the integrated discrimination improvement (IDI) (19). In the multivariable analysis, missing values were accounted for using 10-fold multiple imputations.

Computations were performed using the R software, version 2.15.2, packages ROCR, pROC, Hmisc, and rms (R Foundation for Statistical Computing, Wien, Austria). A p value <0.05 was considered to indicate statistical significance.

RESULTS

The TTM trial investigated 939 patients with no difference in mortality or neurological function between the 33°C and 36°C groups (11). Overall, 700 consecutive patients from 29 different sites participated in the biomarker substudy (**Figure 1A**). A total of 1,823 serum samples from 686 different patients were analyzed (**Figure 1B**).

Main patient characteristics are shown in **Table 1**. There were no significant differences between our study population and the main TTM trial population or in neurological outcome between temperature groups ($p = 0.90$) (**Figure 1A**).

Median NSE values were 18 ng/ml (IQR: 12 to 27 ng/ml) versus 35 ng/ml (IQR: 21 to 58 ng/ml), 15 ng/ml (IQR: 10 to 2 ng/ml 1) versus 61 ng/ml (IQR: 24 to 125 ng/ml), and 12 ng/ml (IQR: 9 to 16 ng/ml) versus 54 ng/ml (IQR: 19 to 132 ng/ml) for good versus poor outcome at 24, 48, and 72 h, respectively ($p < 0.001$). NSE values in both temperature groups were higher in the poor versus the good outcome group at each time point (**Figure 2**). In both good and poor outcome groups, levels of NSE were not significantly affected by the target temperature level.

In the poor outcome groups, we observed a significant increase of median NSE values between 24 and 48 h in both temperature groups: from 35 ng/ml (IQR: 21 to 56 ng/ml) to 60 ng/ml (IQR: 22 to 119 ng/ml) in 33°C ($p < 0.001$) and from 34 ng/ml (IQR: 21 to 62 ng/ml) to 66 ng/ml (IQR: 24 to 137 ng/ml) in 36°C ($p < 0.001$). Between 48 and 72 h, median NSE values decreased in the 33°C group from 60 ng/ml (IQR: 22 to 119 ng/ml) to 52 ng/ml (IQR: 20 to 147 ng/ml) ($p = 0.029$) and in the 36°C group from 66 ng/ml (IQR: 24 to 137 ng/ml) to 56 ng/ml (IQR: 19 to 123 ng/ml) ($p = 0.75$).

In the good outcome groups, we detected a significant decrease of approximately 3 to 4 ng/ml between 2 consecutive time points, with median NSE values at 24, 48, and 72 h at 33°C of 18 ng/ml (IQR: 12 to 27 ng/ml), 15 ng/ml (IQR: 11 to 22 ng/ml), 13 ng/ml (IQR: 9 to 18 ng/ml), respectively ($p < 0.001$), and at 36°C of 18 ng/ml (IQR: 12 to 26 ng/ml), 14 ng/ml (IQR: 10 to 20 ng/ml), 11 ng/ml (IQR: 8 to 15 ng/ml), respectively ($p < 0.001$).

The capacity of NSE to predict CPC at 6 months was first determined using receiver-operating

characteristic curves (Figures 3A to 3C). Twenty-four hours after cardiac arrest, NSE predicted 6-month CPC with an AUC of 0.75. At 48 and 72 h, AUCs were 0.85 and 0.86, respectively. The AUCs obtained at 33°C and 36°C groups were similar.

The change of NSE between 24 and 48 h had an AUC of 0.80 (33°C group) and 0.84 (36°C group), and between 48 and 72 h, the AUC was lower than 0.70 for both groups. An increase of NSE of 6 ng/ml between any of the time points, regardless of the target temperature, was also predictive of a poor outcome (specificity 94% and sensitivity 64% between 24 and 48 h; specificity 93% and sensitivity 39% between 48 and 72 h).

In our cohort, the previously recommended (7) cutoff value of 33 ng/ml at 48 h yielded a specificity of 0.91 and a sensitivity of 0.65.

By maximizing the Youden index, cutoff values for NSE in the pooled patient group were 27, 29, and 23 ng/ml at 24, 48, and 72 h, respectively (Table 2). NSE cutoff values with false positive rates (FPRs) from 5 to 1 range from 49 to 76 ng/ml, 42 to 68 ng/ml, and 33 to 45 ng/ml at 24, 48, and 72 h, respectively (Table 2). No patient with a good outcome had an NSE value at or above the cutoff reported with an FPR of zero ("NSE 0" values in Table 2).

Kaplan-Meier curves showed that survival was significantly lower in groups with higher NSE levels as defined by quartiles (Central Illustration). NSE at each time point was an efficient predictor of survival in both temperature groups (all $p < 0.05$).

MULTIVARIABLE ANALYSIS. In multivariable analysis including serial NSE, target temperature, and baseline variables (age, sex, bystander cardiopulmonary resuscitation, first monitored rhythm, time to ROSC, lactate levels on admission, and circulatory shock), NSE was a strong predictor of neurological outcome at each time point (Table 3). Our model integrating NSE measures at 3 time points had a specificity of 0.88 and a sensitivity of 0.84. Continuous NRI (1.29; $p < 0.001$) and IDI (0.37; $p < 0.001$) showed that NSE significantly improved classification compared with a model with clinical parameters alone. When modeling CPC using the mean and the trend effects of NSE values, which are independent, we found the same results. When adjusted for centers, NSE remained a highly significant outcome predictor, and no site effect was observed (data not shown).

When analyzing the capacity of NSE to predict mRS and death at 6 months as well as death at the end of the trial, we found similar results to those referring to CPC at 6 months and with no influence of target temperature (data not shown).

TABLE 2 Continued

		Cutoff (ng/ml)	Sensitivity	95% CI	Specificity	95% CI
			36°C			
24 h	NSE Youden	23	0.66	0.59–0.74	0.71	0.63–0.78
	NSE 5	50	0.36	0.29–0.44	0.95	0.91–0.98
	NSE 4	57	0.30	0.23–0.37	0.96	0.93–0.99
	NSE 3	57	0.28	0.21–0.35	0.96	0.93–0.99
	NSE 2	65	0.23	0.16–0.30	0.98	0.95–1.00
	NSE 1	76	0.16	0.10–0.21	0.99	0.96–1.00
	NSE 0	108	0.08	0.03–0.12	1.00	0.97–1.00
48 h	NSE Youden	29	0.70	0.63–0.78	0.90	0.85–0.95
	NSE 5	40	0.61	0.53–0.69	0.95	0.91–0.98
	NSE 4	42	0.62	0.54–0.70	0.96	0.93–0.99
	NSE 3	44	0.61	0.53–0.69	0.97	0.93–0.99
	NSE 2	44	0.60	0.52–0.68	0.98	0.96–1.00
	NSE 1	70	0.48	0.40–0.57	0.99	0.96–1.00
	NSE 0	76	0.45	0.37–0.53	1.00	0.97–1.00
72 h	NSE Youden	22	0.68	0.60–0.76	0.89	0.84–0.94
	NSE 5	34	0.61	0.52–0.70	0.95	0.91–0.98
	NSE 4	34	0.62	0.53–0.70	0.96	0.93–0.99
	NSE 3	37	0.59	0.50–0.67	0.97	0.93–0.99
	NSE 2	37	0.58	0.49–0.67	0.98	0.95–1.00
	NSE 1	48	0.51	0.43–0.60	0.99	0.96–1.00
	NSE 0	53	0.52	0.43–0.61	1.00	0.97–1.00

NSE Youden indicates an NSE cutoff that compromises sensitivity and specificity (maximized Youden index). The number following NSE refers to the false positive rate. Sensitivity and specificity are corrected by bootstrap internal validation.

CI = confidence interval; NSE = neuron-specific enolase.

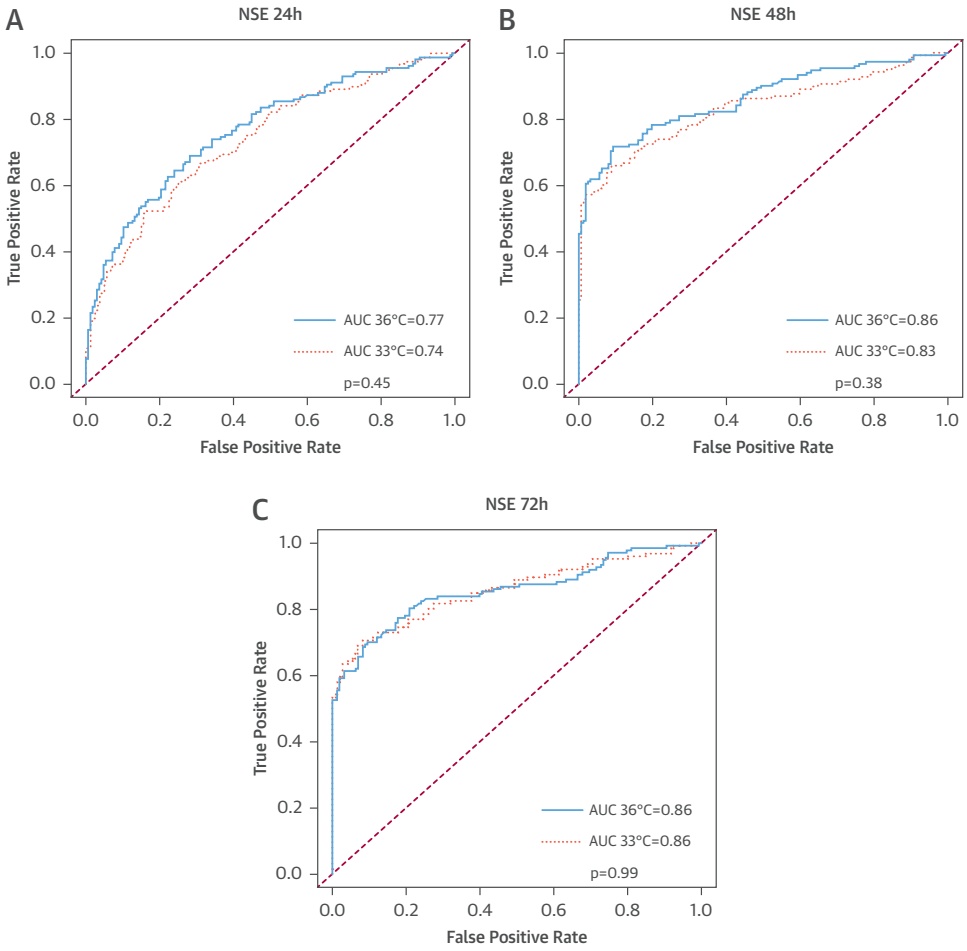
DISCUSSION

In a large international trial of patients treated with targeted temperature after out-of-hospital cardiac arrest, NSE was a strong and robust predictor of outcome (Central Illustration). Target temperature level did not significantly influence NSE values.

Although median NSE values declined between 48 and 72 h in all groups, we confirmed that an increase of NSE between any 2 time points was associated with poor outcome (20–24). There was no significant difference between temperature groups at any time point for any of our outcome measures, substantiating previous studies reporting no statistically significant differences in NSE values between temperatures (21,25–27). Other studies reporting lower NSE values in 33°C-treated patients suffered from limitations, notably due to the comparison of patients treated at 33°C to historical control subjects (28) or to a small sample size in a population without fever management in the control group (20).

The cut-off values at 48 and 72 h after ROSC provided the best capacity to predict outcome when referring to the highest sensitivities and specificities. At 24 h, sensitivity was too low to be

FIGURE 3 NSE Receiver-Operating Curves

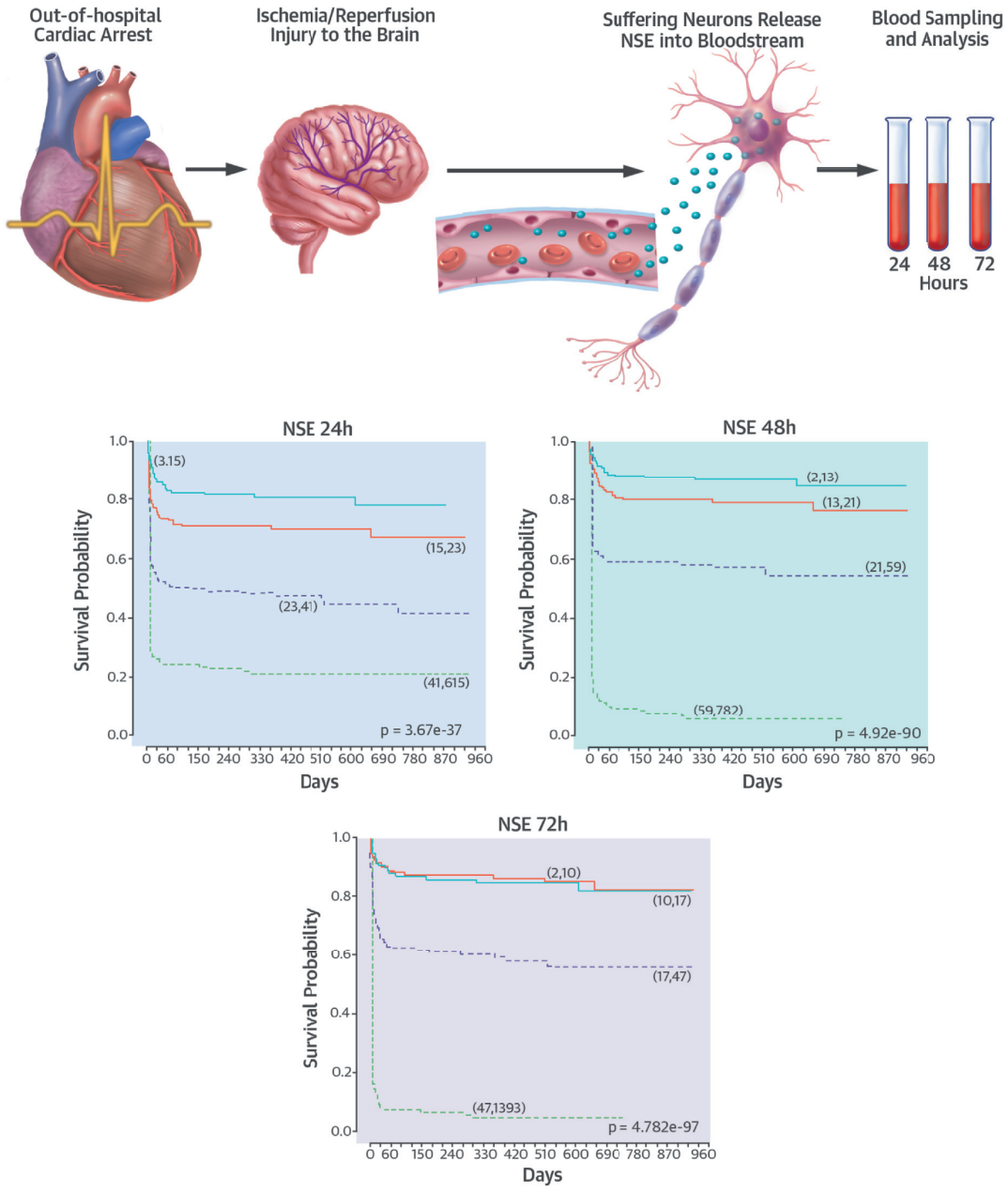


Receiver-operating characteristic curves with areas under the curve (AUCs) for NSE at **(A)** 24, **(B)** 48, and **(C)** 72 h after ROSC for outcome prediction according to CPC at 6 months. Abbreviations as in Figure 2.

of clinical interest. Deliberately, we presented FPRs of 5% or lower, as no compromise in the literature exists that defines the absolute best characteristics of a biomarker cutoff value. As such, we showed that "high" NSE cutoff values (with ≤5% FPR and tight 95% CIs) offer reliable prediction of poor outcome with sufficient sensitivity to remain clinically useful within a multimodal prognostication package, including clinical examination, imaging, neurophysiology, and biomarkers (10,29). Notwithstanding the low FPR and narrow 95% CIs of cutoff values in our sample, no single test, even with high specificity, should be considered for withdrawal of life-sustaining therapies. Also, by looking for cutoffs with an FPR of 0, indicating absolute poor outcome prediction, values around 100 ng/ml might have a too low sensitivity to be of clinical utility.

Our cutoff values are higher than the formerly reported 33 ng/ml at 48 h (6,7). Several explanations exist for discrepancies with previous studies. First, the assay we used differed from some of the previous reports, and variability among NSE assays is well described (30). A recent publication by Rundgren et al. (8) showed that NSE values can vary by 15% to 36% based on the assay used and

CENTRAL ILLUSTRATION **NSE for Prognostication After Cardiac Arrest**



Stammet, P. et al. J Am Coll Cardiol. 2015; 65(19):2104-14.

After an out-of-hospital cardiac arrest (OHCA), neuron-specific enolase (NSE) is released by dying neurons into the blood stream. NSE values determined in blood samples taken at 24, 48, and 72 h after cardiac arrest can guide prognostication after OHCA. No significant difference between target temperature groups was noticed in NSE levels. When taken at these time points, NSE levels, divided into quartiles, showed that survival was significantly lower with higher NSE levels as seen in the Kaplan-Meier curves of pooled data from both target temperature groups.

TABLE 3 Multivariable Analysis With NSE and Clinical Variables

	Effect	Odds Ratio	95% CI Lower	95% CI Upper	p Value
Intercept	−6.853	0.001	0.0001	0.001	<0.0001
NSE 24 h, ng/ml	−0.034	0.966	0.945	0.989	0.0034
NSE 48 h, ng/ml	0.040	1.041	1.012	1.070	0.0044
NSE 72 h, ng/ml	0.066	1.069	1.033	1.105	0.0001
Target temperature	0.247	1.280	0.796	2.059	0.3076
Age, yrs	0.094	1.098	1.073	1.124	<0.0001
Male	−0.307	0.735	0.408	1.326	0.3066
Bystander CPR performed	−0.601	0.548	0.323	0.930	0.0257
First monitored rhythm	−1.276	0.279	0.134	0.580	0.0006
ROSC after bystander defibrillation	−1.460	0.232	0.038	1.421	0.1142
Time from CA to ROSC	0.010	1.010	0.996	1.023	0.1534
Serum lactate level	0.012	1.012	0.950	1.078	0.7051
Shock on admission	0.332	1.394	0.661	2.939	0.3829

Abbreviations as in Tables 1 and 2.

whether fresh or frozen samples were analyzed. Outcome measurements in previous publications also differed in time to follow-up, ranging from ICU discharge to 6 months, and some studies categorized neurological outcome differently (5). We used the most common follow-up period of 6 months, the CPC 1 or 2 score for good outcome, the CPC 3 to 5 scores for poor outcome, and, most importantly, a blinded outcome assessment with face-to-face interviews (11,12,31,32). Another explanation for our reported discrepancies might be that the TTM trial prognostication and, when indicated, subsequent withdrawal of life-sustaining therapy were well codified and delayed. Furthermore, our sample included more than twice the number of patients compared with the 272 normothermic individuals in the PROPAC (PROgnosis after PostAnoxic Coma) trial (6). The latter served as a basis of the American Academy of Neurology guidelines for outcome prediction, which fixed the NSE cutoff as 33 ng/ml with an FPR of 0 (7). In our cohort, an FPR of 0 could not be verified at the 33 ng/ml cutoff, which yielded an FPR of 9%. Zellner et al. (33) had similar findings as ours in patients at 33°C with 10% FPR at cutoff values of 41 ng/ml at 48 h.

Our multivariable model, integrating NSE at the 3 time points, confirmed NSE as a predictor of CPC in this set of patients as shown by the highly significant NRI and IDI. These findings are in line with previous studies and strengthen the position of NSE as a robust and clinically-useful outcome predictor (21).

STUDY STRENGTHS AND LIMITATIONS. Although being a pre-defined substudy of the TTM trial, not all sites enrolling in the main trial participated in biomarker sampling. However, as the trial was stratified for sites, the balanced design tends to be preserved in all comparisons between the temperature groups. Indeed, our population did not differ significantly from the TTM trial population. Not all patients had blood samples taken at every time point, and there was no external quality control at each participating site where samples were collected and preanalytically processed.

Biomarkers, unlike some prognostic neurophysiology tests, are unaltered by sedation and may, therefore, be a more objective marker of brain injury. One general limitation of biomarkers is that their measurement is punctual, whereas production or secretion is a dynamic process, highlighting the importance of serial measurements taking into account the absolute values, their changes over time, and serial cutoffs to best predict outcome (4). Brain biomarkers measured in circulating blood might have some additional weaknesses as the integrity of the blood brain barrier after ischemia-reperfusion injury in individuals cannot be measured and may vary substantially. In the case of NSE, which is predominantly released from neural and neuroendocrine cells, caution is warranted as serum levels might reflect variable degrees of brain damage, disruption of the blood brain barrier, or—albeit rarely—NSE from extracerebral origins as seen in small-cell lung cancer and neuroendocrine tumors (24).

The major strength of this investigation is that it was a pre-defined substudy investigating a serum biomarker for prognostication after OHCA within the largest multicenter randomized clinical trial studying 2 target temperature regimens in comatose cardiac arrest patients. It represents the largest prospective study of its kind. All analyses were performed in a single core laboratory, limiting the influence of assay variability and laboratory processing. The results of NSE values were not available to the treating physicians during the trial and, therefore, did not influence prognostication of patients, reducing the risk of "self-fulfilling prophecy." A unique feature of the TTM trial is that prognostication and withdrawal were standardized, which increases the validity of our results (34).

As is the case with other prognostic tools, the current study demonstrated that the functional consequences of brain injury cannot be predicted by NSE alone. When using NSE, we recommend a dynamic approach with serial measurements within a prognostication protocol including other methods, such as clinical examination, electroencephalogram, brain imaging, and somatosensory-evoked potentials, for the most accurate outcome prediction (10,29).

CONCLUSIONS

Serial, high NSE values have a high predictive value of poor outcome in comatose out-of-hospital cardiac arrest patients. This predictive value of NSE is not significantly affected by target temperature at either 33°C or 36°C.

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PERSPECTIVES

COMPETENCY IN MEDICAL KNOWLEDGE: Cardiac arrest is associated with 50% mortality in those patients admitted to hospital after resuscitation in the field, and the predominant cause of death in these cases is severe neurological injury.

COMPETENCY IN PATIENT CARE: Consistently high levels of brain-oriented biomarkers, like NSE, may identify patients prone to poor outcomes after resuscitation from OHCA.

TRANSLATIONAL OUTLOOK: Identification of combinations of variables that accurately correlate with more or less favorable outcomes could lead to the development of more effective therapeutic strategies for victims of cardiac arrest.

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KEY WORDS biomarker, cerebral performance, neuroprognostication, prognosis

8.2 Paper II:

Protein S100 as outcome predictor after out-of-hospital cardiac arrest and targeted temperature management at 33°C and 36°C. Stammet P, Nielsen N, Fays F, et al. Submitted.

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Conflicts of interest:
TP reports lecture fees from Bard Medical.

Author contribution statement:
PS, NN, YD, CH, HF, MK, MWi, DE conceived this study. NN, HF, CH, YD obtained funding. GG performed the laboratory analysis. OC and FF performed the statistics. PS, HF, NN and YD drafted the manuscript. All others are steering group members and actively recruited patients and participated in blood sampling. All authors have read, critically reviewed and accepted the present manuscript. PS takes the responsibility for the paper as a whole.

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ABSTRACT

Study objective:
To specify protein S100 as neurologic outcome predictor, including the potential effect of targeted temperature management at either 33°C or 36°C after out of hospital cardiac arrest (OHCA), evolution over time and comparison to other outcome predictors, including neuron specific enolase (NSE)**.** Six months outcome was assessed using the cerebral performance category scale (CPC 1-2 = good outcome, CPC 3-5 = poor outcome).

Methods:
This study was a predefined **s**ub study of the TTM trial. Serum levels of S100 were measured *a posteriori* in a core laboratory in samples collected at 24, 48 and 72 hours after return of spontaneous circulation.

Results:
687 patients were included. Median S100 values were significantly higher in poor compared to good outcome patients at 24, 48 and 72 hours respectively: 0.19 [IQR 0.10-0.49] versus 0.08 [IQR 0.06-0.11] µg/ml, 0.16 [IQR 0.10-0.44] versus 0.07 [IQR 0.06-0.11] µg/L and 0.13 [IQR 0.08-0.26] versus 0.06 [IQR 0.05-0.09] µg/L. Over time S100 values decreased in both temperature groups and in both outcome groups. S100 values were not significantly affected by the level of target temperature. The outcome prediction capacity of S100 was best at 24 hours with an area under the curve (AUC) of 0.80 (95% CI: 0.77-0.83). In multivariable analyses including age, gender, bystander cardiopulmonary resuscitation, initial rhythm, time to return of spontaneous circulation, allocated target temperature, lactate level and shock on admission, serial S100 measurements improved the prediction from an AUC of 0.80 to 0.84, but S100 was no independent outcome predictor. Adding S100 to the same model including NSE did not further improve the AUC.

Conclusions:
S100 values are not affected by the level of target temperature. High S100 values are predictive of poor outcome in univariate analysis but do not outperform NSE.

Keywords: biomarker, prognosis, neuroprognostication, cerebral performance

Clinical Trial: NCT01020916

Introduction

Background and importance

Mortality in comatose out-of-hospital cardiac arrest (OHCA) patients admitted to an intensive care unit (ICU) is around 50%. Whilst initial ICU mortality is caused by hemodynamic failure in the majority of cases, later morbidity and mortality are mainly due to hypoxic brain damage [1, 2]. Withdrawal of life-sustaining therapies (WLST) based on presumed poor neurologic prognosis is the predominant cause of death [2, 3]. To better guide therapy and to support decisions on WLST, there is a need for early and accurate outcome prediction tools in this severely ill population.

Amongst biomarkers of brain damage, the protein S100 is a potential marker for outcome prognostication after cardiac arrest [4, 5].

The protein S100 is an approximately 21kDa intracellular calcium-binding dimer implicated in neuronal differentiation, proliferation and apoptosis [6]. Many subtypes of the S100 protein are known but the most studied in humans are the brain specific homodimers A1B (αβ) and BB (ββ) [7, 8].S100 is mainly present in white matter, predominantly in astroglial cells in contrast to neuron specific enolase (NSE) which is largely found in neurons and neuroendocrine cells [9]. Thus in the context of hypoxic-ischemic brain damage after cardiac arrest (CA), serum S100 levels may reflect the degree of brain damage and complement other prognostication tools including the established biomarker NSE.

Goals of this study

Previous studies on the outcome prediction capacity of S100 in patients after cardiac arrest yielded a wide range of cut-off values for a poor outcome and current guidelines do not advocate the use of S100 for this purpose [10]. We aim at investigating the role of S100 as outcome predictor after cardiac arrest. In particular, we want to study the capacity of serial S100 levels to reliably predict outcome after cardiac arrest, the influence of the level of the targeted temperature, and perform a multivariable analysis including NSE, according to the results of our previously published study[11].

The Target Temperature Management after out-of-hospital cardiac arrest trial (TTM-trial), a multicenter clinical trial that included 939 patients randomized to targeted temperature management of 33°C or 36°C, provided an optimal platform to further specify the role of S100 as a prognostic marker of outcome after OHCA [12].

Methods and Measurements

Study design and settings

All patients included in this study were part of the TTM trial (November 2010 to July 2013), comparing two target temperature regimens in adult unconscious patients admitted to an ICU after an OHCA of a presumed cardiac cause [12]. The TTM trial design, statistical analysis plan, and main results have previously been published [12-14](registration at clinicalTrials.gov: NCT01020916). The randomization was centrally performed and stratified by site with adequate allocation concealment and sequence generation. According to allocation a target temperature of 33ºC or 36°C was initiated. Twenty-eight hours after start of the intervention, rewarming to 37°C was started at a maximum speed of 0.5°C/h. The steering committee approved this predefined substudy before trial completion and before starting analysis of S100. In line with the Helsinki declaration, the TTM trial protocol was approved by ethical committees in each participating country and informed consent was waived or obtained from all participants or relatives according to national legislations.

Selection of patients

All patients included in the TTM trial were part of this sub study. The TTM trial included all patients admitted to the ICU after out-of-hospital cardiac arrest. Exclusion criteria were randomization not performed within 240 minutes after return of spontaneous circulation (ROSC), unwitnessed asystole and refractory shock. For this substudy, no patient selection was performed. Seven TTM trial centers did not participate to the biobank part, because of legal concerns. Patients dying before the possible blood sampling respectively with incomplete sampling were treated as missing data.

Sampling and measurements

After return of spontaneous circulation (ROSC), serum blood samples were collected from the patients at 24, 48, and 72 hours. All samples were pre-analytically processed at the different sites, aliquoted, and frozen to -80°C before shipment to the Integrated Biobank of Luxembourg. As analyses were performed *a posteriori*, S100 values were not available to the treating physicians during the trial.

S100 determination was performed 6 months after trial completion at the clinical biology laboratory of the Centre Hospitalier de Luxembourg.

Determination of S100 (S100A1B and S100BB) was performed using a COBAS e601 line with an Electro-Chemi-Luminescent-Immuno-Assay (ECLIA) kit (Roche Diagnostics, Rotkreuz, Switzerland). The measuring range extended from 0.005 to 39 µg/L. Samples with values above the measuring range had to be diluted accordingly. Functional sensitivity was at 0.02 µg/L, and expected normal values were <0.105 µg/L. In our laboratory, between-run precision at concentrations of 0.18 and 2.33 µg/L was 2.6% and 3.6%, respectively.

Outcomes

We aimed to investigate S100 as a predictor of death and cerebral performance after OHCA in both temperature groups. We defined high S100 cutoff values as having a false positive rate for poor outcome ≤ 5%.

The primary outcome in this study was neurological function at 6 months, dichotomized into good or poor outcome according to the Cerebral Performance Category (CPC) scale [15]. The CPC score classifies patients into 5 categories: CPC 1 (no neurological disability); CPC 2 (minor neurological deficit); CPC 3 (severe neurological impairment, dependent in everyday life); CPC 4 (coma); and CPC 5 (brain death).

CPC scores 1 or 2 were considered a good outcome, while CPC 3 to 5 were considered a poor outcome.

Neurological prognostication as well as WLST were standardized and reported according to the trial protocol [12-14].

The effect of target temperature on S100 values is analyzed. Finally, to better discriminate the predictive value of S100, we will perform a multivariable analysis, including NSE.

Statistical analysis

Univariate analysis consisted in plotting receiver operating characteristics (ROC) curves of S100 over time and computing their area under the curve (AUC). Predictive cutoffs were determined by maximizing the Youden index and by reporting 95% to 100% specificity. Multivariable analyses were performed by adding S100 measurements first to a logistic clinical model of CPC adjusted for targeted temperature and for the patients characteristics defined in Table 2, and then to the same model including both those variables and NSE measurements at 24, 48 and 72 hours. Bootstrap internal validation and multiple imputations were further performed to respectively correct sensitivity and

specificity for optimism and to account for missing data. The continuous net reclassification index (NRI) and the integrated discrimination improvement (IDI) were computed in order to evaluate the added predictive value of S100. DeLong's test was used to compare AUCs computed without multiple imputations and likelihood ratio test was performed to compare models' fit. Prediction of survival at the end of the trial was assessed using Kaplan-Meier curves and the Log Rank test.

Multivariable models were calculated for clinical variables with and without S100 as well as with NSE based on our previous publication [11].

The R software (version 2.15.2) with the packages ROCR, pROC, Hmisc, and rms was used to perform the computations. A p value <0.05 was considered statistically significant.

Results

Characteristics of study subjects

The TTM trial investigated 939 patients with no difference in mortality or neurological function between the 33°C and the 36°C groups [12]. Overall, 700 consecutive patients from 29 different sites participated in the biomarker substudy (**Figure 1A**). A total of 1843 serum samples from 687 different patients were analyzed (**Figure1B**). Main patient characteristics are shown in **Table 1**. There were no marked differences between our study population and the main TTM trial population nor in neurological outcome between temperature groups (data not shown).

S100 values by outcome group.

Median S100 values were significantly higher in poor versus good outcome patients at 24, 48 and 72 hours respectively: 0.19 [IQR 0.10-0.49] versus 0.08 [IQR 0.06-0.11] µg/ml, 0.16 [IQR 0.10-0.44] versus 0.07 [IQR 0.06-0.11] µg/L and 0.13 [IQR 0.08-0.26] versus 0.06 [IQR 0.05-0.09] µg/L (**Figure 2**).

Time course of S100.

In both outcome groups, we observed a significant decrease of median S100 over time. For good outcome median values were 0.08 [0.06-0.11], 0.07 [0.06-0.11] and 0.06 [0.05-0.09] µg/L at 24, 48 and 72 hours respectively, compared to 0.19 [0.10-0.49], 0.17 [0.10-0.44] and 0.13 [0.08-0.26] µg/L at 24, 48 and 72 hours respectively in the poor outcome group (**Figure 2**).

Influence of temperature on S100.

When comparing the temperature groups regardless of outcome (pooled data), S100 values were higher at 24 and 72 hours in the 33°C group compared to the 36°C group (0.12 [0.07-0.22] vs 0.10 [0.07-0.21] µg/L, and 0.09 [0.06-0.17] vs. 0.08 [0.05-0.10] at 24 and 72 hours respectively). No significant difference was found at 48 hours. When comparing the groups according to their outcome, we only found higher median values in the good outcome groups in the 33°C arm compared to 36°C: 0.08 [0.07-0.12] vs 0.07 [0.05-0.10] µg/L, 0.08 [0.06-0.12] vs 0.07 [0.05-0.10] µg/L and 0.07 [0.05-0.10] vs 0.06 [0.04-0.08] µg/L at 24, 48 and 72 hours respectively. There was no significant difference in levels of S100 between temperature groups in the poor outcome groups.

Prediction capacity of S100.

The capacity of S100 to predict CPC at 6 months was first determined using ROC curves (**Figure 3A through 3C**). The best performance of S100 was achieved at 24 hours, with an AUC of 0.78 (95% CI:

0.73-0.83) for patients treated at 33°C and 0.82 (95% CI: 0.77-0.87) for patients treated at 36°C and an AUC of 0.80 (95% CI: 0.77-0.83) for pooled data. At 48 hours and 72 hours, AUCs were lower. There was no significant difference for the AUC between the temperature groups.

Cutoff values for poor outcome, with false positive rates (FPR), ranging from 0 (100% specificity) to 5% as well as with a maximized Youden index for pooled data are presented in Table 2. Cutoff values for both temperatures groups were not markedly different except for those with a FPR of zero because of outliers (data not shown).

Survival was associated to S100 levels and was significantly lower in groups with higher levels as defined by quartiles (**Figure 4**). At each time-point S100 was a significant predictor of survival in both temperature groups.

Multivariable analysis.

In multivariable analysis including the allocated target temperature and baseline variables (age, gender, bystander CPR, first monitored rhythm, time to ROSC, lactate levels on admission, and circulatory shock), all variables except target temperature, gender and shock on admission were independent neurological outcome predictors (AUC 0.80, 95%CI: 0.76-0.83, sensitivity 0.73, specificity 0.76). When serial S100 values were added to this model, S100 was not an independent outcome predictor, but the AUC improved to 0.84 (95%CI: 0.81-0.87, sensitivity 0.75, specificity 0.81, DeLong test $p<0.001$) (**Table 3a**). Adding S100 improved the reclassification of patients significantly as demonstrated by continuous NRI (0.53, $p<0.001$) and IDI (0.08, $p<0.001$). When adding serial S100 values to a previously published model on the same population and including the same clinical characteristics with serial NSE values at 3 time-points (AUC 0.92, 95%CI: 0.90-0.94) [11], S100 did not further improve the AUC (0.92, 95%CI: 0.90-0.94, sensitivity 0.81, specificity 0.92, DeLong test $p=0.13$) (**Table 3b**). We performed the same multivariable analysis without multiple imputations on the whole cohort (479 patients) and in unconscious patients at day 3 (374 patients without multiple imputation and 545 patients with multiple imputation), with and without including NSE. S100 always failed to turn out as independent outcome predictor. A Spearman test showed that S100 and NSE were strongly correlated.

Limitations

Biomarkers are unaltered by sedation unlike some neurophysiology tests or clinical examination, and therefore may be more objective markers of brain injury. However, their measurement is intermittent, while their production or secretion and metabolism are dynamic processes, underscoring the importance of serial measurements. Not all patients included in the TTM trial participated in the sampling and not all patients had a sample drawn at each time-point. Because of a randomization stratified by site, we believe that this did not have a significant influence on the results as there was no difference between our study cohort and the main TTM trial cohort. Another limitation is that we had no external quality control at the participating sites where samples were collected and pre-analytically processed.

S100 is mainly found in astroglial cells but can also be found in adipocytes chondrocytes, the heart and muscle cells[16-18]. As S100 serum values after cardiac arrest are relatively low, even small amounts originating from extracerebral sources might lead to an overestimation of the actual brain damage.

Discussion

In this large international trial which compared two targeted temperature regimens in patients who remained unconscious after OHCA, S100 was a predictor of outcome. Targeted temperature level had a trivial effect on S100 values without clinical relevance. In multivariable analysis, S100 measurements at 24, 48h and 72h were no significant single predictors of outcome. Although serial S100 values

improved the model's fit as compared to the clinical model, the actual real clinical impact of the added value of S100 compared to NSE must be questioned.

S100 has the ability to distinguish good and poor outcome patients after OHCA as median values were higher in the poor outcome group, which has been described in previous reports [19-25]. It is noteworthy that S100 values declined over time in both temperature groups and for both outcome groups, indicating an early peak of this biomarker which might explain its best performance at the 24 hour time-point [26]. A clear peak earlier than 24 hours could however not be determined in a previous study investigating the kinetic profile of S100 [22]. Other studies confirmed the uniform decline over time after 24 hours in both outcome groups [21, 25]. We did not collect blood samples before 24 hours after ROSC. The early release and subsequent decline may be explained by the low molecular weight allowing a faster transition through the blood brain barrier and the short half-life of approximately two hours [27]. This differentiates S100 from other biomarkers, e.g. NSE, where the kinetics between 24 and 72 hours after cardiac arrest are indicative of outcome [11].

Few studies compared S100 in two target temperature groups and none could detect a significant influence of temperature on S100 levels [24, 28]. In pooled data, we observed significantly higher S100 values at 24 and 72 hours in the 33°C arm vs. 36°C, and at all time-points, only in the good outcome groups. Thus, the difference observed in the pooled data can be attributed to the sole statistical significant difference in the good outcome groups. We hypothesize that this difference has no clinical relevance and no influence on clinical practice since the observed values were well below the suggested cutoff levels, showed large overlaps and were evident only in the good outcome group.

The cutoff values for S100 in this study are in line with those described previously [4, 5, 25, 29]. According to our results, and the supposed kinetics of S100 after CA, the best time-point for outcome prediction would be 24 hours after ROSC. As with other biomarkers, an absolute cutoff value with a FPR of zero for poor outcome may be unrealistic because of absence of absolute values in clinical medicine. Some might argue that, a FPR close to 5% would be acceptable when used in combination with other prediction tools [30]. Discrepancies between previously reported cutoffs for S100 and ours might be due to different assays [19, 21, 22], different outcome measures [29] as well as sample size [22]. With this in mind, we suggest that S100 and other biomarkers like NSE are useful in clinical practice and may assist in outcome prediction after CA. Like with any other prognostication method, prediction should be based on a protocol including a holistic approach and with multiple tests and parameters [30, 31]. Clearly, NSE outperformed S100 for outcome prediction after CA [11]. Adding S100 to our model including clinical characteristics and NSE did not further improve the accuracy of the model. Similar results have also been described by Larsson et al. when S100 was added to NSE [25]. In a multivariable model with fewer variables than ours, another study suggested the usefulness of S100 over NSE on admission [21]. This highlights the potential improvement in outcome prediction when using a combination of (brain) biomarkers with different origins and kinetics, e.g. NSE as marker for neurons and neuroendocrine cells in combination with S100 as a marker for astroglial cells. The impact of a combined approach for outcome prediction after CA remains to be investigated as well as the exact role of the biomarker S100 [32].

The main strength of our study is the large sample size of a predefined substudy of a multicenter clinical trial investigating two target temperatures in comatose patients after OHCA. The TTM trial had strict rules and protocols regarding prognostication and how withdrawal of life supporting therapy was conducted [13]. In addition, all the samples were analyzed in one single core laboratory after the completion of the study, ruling out the problem of variation between laboratories and limiting the risk of “self-fulfilling prophecy” due to having bedside access to the biomarkers.

In summary, although high S100 values in comatose patients after out-of-hospital cardiac arrest are predictive of a poor neurological outcome, they do not provide an added prognostic value over a multivariable clinical model including NSE. Adding S100 to clinical variables without NSE results in a statistical significant improvement of prediction, but probably without relevant clinical impact. The predictive value of S100 is not significantly affected by target temperature at either 33°C or 36°C.

Acknowledgement

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Figure legends:

FIGURE 1: Study flow chart

Number of patients enrolled in the TTM trial and included in this substudy (A); Number of samples included in this study and reasons for eliminating serum samples from analysis (B.). TTM = Target Temperature Management after out-of-hospital cardiac arrest trial, CPC = Cerebral Performance Category.

FIGURE 2: S100 Time Course

Boxplots of S100 over the first 72 hours after ROSC, data are represented as median, quartile 1, quartile 3, and lower fence (i.e. lowest value above (quartile 1-1.5(quartile3-quartile1))) and upper fence (i.e. greater value below (quartile 3 + 1.5(quartile3-quartile1))). A statistical difference was only found in S100 values for good outcome patients, with higher values in the 33°C group (all $p < 0.005$) and between good and poor outcome groups (all $p < 0.001$). * = statistical difference with $p < 0.05$. CPC = cerebral performance category.

FIGURE 3: S100 Receiver Operating Curves

Receiver operating characteristic curves with areas under the curve (AUC) for S100 at 24 (**A**), 48 (**B**), and 72 (**C**) h after ROSC for outcome prediction according to CPC at 6 months. ROSC = return of spontaneous circulation; CPC = cerebral performance category

FIGURE 4: Kaplan-Meier curves

Kaplan-Meier curves for prediction of survival at the end of the trial (primary endpoint of the TTM trial) for S100 values at 24, 48 and 72 hours after return of spontaneous circulation. Separation into quartiles of serum S100 levels.

Table 1 Main Demographic and Utstein Data

	33° (n= 344)	**36° (n=343)**
Male gender, n (%)	292 (83)	273 (79)
Age, mean (SD)	64.2 (11.8)	63.4 (12.9)
First monitored rythm, n (%):		
* Asystole or PEA	67 (19)	64 (18)
* Non perfusing VT or VF	273 (77)	272 (78)
* ROSC after bystander defibrillation	6 (2)	3 (1)
* Unknwon initial rhythm	6 (2)	8 (2)
Time CA to ROSC, mean (SD)	30.5 (21.5)	31.1 (23.8)
Lactate (mmol/l), mean (SD)	6.6 (4.4)	6.6 (4.4)
Shock on admission, n(%)	45 (13)	43 (12)

Values are mean and SD or n (%).

CA = cardiac arrest; CPR = cardiopulmonary resuscitation; PEA = pulseless electric activity; ROSC = return of spontaneous circulation; VF = ventricular fibrillation; VT = ventricular tachycardia.

Table 2 S100 Cutoff Values

		cut-off (µg/L)	Sensitivity	95% confidence interval	Specificity	95% confidence interval
24h	S100 Youden	0.12	0.68	0.63 - 0.73	0.77	0.73 - 0.82
	S100_5	0.25	0.41	0.35 - 0.46	0.95	0.93 - 0.97
	S100_4	0.28	0.40	0.34 - 0.45	0.96	0.94 - 0.98
	S100_3	0.32	0.35	0.30 - 0.40	0.97	0.95 - 0.99
	S100_2	0.36	0.32	0.26 - 0.37	0.98	0.96 - 0.99
	S100_1	0.72	0.22	0.17 -0.26	0.99	0.97 - 1.00
	S100_0	2.59	0.10	0.07 - 0.13	1.00	0.99 - 1.00
48h	S100 Youden	0.13	0.63	0.57 - 0.68	0.82	0.78 - 0.86
	S100_5	0.25	0.36	0.30 - 0.41	0.95	0.93 - 0.98
	S100_4	0.25	0.36	0.30 - 0.41	0.96	0.94 - 0.98
	S100_3	0.27	0.34	0.28 - 0.39	0.97	0.95 - 0.99
	S100_2	0.28	0.34	0.28 - 0.39	0.98	0.96 -0.99
	S100_1	0.36	0.28	0.23 - 0.34	0.99	0.97 - 0.99
	S100_0	3.67	0.05	0.03 - 0.08	1.00	0.99 - 1.00
72h	S100 Youden	0.10	0.65	0.59 - 0.71	0.80	0.75 - 0.84
	S100_5	0.19	0.35	0.29 - 0.40	0.95	0.92 - 0.97
	S100_4	0.23	0.29	0.24 - 0.35	0.96	0.94 - 0.98
	S100_3	0.26	0.25	0.20 - 0.30	0.97	0.95 - 0.99
	S100_2	0.35	0.20	0.15 - 0.24	0.98	0.96 - 0.99
	S100_1	0.52	0.15	0.11 - 0.19	0.99	0.97 - 0.99
	S100_0	1.83	0.05	0.02 - 0.08	1.00	0.98 - 1.00

S100 cutoff values with sensitivity and specificity (95% confidence interval) for poor outcome prediction (CPC 3-5). Data pooled for target temperature.

S100 Youden indicates S100 cutoff with the compromise of the best sensitivity and specificity (maximized Youden index). The number following S100 refers to the false positive rate. Sensitivity and specificity are corrected by bootstrap internal validation.

Table 3 Multivariable Analysis

Table 3A: clinical variables and S100

Multivariate analysis (with multiple imputation)			95% CI	
S100 + clinical data	Effect	Odds ratio	Lower	Upper
Intercept	-3.670	0.025	0.01	0.11
S100 at 24h	1.828	6.221	0.77	50.55
S100 at 48h	0.873	2.395	0.13	45.81
S100 at 72h	1.594	4.926	0.21	117.39
Target temperature	0.085	1.089	0.75	1.59
Age	0.062	1.064	1.05	1.08
Time CA to ROSC	0.022	1.022	1.01	1.03
Lactate level on admission	-0.001	0.999	0.95	1.05
Gender	-0.271	0.762	0.47	1.24
Bystander CPR performed	-0.527	0.590	0.39	0.90
VT/VF vs PEA/asystole	-1.431	0.239	0.13	0.43
ROSC after bystander defibrillation	-1.560	0.210	0.05	0.88
Shock on admission	0.160	1.173	0.62	2.21

Table 3B: clinical variables, S100 and NSE

Multivariate analysis (with multiple imputation)			95% CI	
S100 + NSE + clinical data	Effect	Odds ratio	Lower	Upper
Intercept	-6.480	0.002	0.00	0.01
S100 at 24h	1.012	2.751	0.49	15.33
S100 at 48h	-1.808	0.164	0.00	6.89
S100 at 72h	2.284	9.820	0.24	401.61
NSE at 24h	-0.041	0.960	0.93	0.98
NSE at 48h	0.065	1.068	1.04	1.10
NSE at 72h	0.026	1.026	1.00	1.05
Target temperature	0.187	1.206	0.76	1.91
Age	0.091	1.095	1.07	1.12
Time CA to ROSC	0.009	1.010	1.00	1.02
Lactate level on admission	0.003	1.003	0.94	1.07
Gender	-0.400	0.671	0.38	1.20
Bystander CPR performed	-0.706	0.494	0.29	0.83
VT/VF vs PEA/asystole	-1.062	0.346	0.17	0.72
ROSC after bystander defibrillation	-0.926	0.396	0.07	2.11
Shock on admission	0.356	1.428	0.68	2.99

Abbreviations as in Tables 1 and 2.

Figure 1A (study flow chart)

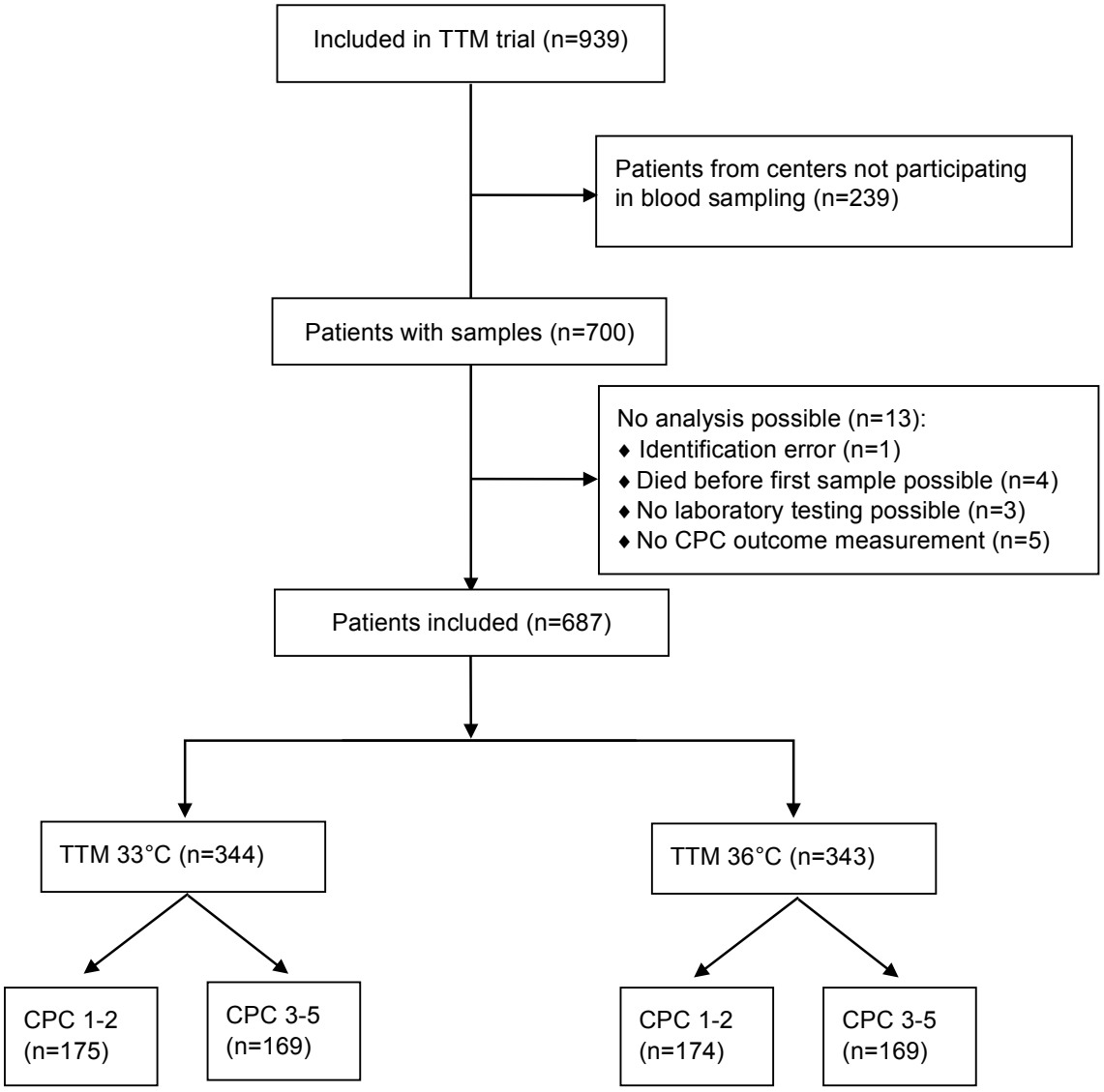


Figure 1B.

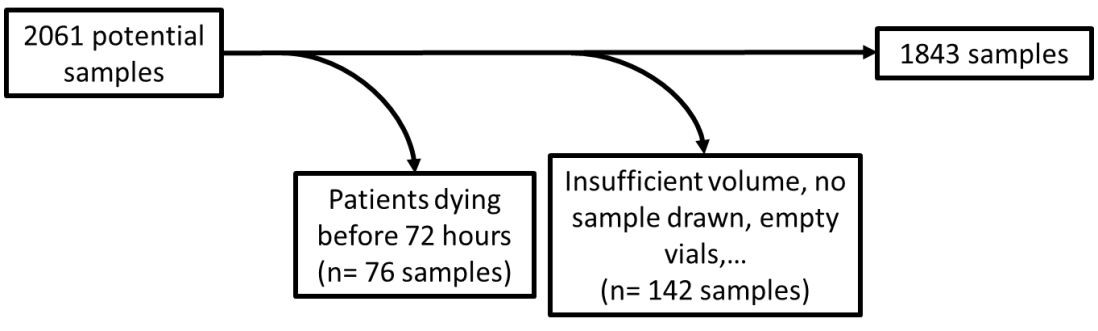


Figure 2: S100 Time Course

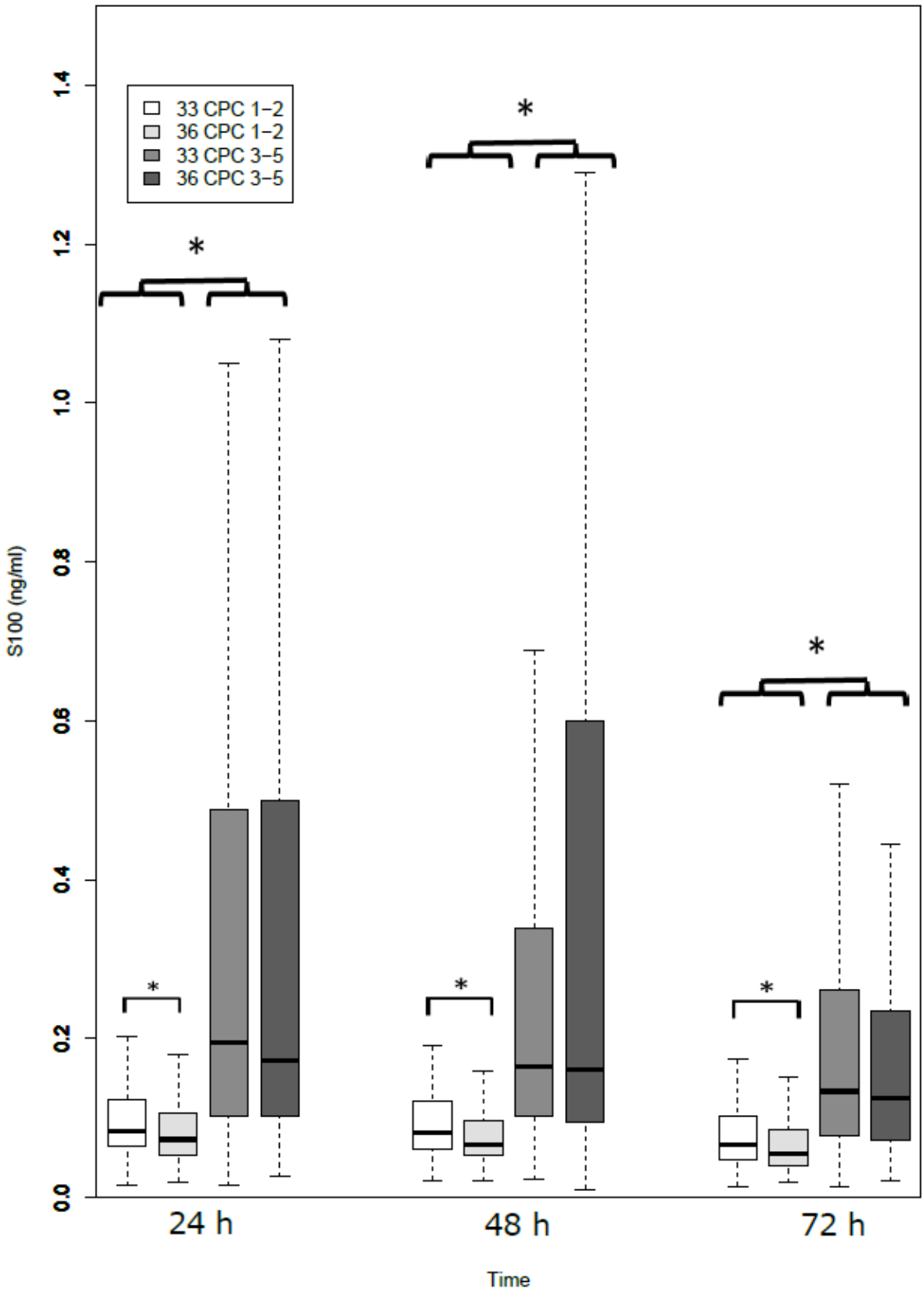
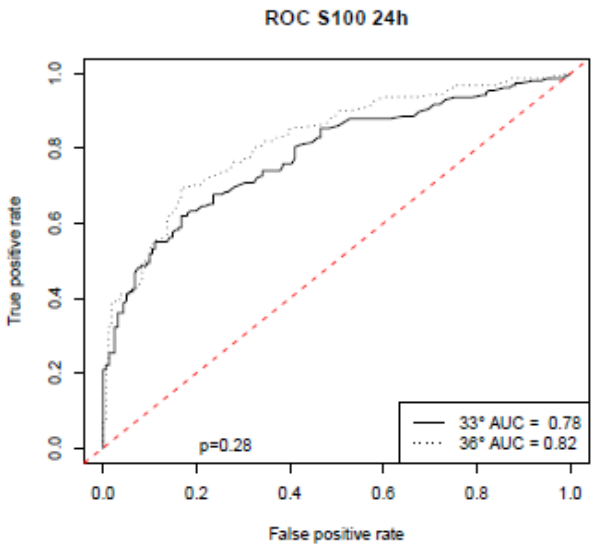
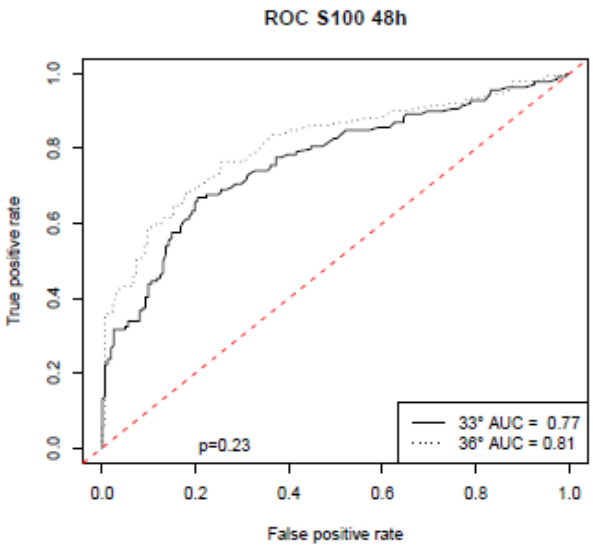


Figure 3: S100 Receiver Operating Curves





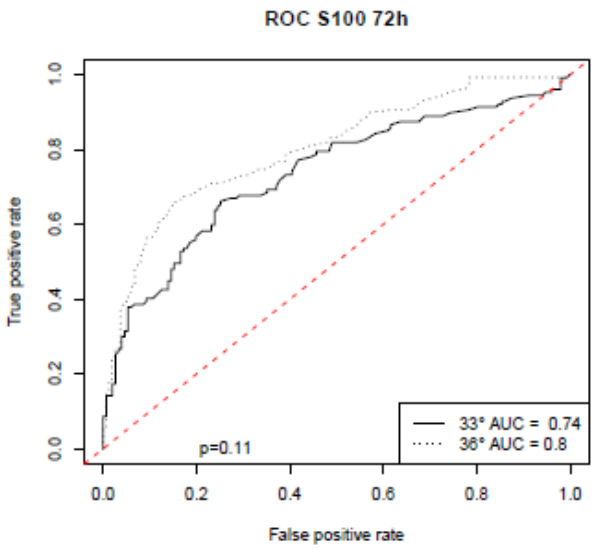
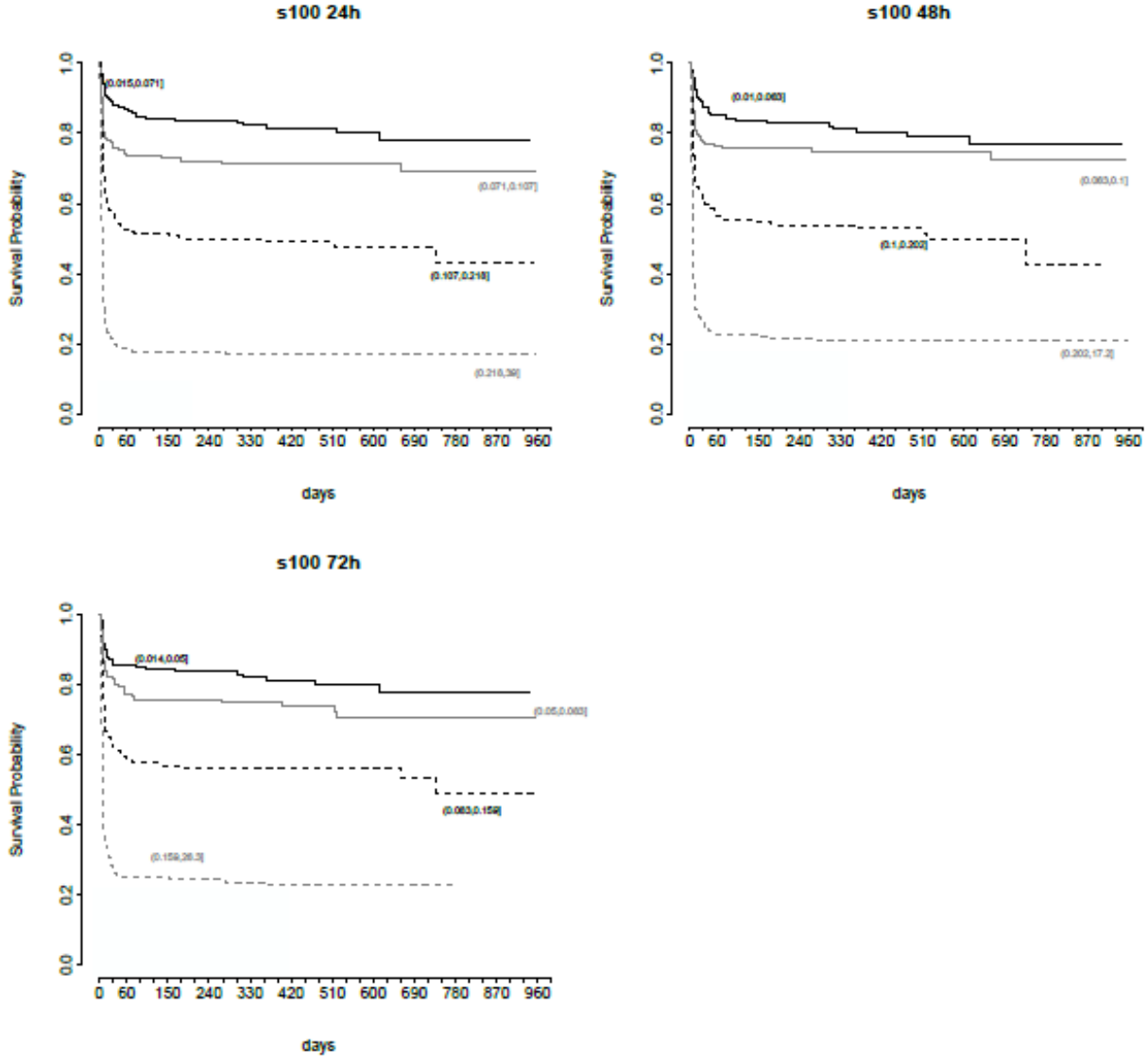


Figure 4: Kaplan-Meier curves



8.3 Paper III:

Circulating microRNAs after cardiac arrest

Pascal Stammet, MD; Emeline Goretti, MSc; Mélanie Vausort, MSc; Lu Zhang, MSc; Daniel R. Wagner, MD, PhD; Yvan Devaux, PhD

Objective: **Prediction of clinical outcome after cardiac arrest is clinically important. While the potential of circulating microRNAs as biomarkers of acute coronary syndromes is an active field of investigation, it is unknown whether microRNAs are associated with outcome in cardiac arrest patients.**

Design: **Prospective, single-center proof-of-concept study.**

Setting: **Eighteen-bed adult general intensive care unit of an academic tertiary care hospital in Luxembourg.**

Patients: **Twenty-eight patients with cardiac arrest treated by therapeutic hypothermia after cardiac resuscitation were enrolled.**

Measurements and Main Results: **Blood samples were obtained at 48 hrs after cardiac arrest for the determination of microRNA levels and neuron-specific enolase. Neurological outcome was determined by the cerebral performance category at discharge from the intensive care unit and at 6-month follow-up. Analysis of microRNA arrays and quantitative assessment by polymerase chain reaction (PCR) identified two microRNAs, miR-122 and miR-21, overexpressed in patients with poor neurological outcome (cerebral performance category 3–5, n = 14) compared to patients with favorable neurological outcome (cerebral performance category 1–2, n = 14) (48-fold and three-fold, respectively).** ***In vitro*** **experiments showed that both miR-122 and miR-21 are produced by neuronal cells, indicating that the elevation of circulating levels of these microRNAs after cardiac arrest may reflect brain damage. miR-122 and miR-21 predicted neurological outcome with areas under the receiver operating characteristic curve of 0.73 and 0.77, respectively. Patients within the highest third of miR-122 or miR-21 values had elevated mortality rate (p = .02). Neuron-specific enolase was an accurate predictor of neurological outcome (areas under the receiver operating characteristic curve = 0.98) and mortality (p < .001). MicroRNA levels were not associated with myocardial damage or activation of inflammation.**

Conclusions: **As compared to neuron-specific enolase, circulating microRNAs are modest but significant predictors of neurological outcome and mortality in this small group of patients with cardiac arrest. This motivates assessing the prognostic value of microRNAs in larger cohorts of cardiac arrest patients. (Crit Care Med 2012; 40:0–0)**

Key Words: biomarkers; cardiac arrest; microRNAs; neurological manifestation; prognosis; survival

During the past 30 yrs, the overall prevalence of all-rhythm cardiac arrest (CA) was 38 patients per 100,000 per year in Europe (1). In the United States, the prevalence of out-of-hospital CA was 124 patients per 100,000 in 2010 (2). Survival after in-hospital CA is <20% (3) and <10% after out-of-hospital CA (4).

The protective effect of therapeutic hypothermia on neurological sequelae and mortality has been demonstrated (5–7) and is now commonly used (8). However, despite hypothermia, outcome remains variable. The interest for early and reliable neurological prognostication after hypothermia in patients resuscitated from CA is major to avoid prolonged treatment to patients with low chance of good recovery (especially persisting coma or vegetative state) and to assure maximal treatment to those expected to have good outcome. However, the research for early clinical or biological markers to reliably predict outcome in these patients has not yet been completely successful (9). Neuron-specific enolase (NSE) seems to be the most promising biomarker for outcome prediction, but no well-designed large-scale trial investigated this marker since the implementation of induced hypothermia.

Recently, microRNAs (miRNAs) have been at the center of investigations in the heart. Their functional roles in cardiac development, homeostasis, remodeling, and dysfunction are being actively deciphered. The value of circulating miRNAs as diagnostic biomarkers of acute coronary syndromes is currently under investigation (10). The performance of miRNAs to predict outcome after acute coronary syndrome has also been investigated (11–13). In CA, the prognostic value of miRNAs has not been studied. We hypothesized that miRNAs may be released in the blood stream as a result of post-CA neurological damage. Therefore, the aim of the current study was to determine whether circulating miRNAs can be used to predict outcome after CA.

MATERIALS AND METHODS

An expanded version of Methods in the online supplemental data (Supplement Digital Content 1, http://links.lww.com/CCM/A484) includes detailed methods for the following: culture of neuronal cells, measurement of gene and miRNAs expression, microarrays, and biochemical assays.

Patients. Twenty-eight patients after resuscitated CA that survived >48 hrs were enrolled in this prospective study. The protocol

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has been approved by the National Research Ethics Board (National Committee for Ethics in Research of Luxembourg), and informed consent has been obtained from all subjects or their legal representatives. Over an 18-month period, patients admitted with CA to the intensive care unit of an academic tertiary care center were enrolled. Patients were treated by hypothermia at 33°C during 24 hrs after CA. The Coolgard System (Zoll Circulation, Sunnyvale, CA) was used for maintenance and controlled rewarming at a maximal rate of 0.5°C/hr. Blood samples for the determination of miRNA expression levels by microarrays and PCR were harvested in citrated tubes at 48 hrs after CA, in normothermic conditions. Creatine phosphokinase and cardiac troponin T were determined for the assessment of acute myocardial injury. NSE and procalcitonin (PCT) were measured 48 hrs after CA. Neurological evaluation was performed before intensive care unit discharge and at 6-month follow-up. Each patient was classified according to the cerebral performance category (CPC) score—favorable outcome: CPC 1–2 = no or minor neurological sequelae; poor outcome: CPC 3–4 = severe neurological sequels or coma, CPC 5 = death.

Culture of Neuronal Cells. The SH-SY5Y neuroblastoma cell line was differentiated with all-trans retinoic acid (Sigma, Bornem, Belgium) and human brain-derived neurotrophic factor (Sigma).

Microarrays. Plasma samples obtained at the end of hypothermia were used for microarrays. Identical volumes of plasma from two groups of 14 patients, one group of patients with CPC 1–2 and one group of patients with CPC 3–5, were pooled (see Table 1 for patient characteristics). Total RNA was extracted from these two pools of plasma, and miRNAs were hybridized on Human Microarray Release 12.0 slides covering 695 miRNAs (Agilent Deigem, Belgium). Five arrays per pool were hybridized. Data are available at the Gene Expression Omnibus under the accession number GSE 34643.

Measurement of Gene and miRNAs Expression. Quantitative PCR was used to measure the expression of miRNAs in plasma samples and conditioned medium from cultured SH-SY5Y cells, and gene expression in SH-SY5Y cells.

Statistical Analysis. Mann-Whitney rank sum test was used to compare two groups of continuous variables. Fisher's exact test or the chi-square test was used to compare categorical variables. Kruskal-Wallis one-way analysis of variance on ranks was used for multiple group comparisons. Correlations were determined using Spearman's test on ranks. Prognostic performances were evaluated using receiver operating characteristic curves and the area under the receiver operating characteristic curves (AUC).

Kaplan-Meier curves and the log-rank statistic were used for survival analysis, in which patients were distributed in thirds of biomarker values. To isolate the thirds that differ from each other, all pairwise multiple comparison procedure was applied (Holm-Sidak method). The SigmaPlot v11.0 software was used for statistical testing. $p < .05$ was considered statistically significant.

Table 1. Demographic features

	CPC 1–2 (n = 14)		CPC 3–5 (n = 14)		*p*
Age, (yrs, mean-range)	64	45 – 82	63	47 – 78	.724
Sex (male, n, %)	9	64.3	9	64.3	1.000
Simplified Acute Physiology Score II (mean-range)	62	47 – 83	74	56 – 98	.009
Time cardiac arrest to return of spontaneous circulation (min, median-range)	20	5 – 35	30	22 – 76	<.001
Initial rhythm (n, %)					.038
Asystole	0	0	6	42.9	
Pulseless electric activity	2	14.3	0	0	
Ventricular fibrillation/ventricular tachycardia	11	84.6	8	57.1	
Associated factors (n, %)					
Cardiogenic shock	3	21.4	8	57.1	.120
Acute myocardial infarction	9	64.3	10	71.4	1.000
Electroencephalographic status epilepticus	0	0	5	35.7	.041
Seizures	0	0	6	42.9	.016
Medical history (n, %)					
Tobacco	6	42.9	2	14.3	.209
Alcohol abuse	0	0	1	7.1	1.000
Hepatic disease	0	0	2	14.3	.481
Renal impairment	2	14.3	2	14.3	1.000
Hypertension	8	57.1	7	50	1.000
Heart failure	5	35.7	7	50	.704
Coronary disease	5	35.7	5	35.7	1.000
Diabetes					
Insulin dependent	3	21.4	0	0	.222
Noninsulin dependent	2	14.3	2	14	1.000
CPC end intensive care unit (n, %)					<.001
CPC 1	10	71	0	0	
CPC 2	4	29	0	0	
CPC 3	0	0	3	21	
CPC 4	0	0	3	21	
CPC 5	0	0	8	58	
CPC 6 months (n, %)					<.001
CPC 1	13	93	0	0	
CPC 2	1	7	0	0	
CPC 3	0	0	1	7	
CPC 4	0	0	1	7	
CPC 5	0	0	12	86	

CPC, cerebral performance category.
Note: CPC 3–5 denotes CPC 3, 4, or 5.

RESULTS

Clinical Characteristics. Main clinical and demographic data are shown in Table 1. Two groups of 14 age- and sex-matched patients were formed: on one hand, patients with a favorable neurological outcome (6-month CPC 1–2), and on the other patients with a poor neurological outcome (6-month CPC 3, 4, or 5, thereafter noted CPC 3–5). Parameters related to the severity of illness, like Simplified Acute Physiology Score, and time from CA to return of spontaneous circulation were significantly higher in the CPC 3–5 group. However, the groups did not differ significantly regarding preexisting diseases.

No patient evolved from the poor neurological outcome group to the favorable neurological outcome group between discharge and follow-up. Three patients from the favorable neurological outcome group improved their CPC from 2 to 1 during the follow-up period. Patients with poor neurological outcome either remained stable or died during the follow-up period.

Expression Profiles of Circulating miRNAs After CA. Total RNA samples from the two groups of patients were pooled and used to determine miRNA expression profiles by microarrays. Of the 695 miRNAs represented on the microarrays, 115 were consistently detected (at least in three of the five arrays performed per group; see Materials and Methods section for details). Figure 1*A* shows a heat-map

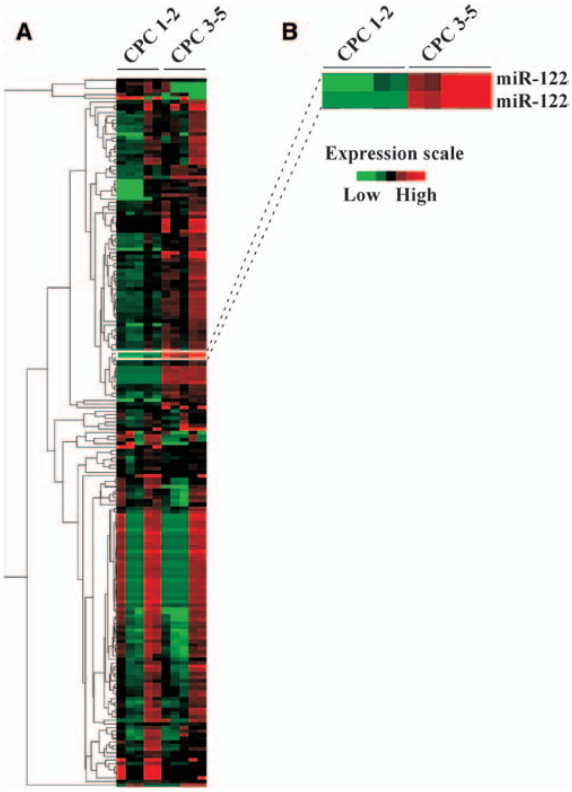

Figure 1. Expression profiles of circulating microRNAs (*miRNAs*) in cardiac arrest patients. Plasma samples from patients with favorable neurological outcome (cerebral performance category [*CPC*] 1—2, n = 14) and patients with poor neurological outcome (CPC 3–5, n = 14) were pooled in two groups. *A*, Clustering of the 115 miRNAs detected on microarrays in at least three arrays. Five arrays per pool were hybridized. *B*, Expression level of miR-122 in the two groups of patients. Color indicates expression level. Note that miR-122 was recognized by two probes on the arrays.

of these 115 miRNAs. Visual inspection of this heat-map allowed identifying miR-122 as differently expressed between the two groups of patients (Fig. 1*B*).

Expression of miRNAs in Neuronal Cells. To confirm that circulating miRNAs may reflect neuronal damage after CA, we investigated the expression of several miRNAs in the SH-SY5Y neuroblastoma cell line. Cells were differentiated by a 5-day incubation with retinoic acid followed by a 7-day incubation with brain-derived neurotrophic factor (Fig. 2*A*). Microscopic examination of the cells showed a characteristic change in morphology. Differentiated cells were rounded, had bright phase bodies, reduced size, and displayed extended neuritis. Complete cell differentiation was verified by increased expression of the neuronal markers, CNR1, GABBR1, and SYT-5 (Fig. 2*B*). Measure of the expression of several miRNAs in conditioned medium of differentiated cells revealed that miR-21/-122/-150*/-451 were reliably detectable (Fig. 2*C*). This observation indicated that miRNAs, detected in the blood stream after CA, may originate from neurons.

Expression of Selected Circulating miRNAs After CA. Quantitative PCR was used to accurately measure expression levels of miR-122, as identified by microarrays, and miR-21, because it displayed a high expression level in cultured neuronal cells. Both miRNAs were up-regulated in patients with poor neurological outcome (48-fold and three-fold for miR-122 and miR-21, respectively; Fig. 3).

Prediction of Neurological Outcome. We then determined the ability of miR-122 and miR-21 to predict 6-month neurological outcome. This prediction was compared to NSE and PCT measured 48 hrs after CA. First, we observed positive correlations among miR-122, NSE, and PCT. miR-21 did not correlate with any marker (Supplementary Table I; see Supplemental Digital Content 1, http://links.lww.com/CCM/484). Analysis of receiver operating characteristic curves reported an AUC of 0.73 (95% confidence interval, 0.54–0.93) for miR-122 and an AUC of 0.77 (95% confidence interval, 0.58–0.95) for miR-21 to predict neurological outcome after CA (Fig. 4). Combining miR-122 and miR-21 did not improve prediction accuracy (not shown). NSE, and to a lesser extent PCT, was accurate predictor of neurological outcome. A maximal AUC of 0.98 was obtained for NSE (Fig. 4). Lower AUCs were obtained when NSE was measured 12 and 24 hrs after CA (AUC = 0.89 and 0.94, respectively).

Prediction of Mortality. Eight patients died in the intensive care unit and four patients (total 43%) died during the 6-month follow-up period. Mean survival time was 116 days. Patients within the highest third of miR-122, miR-21, or NSE values had elevated mortality rate (Fig. 5, *A*–*C*). The performance of NSE to predict mortality was more significant ($p < .001$) than miR-122 and miR-21 ($p = .02$ for both). PCT did not have a significant predictive value for mortality (Fig. 1*C*). Therefore, miR-122 and miR-21 are significant predictors of mortality but do not outperform NSE.

Association Between miRNAs and Preexisting Pathologies. We searched for confounding factors that may affect miRNA plasma levels. No significant correlation between miRNAs and any risk factor was detected (Supplementary Table II; see Supplemental Digital Content 1, http://links.lww.com/CCM/484). Because miR-122 is enriched in the liver, we investigated whether elevated miR-122 levels in CA patients could be consecutive to hepatic problems. Two patients had hepatic disease prior to their CA and their levels of miR-122 were below the average value, suggesting that elevated miR-122 levels after CA are not associated with hepatic problems.

Impact of Cardiac Damage on miRNA Plasma Levels in CA Patients. In 19 of the 28 patients of our cohort, CA was due to acute myocardial infarction (AMI). miR-122 levels were not different between patients with AMI and patients without AMI. miR-21 was lower in patients with AMI compared to patients without AMI (Supplementary Figure IA; see Supplemental Digital Content 1, http://links.lww.com/CCM/484). miRNA levels did not correlate with the markers of myocardial injury creatine phosphokinase and cardiac troponin T or with left ventricular ejection fraction

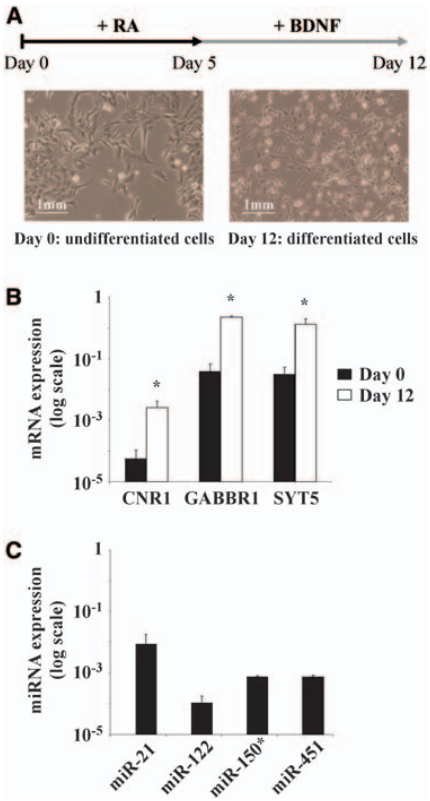

Figure 2. MicroRNAs (*miRNAs*) production by neuronal cells. *A*, Experimental protocol used to differentiate SH-SY5Y cells. Cells were cultured during 5 days with retinoic acid (*RA*) and then during 7 days with brain-derived neurotrophic factor (*BDNF*). Pictures display the microscopic aspect of cells, before and after differentiation. *B*, Total RNA was extracted from undifferentiated (day 0) and differentiated cells (day 12), and mRNA expression of markers of differentiation was assessed by polymerase chain reaction (PCR). Data are mean ± SD (n = 3). *$p < .001$. *C*, Total RNA was extracted from conditioned medium of differentiated cells cultured for 24 hrs, and miRNA expression was assessed by PCR. Data are mean ± SD (n = 3). *CNR1*, cannabinoid receptor 1; *GABBR1*, gamma-aminobutyric acid type B receptor 1; *SYT-5*, synaptogamin-5.

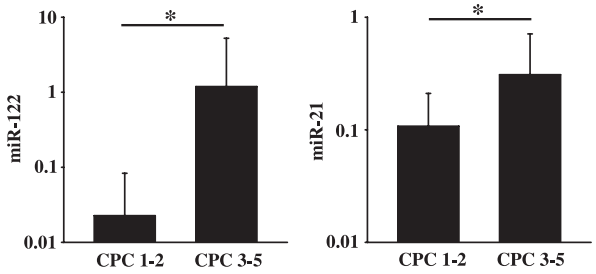

Figure 3. miR-122 and miR-21 in patients with cardiac arrest (CA). Circulating levels of microRNAs (*miRNAs*) in 28 patients with CA were assessed by quantitative polymerase chain reaction and dichotomized into favorable neurological outcome (cerebral performance category [*CPC*] 1—2), n = 14) and poor neurological outcome (CPC 3–5, n = 14). Data are mean ± SD. *$p < .05$.

measured by transthoracic echocardiography (Supplementary Figure IB–D; see Supplemental Digital Content 1, http://links.lww.com/CCM/484). These data show that miRNA plasma levels do not correlate with myocardial damage in patients with CA.

Association Between miRNAs and Inflammation. CA is associated with a strong inflammatory response. White blood cell count, CRP, and PCT are markers of inflammation. Neither miR-122 nor miR-21 levels correlated with white blood cell count and CRP, but both miRNAs positively correlated with PCT levels (Supplementary Figure II; see Supplemental Digital Content 1, http://links.lww.com/CCM/484). Even after removal of a patient with highly elevated PCT (135.5 ng/mL) due to a very long resuscitation period and immediate multiorgan failure, the correlation between miR-21 and PCT remained significant ($r = .48$, $p = .02$).

DISCUSSION

Having characterized miRNA expression profiles in the plasma of patients with CA, we observed that neurological outcome of these patients is associated with an miRNA biosignature. Circulating levels of miR-122 and miR-21, measured in normothermia 48 hrs after hypothermia-treated CA, are elevated in patients with poor outcome and provide some prognostic value. In addition, we provide evidence that miR-122 and miR-21 are produced by neuronal cells, suggesting that up-regulation of their plasma levels after CA may arise from injured neurons.

We dichotomized the patients into two groups according to their neurological outcome, age, and sex. Not surprisingly, Simplified Acute Physiology Score II was higher and time to return of spontaneous circulation longer in the CPC 3–5 group. This is also true for the initial rhythm, as asystole is more prone to lead to poor outcome than a shockable rhythm (4). In the CPC 3–5 group, clinical and/or electroencephalographic seizures or status epilepticus were more often present, indicating that cerebral damage was more extensive. Regarding medical history, we did not find any significant differences between the two groups. Overall, baseline characteristics were comparable.

We used a pooling strategy to perform microarrays. Identical volumes of plasma samples from two groups of patients with preserved (CPC 1–2) and impaired (CPC 3–5) cerebral performance after CA were pooled before analysis by miRNA microarrays. Although the effectiveness of this approach to screen miRNA biomarker candidates has been shown elsewhere (14), it prevented from performing statistical analysis to identify differentially expressed miRNAs. Indeed, the replicate data obtained by microarrays were technical and not biological replicates. This limitation was however compensated in a

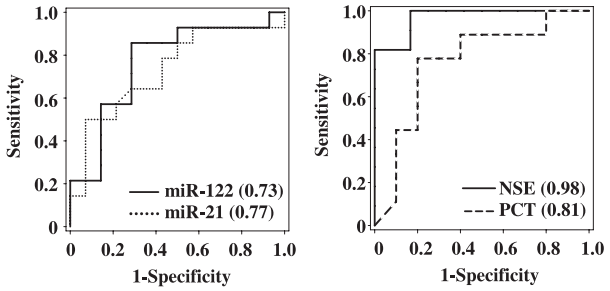

Figure 4. Prediction analyses. Circulating levels of miR-122 and miR-21 were determined by quantitative polymerase chain reaction at the end of hypothermia in 28 cardiac arrest (CA) patients. Neuron-specific enolase (*NSE*) and procalcitonin (*PCT*) were measured 48 hrs after CA. Displayed are receiver operating characteristic curves showing the value of biomarkers to predict neurological outcome (cerebral performance category [CPC] 1–2 vs. CPC 3–5). Areas under the receiver operating characteristic curve are indicated.

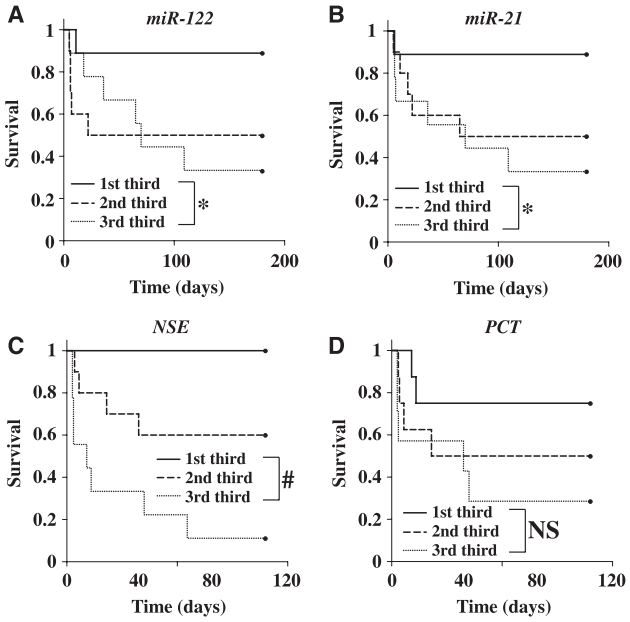

Figure 5. Kaplan-Meier curves showing the survival of patients according to thirds of (*A*) miR-122, (*B*) miR-21, (*C*) neuron-specific enolase (*NSE*), and (*D*) procalcitonin (*PCT*). Patients in the highest third of miR-122 or NSE values had elevated mortality rate. *$p < .05$; #$p < .001$. *NS*, not significant ($p = .08$).

second step by quantitative assessment of miRNAs expression in each patient individually by PCR. All prediction and association analyses were performed using these individual data. The pooling strategy can lead to high false discovery rate in some circumstances, notably in the presence of outliers. In the present study, one patient from the group of patients with poor outcome had a very high level of miR-122. This patient had impaired cerebral performance after hypothermia (CPC = 3) and died 3.5 months after discharge. However, exclusion of this patient did not affect the prognostic value of miR-122 (AUC = 0.73 and 0.71 in the presence and absence of the outlier, respectively).

Cardiac injury may influence plasma levels of miRNAs (15). After AMI notably, there is a massive release of cardiomyocyte-enriched miRNAs in the circulation as a result of cardiac ischemia (10, 16). In our study, miR-122 and miR-21 levels were not influenced by cardiovascular risk factors and known coronary artery disease. In addition, levels of both miRNAs were similar in patients with CA after AMI and in patients with CA not related to AMI. Finally, miRNA levels did not correlate with the markers of myocardial injury creatine phosphokinase and cardiac troponin T. These observations strongly suggest that the elevation of miR-122 and miR-21 levels after CA is not linked to myocardial damage.

Because miR-122 is enriched in the liver (17), it was plausible that high circulating levels of miR-122 could be consecutive to liver damage. In the present study, two patients had impaired liver function, and their levels of miR-122 were not distinct from patients with normal liver function. These observations suggest that our findings are not critically affected by hepatic failure.

The observation that miRNA levels did not correlate with white blood cell count and CRP suggests that the elevation of miRNA levels is not a direct consequence of activation of inflammation, which is induced after CA (18). However, we observed a positive correlation between both miR-122 and miR-21, and PCT. The reason for this association is not clear.

PCT was found to predict neurological outcome with an AUC of 0.81, in line with other reports (18–20), including ours (21) showing that elevated levels of PCT in CA patients are related to the severity of the postresuscitation phase and are associated with a poor outcome (18–20). NSE was very accurate at predicting both neurological outcome and mortality, better than PCT and miRNAs. When measured 48 hrs after CA, NSE was an excellent predictor of neurological outcome (AUC = 0.98), and this although our study population is more heterogeneous than in most reports on NSE. Of note, NSE measured at earlier timepoints (12 and 24 hrs after CA) also provided an accurate prognostic, albeit lower than that measured at 48 hrs. Treating physicians were not blinded to NSE values, but we observed patients over a sufficiently long time period before withdrawing treatment (21, 22). It has to be noted that NSE was not used as sole indicator for decision making, and treatment withholding or withdrawal was based on solid electrophysiological findings and clinical examination. Previous studies reported that NSE is especially a good predictor of poor outcome, but a relatively poor predictor of favorable outcome (23–26). Predicting patients who survive cardiac resuscitation is of major clinical interest (27). In our study, a level of NSE >33 μg/L, which perfectly predicted a poor prognosis in another study (28), predicted survival with 94% sensitivity. The low number of patients included here

prevented an accurate determination of cutoff values and sensitivity values for miRNAs to predict survival.

Because our initial hypothesis was that prognostic markers of neurological damage after CA may be isolated from the blood stream as a result of neuronal death, we verified that miR-122 and miR-21 were indeed produced by neuronal cells. Both miRNAs were detected in conditioned medium of SH-SY5Y neuronal cells. This observation is consistent with our working hypothesis and suggests that miRNAs may be used to evaluate brain damage. The presence of miRNAs in the blood implies a disruption of the blood-brain barrier. Such phenomenon has been observed following cerebral ischemia (29).

This study is limited by a multiplicity issue due to the search of miRNAs among the high number of miRNAs recognized by microarrays. Nevertheless, the association between miR-122 and outcome after CA was confirmed using an independent and quantitative technique, and miR-21 was not identified by microarrays but in cultured neuronal cells. It is acknowledged that the second part of this study, that is, quantitative assessment of miRNAs in individual patients, is not an independent validation of the first part, that is, characterization of miRNA profiles in two pools of CA patients. Finally, the present findings will have to be independently replicated before miR-122 and miR-21 can be considered potential biomarkers after CA.

CONCLUSIONS

We report the first characterization of circulating miRNA profiles in patients with CA. These profiles are attractive sources of miRNA biomarker candidates. In this pilot study, miR-122 and miR-21 were identified as potential biomarkers of neurological outcome and survival after CA. Their clinical relevance remains to be determined in larger patient cohorts.

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Clinical Paper

Bispectral Index to Predict Neurological Outcome Early After Cardiac Arrest☆

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ABSTRACT

Aim of the study: To address the value of continuous monitoring of bispectral index (BIS) to predict neurological outcome after cardiac arrest.
Methods: In this prospective observational study in adult comatose patients treated by therapeutic hypothermia after cardiac arrest we measured bispectral index (BIS) during the first 24 hours of intensive care unit stay. A blinded neurological outcome assessment by cerebral performance category (CPC) was done 6 months after cardiac arrest.
Results: Forty-six patients (48%) had a good neurological outcome at 6-month, as defined by a cerebral performance category (CPC) 1-2, and 50 patients (52%) had a poor neurological outcome (CPC 3-5). Over the 24 h of monitoring, mean BIS values over time were higher in the good outcome group (38 ± 9) compared to the poor outcome group (17 ± 12) ($p < 0.001$). Analysis of BIS recorded every 30 minutes provided an optimal prediction after 12.5 h, with an area under the receiver operating characteristic curve (AUC) of 0.89, a specificity of 89% and a sensitivity of 86% using a cut-off value of 23. With a specificity fixed at 100% (sensitivity 26%) the cut-off BIS value was 2.4 over the first 271 minutes. In multivariable analyses including clinical characteristics, mean BIS value over the first 12.5 h was a predictor of neurological outcome ($p = 6E-6$) and provided a continuous net reclassification index of 1.28% ($p = 4E-10$) and an integrated discrimination improvement of 0.31 ($p = 1E-10$).
Conclusions: Mean BIS value calculated over the first 12.5 h after ICU admission potentially predicts 6-months neurological outcome after cardiac arrest.

1. Introduction

Successfully resuscitated cardiac arrest remains a condition with a high mortality rate, even when applying the best medical care to the patients.[1–4] This high mortality of about fifty percent of the patients admitted to the hospital after successful pre-hospital resuscitation is mainly due to neurological impairment consecutive to the anoxic period during cardiac arrest and possibly during reperfusion.[5,6] The ability to predict outcome early after cardiac arrest would represent a major breakthrough towards personalized medicine by adapting the treatment strategy individually to the patient. This early prediction would allow avoiding futile healthcare to patients with irreversible neurological damage while maintaining resources in patients most likely to benefit. However, neurological prognostication of comatose patients after successful resuscitation and admission to the intensive care unit (ICU) remains a challenge.[7]

Several studies have focused on the prognostic value of biomarkers and electrophysiological parameters, but none could demonstrate an accurate prediction of outcome within the first 24 hours after cardiac arrest in hypothermia-treated patients.[7–10] Thus, there is an unmet need for early prediction tools. The actual lapse of time to reliably predict outcome, generally 24, 48 or even 72 hours after cardiac arrest, might be too lengthy if therapeutic decisions on highly invasive acute cardiac assistance have to be taken.[11,12] In previous reports, bispectral index (BIS), a processed electroencephalogram, initially designed to assess the depth of

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anesthesia, has potential to predict outcome early after cardiac arrest.[13–17] In these studies, either BIS value monitored at a single time-point or the lowest recorded BIS value were considered in prediction analyses.

The aim of this study was to refine the value of BIS as an early predictor of outcome after cardiac arrest. Using serial measurements of BIS over the first 24 hours after admission to the ICU, we determined BIS cut-off values and time of recording providing optimal prediction of outcome.

2. Methods

2.1. Patients

We included all successfully resuscitated adult cardiac arrest patients enrolled in a prospective local registry admitted from February 2009 to June 2013 to our general ICU. Part of the patients have been involved in previous studies, although none of these addressed the prognostic value of continuous BIS monitoring.[16,18–20] All patients were older than 18 years, unconscious (Glasgow coma score below or equal to 8) and received induced hypothermia at 33 °C with sedation and neuromuscular blockade according to our standard protocol (midazolam, max. 0.25 mg/kg/h; fentanyl, max. 2.5 μg/kg/h and cisatracurium 0.15 mg/kg/h).[14,16] This sedation regimen allows suitable sedation with minimal electromyogram (EMG) artefacts on the electroencephalogram (EEG) signal. After 24 h, patients were rewarmed to 36 °C at a maximum rate of 0.5 °C/h and sedation was tapered.

Patients' relatives were asked for informed consent according to the requirements of the National Committee for Ethics in Research (which approved the protocol). Patients regaining consciousness were also re-consented a posteriori. Only data sets with approved consents were considered in this study. Decisions to withdraw life support or to limit care were never taken by considering BIS values. Only clinically relevant parameters like absence of awakening after complete cessation of sedation, signs of brain death or early myoclonus or status epilepticus in combination with bilaterally absent N20 peak on somato-sensory evoked potentials or imaging findings compatible with irreversible brain damage were taken into account.[21,22]

2.2. Bispectral index monitoring

The BIS (BIS XP Quatro, COVIDIEN, Mansfield, Massachusetts, USA) monitor was routinely applied to every patient by the nursing staff: application of the electrodes on the dry skin of the forehead according to the manufacturer's instructions. Signal quality was immediately assessed by the treating nursing personnel, using the build-in tools, to obtain a stable and high signal quality index (SQI) and low EMG artefacts. BIS values were continuously registered via the patient monitoring system (IntelliVue, Philips, Böblingen, Germany), recorded in the patient data management system (Metavision, IMDsoft, Tel Aviv, Israel) and retrospectively exported as excel files into R. Throughout the 24 hour study period, only BIS values that fulfilled our quality criteria of low EMG artefacts (<30 dB) and high SQI (>80) were retained for the analysis. Patients without a BIS monitoring or without BIS data meeting these quality criteria were excluded from the study.

2.3. Neurological evaluation

The endpoint of this study was neurological outcome as defined by the cerebral performance category (CPC) score at 6 months,[23] CPC 1-2 being considered as good outcome and CPC 3-5 being considered as poor outcome. CPC score 1 or 2 indicates no or minor neurological sequelae, 3-4 indicates severe neurological sequelae or coma, and 5 indicates death. Evaluation of the neurological status using the CPC scale was performed by physicians unaware of BIS data.

Table 1
Study population.

Characteristic	Good outcome (CPC 1-2, n = 46)	Poor outcome (CPC 3-5, n = 50)	P value
Age, years	57 (21-81)	67 (24-83)	0.023
Gender			
Male	42 (91%)	36 (72%)	0.016
SAPS II	61 (43-83)	71 (48-100)	<0.001
Time to ROSC, min	19 (4-60)	30 (3-104)	<0.001
Initial rhythm			<0.001
VF/VT	42 (91.3%)	19 (38%)	
PEA	2 (4.3%)	7 (14%)	
Asystole	2 (4.3%)	24 (48%)	
Associated factors			
Shock	11 (24%)	19 (38%)	0.14
AMI	37 (80%)	26 (52%)	0.003
EEG Status epilepticus	1 (2%)	13 (26%)	<0.001
Seizures	3 (7%)	27 (54%)	<0.001
Medical history			
Tobacco	18 (39%)	21 (42%)	0.78
Hypertension	17 (37%)	26 (52%)	0.12
Heart failure	8 (17%)	15 (30%)	0.13
Coronary disease	12 (26%)	21 (42%)	0.09
Pulmonary disease	4 (9%)	9 (18%)	0.17
Diabetes	4 (9%)	12 (24%)	0.08

Continuous characteristics are indicated as median (range) and categorical characteristics are indicated as number (frequency). AMI: acute myocardial infarction; EEG: electro-encephalogram; ICU: intensive care unit; PEA: pulseless electric activity; SAPS: simplified acute physiology score; ROSC: return of spontaneous circulation; VF: ventricular fibrillation; VT: ventricular tachycardia.

2.4. Statistical analysis

Demographic and clinical data. Comparisons between patients with good and poor outcome were performed using the Wilcoxon test for continuous data and the Chi-square test or Fisher exact test when the validity constraints of the former were not met for categorical data. A p value <0.05 was considered significant.

Prediction analyses. First, partial least square discriminant analysis (PLS-DA) was performed.[24] At each time-point during the first 24 h after ICU admission, the first factor of PLS-DA was computed using all available measurements from time 1, which corresponds to the first BIS measurement after arrival in the ICU. Second, average BIS over the first 24 h of ICU stay was evaluated as a predictor of CPC at each time-point. Third, in order to evaluate prediction performances, a receiver operating characteristic (ROC) curve was plotted at each time-point using either the average BIS or the PLS-DA first factor. The best cut-off for CPC prediction was selected to maximize sensitivity and specificity using Youden index (sensitivity + specificity -100%). The optimal time and cut-off for CPC prediction are the ones maximizing Youden index over all BIS time course. Time offset in BIS curves were corrected by averaging values in a window from 5 min before to 5 min after the actual measurement. Missing BIS measurements at time 1 and 1441 were filled by the average BIS at these times. Other missing values were filled using linear interpolation.

In order to avoid overfitting, sensitivity and specificity were corrected for optimism using bootstrap internal validation (see Supplementary Material).[25]

To evaluate the added predictive value of BIS to a reference model, relevant clinical patient characteristics depicted in Table 1 were first selected using L^1 penalization[26] to fit a multivariable logistic model of CPC. Briefly, this method selects forward the most useful clinical variables to predict CPC until all the remaining ones

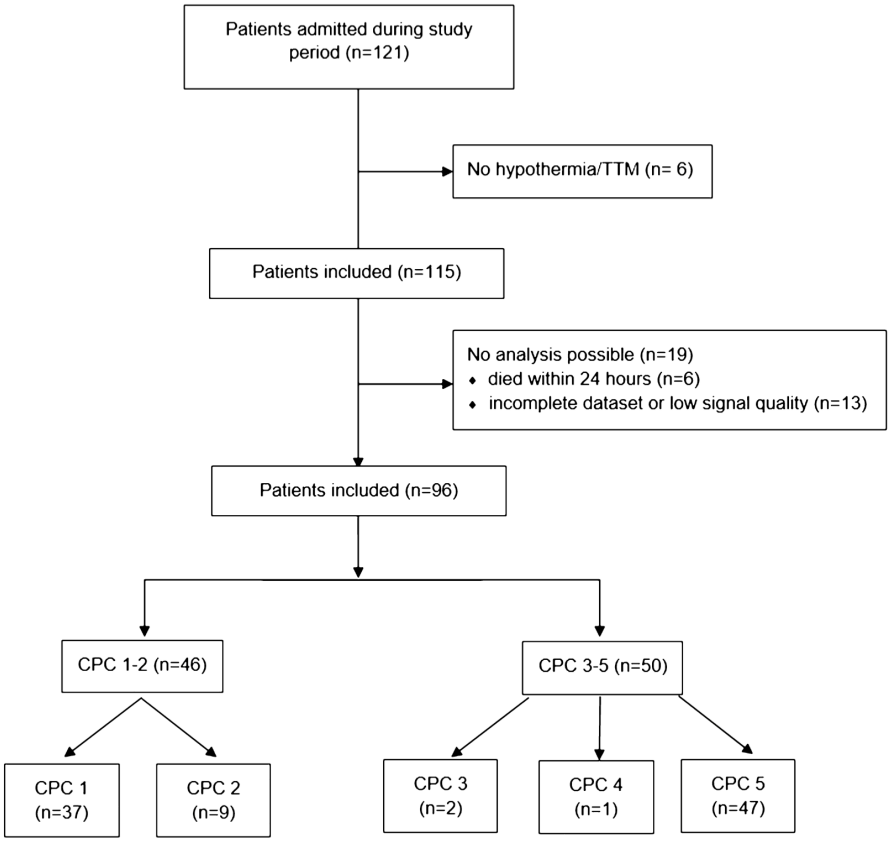

Figure 1. Study flow chart of patients. CPC = Cerebral performance category; TTM = targeted temperature management.

have no effect anymore on the outcome, giving them a zero coefficient in the regression model. Then, supplementary predictive value of BIS was evaluated adding mean BIS at optimal time to the previous model and computing continuous net reclassification index (NRI) and integrated discrimination improvement (IDI).[27] Details on the statistical analysis can be found in the supplemental electronic data.

Data are presented as mean ± standard deviation and median with interquartile range unless stated otherwise. Confidence intervals were computed using the normal approximation formula when possible or with the Wilson formula otherwise.

All computations were performed using the R statistical environment (2.15.2) with the packages ROCR, penalized, Hmisc, and mixOmics (http://cran.r-project.org/).

3. Results

3.1. Patients

Over the four year enrollment period, 121 patients were admitted to our ICU after cardiac arrest. Twenty-five patients had to be excluded (Figure 1). Thus, 96 patients were enrolled in this study. Median time from ROSC to first BIS measurement was 197 [166–246] minutes and time to target temperature was 300 [237–391] minutes. Median doses of midazolam, fentanyl and cisatracurium were respectively: 0.16 [0.12-0.25] mg/kg/h, 1.69 [0.99-2.4] μg/kg/h and 0.11 [0.08-0.12] mg/kg/h. There was no difference in median times from cardiac arrest to target temperature and sedation doses between the two outcome groups. Main demographic and clinical characteristics are displayed in Table 1. Patients were dichotomized according to their 6-months outcome: 46 (48%) patients had good outcome and 50 patients (52%) had poor outcome (Figure 1). Only 1 patient with a CPC 2 during ICU stay died of sepsis on day 36 (included in the CPC 3-5 group). No patient with a CPC score 1-2 at ICU discharge died within the 6 months observation period. Three CPC 3 patients improved their outcome to CPC 2 within 6 months and only 1 CPC 4 increased to CPC 2 within 6 months. All others died or stayed in the same group.

3.2. Time-course of BIS according to neurological outcome

We analyzed the evolution of BIS values in 96 patients during the first 24 hours of ICU stay. BIS was recorded every 30 minutes. Evolution of BIS values of the 2 groups of patients are displayed in Figure 2. At the beginning of BIS recording, BIS values were higher in the good outcome group and remained relatively stable over 24 hours. In the poor outcome group, BIS values were close to 0 from the 1st hour to the 5th hour, and increased thereafter, without reaching those from the good outcome group. Furthermore, the variability of BIS values was lower in patients with good compared to patients with poor outcome.

3.3. Prediction of neurological outcome by BIS

As shown in Figure 3A, mean BIS over 24 hours was higher in the good outcome group (38 ± 9) compared to the poor outcome group

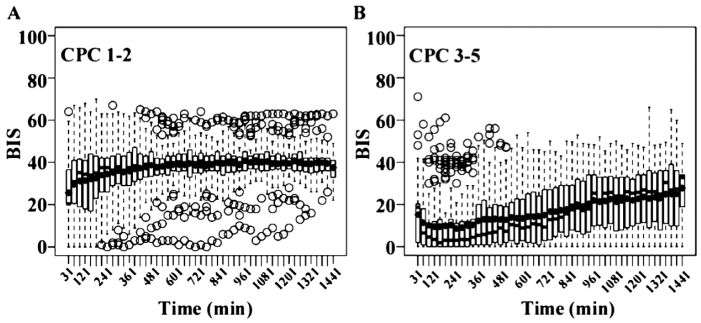

Figure 2. Time-course of BIS values according to patient outcome. BIS values were recorded every 30 min in 96 patients with cardiac arrest, during 24 hours of ICU stay. 46 patients had a good neurological outcome at 6-month (CPC 1-2) and 50 patients had a poor neurological outcome (CPC 3-5). Displayed are boxplot of BIS values at each time-point in good outcome (A) and poor outcome (B) patients. Black dots represent the mean, length of the squares are marked out by the first and third quartile, dotted lines are drawn from the lower to the upper fence (q1-1.5*(q3-q1) and q3 + 1.5*(q3-q1))and white circles represent outliers.

(17 ± 12) ($p < 0.001$). Neurological outcome was predicted by mean BIS with an AUC above 0.80 (Figure 3B). The optimal specificity (89%, 95% CI [80%,98%]) and sensitivity (86%, 95% CI [76%,96%]) for 6-months neurological outcome prediction was obtained from the 751st minute (12.5 hours) using a cut-off value of BIS of 23 (AUC: 0.88, Figure 3 C).

The tight gap between solid and dotted lines in Figure 3 C, representing sensitivity and specificity before and after correction for optimism using bootstrap internal validation, attests for the robustness of predictive models whose performances are unlikely to decrease markedly when applied to other patients.

Prediction analyses were also performed using the more elaborated PLS-DA statistical method. PLS-DA did not show any important differences with mean BIS measurements, the latter even outperforming PLS-DA at some points. Detailed PLS-DA results are shown in the online supplement (Figure S1).

3.4. Determination of cut-offs allowing absolute poor outcome prediction

In order to allow accurate detection of all patients with poor outcome with certainty, we fixed specificity to 100%, 95% CI [92%,100%] and determined a mean BIS cut-off value of 2.40 over 271 minutes after ICU admission (sensitivity 26%, 95% CI [14%,38%]). Thirteen patients (14%) had a mean BIS below 2.4 over the first 271 minutes and all evolved to CPC 3-5.

3.5. Prediction of neurological outcome by BIS measured every minute

In a subgroup of 50 patients we determined the predictive value of BIS measured minute-by-minute. Twenty-four (48%) patients had good outcome (CPC 1-2) and 26 patients (52%) had a poor

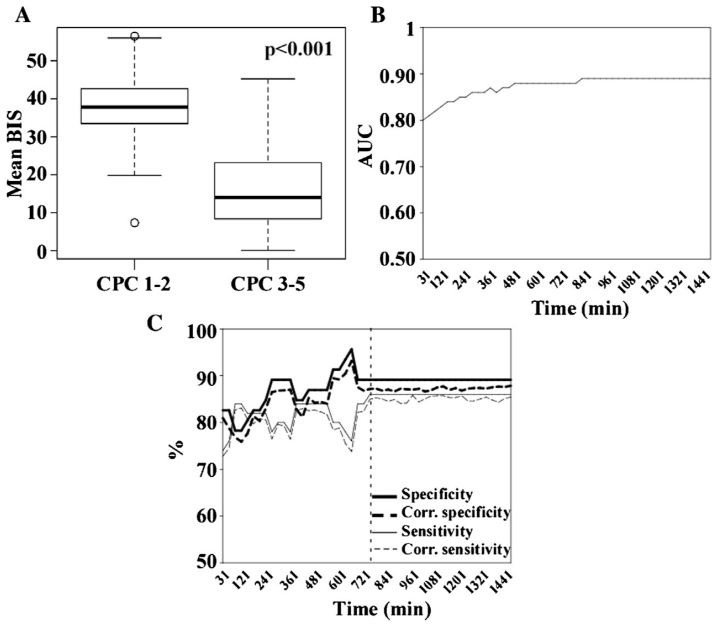

Figure 3. Determination of the value of mean BIS to predict neurological outcome. The mean BIS value recorded in 96 patients with cardiac arrest over the first 24 hours of ICU stay was used in this analysis. A. Box-plot showing the comparison of mean BIS values between patients with good (CPC 1-2) and patients with poor (CPC 3-5) neurological outcome at 6-month. B. ROC curve for the prediction of neurological outcome by mean BIS. C. Sensitivity and specificity of the prediction. Solid lines represent raw performances obtained with all 96 patients. Dotted lines represent the performances corrected for optimism after bootstrap internal validation (see Methods section for details). The vertical line indicates that the maximal prediction is obtained from the 751st min, providing a specificity of 89% and a sensitivity of 86%.

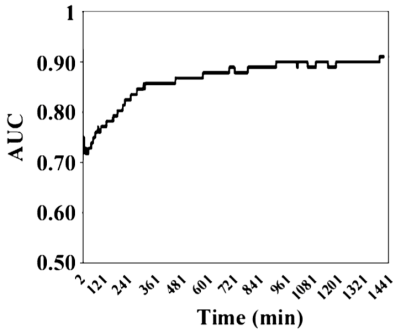

Figure 4. Predictive value of BIS recorded every minute. BIS data obtained every min during the first 24 h of ICU stay in 50 patients with cardiac arrest were included. Predictive value was determined using mean BIS values and corresponding AUC of ROC curves are displayed.

Table 2
Multivariate analysis.

Without mean BIS				
	estimate	CI 2.5%	CI 97.5%	p-value
(Intercept)	5.7244	2.6804	8.7683	2.28E-04
Age	−0.0605	−0.0990	−0.0219	0.002
time to ROSC	−0.0816	−0.1277	−0.0354	0.001
With mean BIS				
(Intercept)	1.3122	−2.236	4.861	4.69E−01
Age	−0.0497	−0.094	−0.005	0.029
time to ROSC	−0.0437	−0.098	0.011	0.116
mean BIS at 12.5 h	0.114844844	0.068	0.162	1.70E-06

outcome (CPC 3-5). The analysis of the mean BIS showed an AUC of 0.80 after 166 minutes (Figure 4). The AUC of 0.89 was reached after 925 minutes and a maximum AUC of 0.90 was obtained after the 1420th minute. The best compromise between specificity (92%, 95% CI [75%;98%]) and sensitivity (81%, 95% CI [62%;92%]) was obtained at the 1353rd minute using a cut-off value of 23. The same analysis performed with PLS-DA showed comparable results (Figure S2 in the online supplement).

3.6. Multivariable analyses–added value of BIS to clinical characteristics

Among all variables of Table 1, we could only determine age and time to ROSC to be useful clinical predictors of neurological outcome in the 96 patients using penalization. Both characteristics were significant clinical predictors of CPC ($p = 0.002$ and $p = 0.001$, respectively) using logistic regression (Table 2). After addition of the mean BIS value over the first 12.5 hours to this model, only age remained a significant predictor ($p = 0.029$). In these analyses, the mean BIS after 12.5 hours was significantly associated with neurological outcome ($p = 1.7E-6$). Continuous NRI (1.28, $p = 4E-10$) and IDI (0.31, $p = 1E-10$) showed that mean BIS significantly improved patients classification in regards to the clinical model including age and time to ROSC. As SAPS II is only obtained after 48 hours, thus extending well over the BIS measurement period, it was not considered in this multivariable analysis.

4. Discussion

This study supports the hypothesis that continuous monitoring and calculation of mean BIS during the first 24 hours after ICU admission allows an early and accurate prediction of outcome. We determined cut-off values for BIS and duration of monitoring that allow optimal prediction. BIS monitoring had an additive prognostic value to standard clinical parameters.

Patients with good and poor neurological outcome differed in terms of age, time to ROSC, illness severity score (SAPS II) and initial rhythm. This was expected considering the dichotomization of the groups according to neurological outcome.

Two different statistical methods were used to determine the prognostic value of BIS. The PLS-DA is the method of choice to compensate for the higher number of measurements (1440 measurements for monitoring every minute) compared to the number of patients and because these measurements were highly correlated. Hence, bootstrap internal validation was performed to correct for optimism and avoid model overfitting. Averaging BIS was used as a more classical and simple method.

Overall, our results showed that mean BIS values monitored every 30 minutes predicted outcome as accurately as the more elaborated statistical method PLS-DA.

By calculating mean BIS values on a half-hour basis during the first 24 hours in ICU, we could determine a cut-off value of 23 after 12.5 hours for poor neurological outcome, with a specificity of 89% and a sensitivity of 86%. Seder and coworkers determined a cut-off BIS value of 22 in intermittently neuromuscular blocked patient upon admission to the ICU.[13] This measurement called "BIS1" was performed after a mean of 280 minutes after cardiac arrest based on a stable BIS signal during the action of neuromuscular blockade. These findings are confirmed by ours and highlight the potential of BIS for very early prediction. We could show that using a larger timeframe, with continuous neuromuscular blockade, the ideal time-period for outcome prediction with BIS covers the first 12.5 hours after ICU admission.

On the other hand, a mean BIS value lower than 2.4 calculated over the first 6.5 hours of ICU stay was predictive of a certain bad outcome. These findings are in line with previous reports and confirm the ability of BIS to be an early predictor of outcome after cardiac arrest.[14–17]

We also showed that there is no improved accuracy for outcome prediction using minute-by-minute data as neither specificity, nor sensitivity, nor earliness of prediction were improved compared to the method referring to BIS monitoring every 30 minutes. An explanation might be that minute-by-minute values do not add further information as there are fluctuations without relevance that nevertheless render calculations of prediction models more laborious. Also, it is possible that the lower number of patients with minute-by-minute data may have prevented finding an added value to recordings every 30 minutes.

Compared to previous studies on BIS, ours is not limited to a short period of measurement or even a single time-point, more prone to potential artefacts due to patient examination and manipulation.[13–15] As shown in Figure 2AB, profiles of BIS values are different in both outcome groups, vary over time and outliers are common. Thus, continuous monitoring of brain function may be more accurate. Furthermore, patients were subjected to continuous neuromuscular blockade and BIS data were cleaned for low signal quality and EMG artefacts.

In a recent paper, BIS, at 7 hours after ICU admission, combined with sedation requirements, was predictive of neurological outcome after cardiac arrest.[28] Using a different approach, we found similar results, strengthening the assumption that BIS monitoring can be an early prognostic indicator in cardiac arrest patients.

BIS, although initially designed for monitoring the depth of anesthesia, is simple to use and the electrodes are easily applicable. Interpretation of data does not require expertise from neurophysiologists or specially trained personnel like for EEG, amplitude integrated EEG or SSEP.[10,29] This provides the premises for a widespread use. 13 patients could not have reliable datasets, mainly because of technical reasons (low SQI, high EMG) emphasizing the

need to screen for artefacts rendering calculations unreliable and the need of correct electrode placement and surveillance.

We acknowledge some limitations of our study. It is a single center study with a small sample size and no validation cohort, but to avoid optimism and model overfitting due to multiple predictive variables in a small sample set, sensitivity and specificity were corrected using bootstrap internal validation. All BIS data were acquired in the ICU and time from cardiac arrest to ICU admission may vary. As in all other BIS trials, the treating physicians were not blinded to the BIS values. Although technically feasible, this blinding was not performed in the present study. We must emphasize that in our protocol limitation of care or withdrawal of life supporting therapies were not applied before complete cessation of sedation. Only bundles of arguments, including clinical neurological examination, electrophysiology (SSEP and conventional EEG), biomarkers and neuroimaging eventually lead to termination of active treatment. Despite our present results, we advise to never use a single parameter for outcome prediction, especially within the first 24 hours. Our actual sedation protocol, including continuous neuromuscular blockade, is tailored for sedation in patients receiving hypothermia at 33 °C. As the real impact of induced hypothermia after cardiac arrest is questioned, this sedation protocol might need to be adapted and our findings to be reconsidered.[1,30] Adherence to the sedation protocol is of utmost importance as over-sedation as well as hypothermia might lead to lowering BIS. However, as all patients benefitted from the same sedation and target temperature regimen with the same time to target temperature, these parameters are unlikely to have significantly affected our results. Other factors such as local brain perfusion and metabolic disturbances might also influence BIS.

A larger prospective multi-center trial is warranted to confirm the value of BIS as early outcome predictor after cardiac arrest, and to determine the earliest time-point for the safest outcome prediction.

5. Conclusions

Calculation of the mean BIS value over the first 12.5 hours after ICU admission might be another potential predictor of neurological outcome after cardiac arrest. Further studies are warranted to confirm and refine these findings.

The data of this paper do not overlap with previous publications and the manuscript, including related data, figures and tables, have not been published previously and the manuscript is not under consideration elsewhere.

Conflicts of interest: None.

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Conception and design of the study: PS, YD, OC; Acquisition of data: PS, CW, CS; Analysis and interpretation of data: PS, OC, YD; Writing of the article: PS, YD; Critical review and final approval of the content by all authors.

6. Conflict of interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.resuscitation.2014.09.009.

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8.5 Paper V:

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Biomarkers

Modeling Serum Level of S100β and Bispectral Index to Predict Outcome After Cardiac Arrest

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Objectives This study was designed to evaluate multimodal prognostication in patients after cardiac arrest (CA).

Background Accurate methods to predict outcome after CA are lacking.

Methods Seventy-five patients with CA treated with therapeutic hypothermia after cardiac resuscitation were enrolled in this prospective observational study. Serum levels of neuron-specific enolase (NSE) and neuron-enriched S100 beta (S100β) were measured 48 h after CA. Bispectral index (BIS) was continuously monitored during the first 48 h after CA. The primary endpoint was neurological outcome, as defined by the cerebral performance category (CPC) at 6-month follow-up: scores 1 or 2 indicated good outcome, and scores 3 to 5, poor outcome. The secondary endpoint was survival.

Results A total of 46 (61%) patients survived at 6 months and 41 (55%) patients had CPC 1 or 2. Levels of NSE and S100β were higher in patients with poor outcomes compared with patients with good outcomes (4-fold and 10-fold, respectively; $p < 0.001$). BIS was lower in patients with poor outcomes (10-fold; $p < 0.001$). NSE, S100β, or BIS alone predicted neurological outcome, with areas under the receiver-operating characteristic curve (AUC) above 0.80. Combined determination of S100β and BIS had an incremental predictive value (AUC: 0.95). S100β improved discriminations based on BIS ($p = 0.0008$), and BIS improved discriminations based on S100β ($p < 10^{-5}$). Patients with S100β level above 0.03 μg/l and BIS below 5.5 had a 3.6-fold higher risk of poor neurological outcome ($p < 0.0001$). S100β and BIS predicted 6-month mortality (log-rank statistic: 50.41; $p < 0.001$).

Conclusions Combined determination of serum level of S100β and BIS monitoring accurately predicts outcome after CA. (J Am Coll Cardiol 2013;62:851–8) © 2013 by the American College of Cardiology Foundation

According to the Declaration of the European Parliament of June 14, 2012, on establishing a European Cardiac Arrest (CA) Awareness Week, it is estimated that some 400,000 people in Europe experience a sudden out-of-hospital CA each year, with a survival rate around 10% (1). Survival rate after successful cardiopulmonary resuscitation largely depends on residual function of the heart and the degree of permanent brain damage. CA is therefore a devastating disease in terms of both morbidity and mortality.

The ability to accurately predict outcome within 48 h of admission to the intensive care unit (ICU) in patients resuscitated from CA would be a major achievement. Health care providers consider that this prediction would allow a personalized therapy that would benefit the patient. Maximal cardiac supportive treatment could be applied to patients with a possible good neurological outcome. On the other hand, treatment could be alleviated in those with a futile neurological prognosis. However, accurate methods for early outcome prediction after CA are still lacking.

Initial reports of the out-of-hospital CA score (2), which used several variables readily available at admission to the ICU, have not been replicated (3). Neurophysiological tests such as electroencephalography (EEG) (4–6) or somatosensory evoked potentials (7,8) have been suggested to predict outcome after CA. However, while useful in some situations, these methods are not universally applicable in clinical practice because they require specially trained consultants. Bispectral index (BIS) monitoring, an electroencephalographic monitoring method initially designed to measure the depth of anesthesia, has some potential in predicting brain damage after therapeutic hypothermia in CA patients (9–11). Recently, Riker et al. (12) suggested that BIS monitoring may aid in the identification of patients

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Abbreviations and Acronyms

BIS = bispectral index
CA = cardiac arrest
CPC = cerebral performance category
EEG = electroencephalography
ICU = intensive care unit
IDI = integrated discrimination improvement
NSE = neuron-specific enolase
ROSC = return of spontaneous circulation
S100β = neuron-enriched S100 beta
SAPS = simplified acute physiology score

susceptible to waking up during therapeutic hypothermia. The main advantages of BIS monitoring are its ease of use and therefore its potential for widespread use.

Circulating levels of the biomarkers neuron-specific enolase (NSE) and neuron-enriched S100 beta (S100β) may aid in the prognostication of outcomes after CA (13–15). The use of NSE in this context is recommended in the guidelines of the American Academy of Neurology to predict outcomes in survivors of CA (16). These guidelines stated that, due to insufficient data, the use of other markers cannot be either supported or rejected. Furthermore, most data were based on non–hypothermia-treated patients. The 2010 European Resuscitation Council guidelines also stipulated that, due to a lack of evidence, biomarkers should not be used as the sole predictors of outcome (17). For the same reason, these guidelines also advocate greatest care when using electrophysiological tests for prognostication.

We hypothesized that combined biomarker determination and BIS monitoring may improve outcomes prediction in patients resuscitated from CA.

Methods

Patients. From April 2008 to July 2011, 87 patients with CA were admitted to the 18-bed adult general ICU of an academic tertiary care hospital in Luxembourg. Of these, 75 patients were enrolled in this prospective observational study. The remaining 12 patients had either no blood sample from which to measure biomarkers or were enrolled in another ongoing study. All patients were unconscious on admission, with a Glasgow coma score below 8. The biomarker research protocol was approved by the national research ethics board (National Committee for Ethics in Research of Luxembourg). Informed consent was obtained from all survivors or their legal representatives. Patients were treated with hypothermia at 33°C for 24 h after successful resuscitation. All patients were sedated according to a standard sedation protocol using midazolam (maximum dosage: 0.2 mg/kg/h) and fentanyl (maximum dosage: 1.5 μg/kg/h). A continuous infusion of cisatracurium (0.1 mg/kg/h) was used to avoid shivering and muscular artefacts on BIS monitoring. No patient woke up during the first 48 h, as all patients were sedated for at least 36 h (24 h of hypothermia and 12 h of rewarming phase) with long-acting sedatives (midazolam). Blood samples for biomarker determination were drawn in citrated tubes 48 h after CA, when patients had returned to normothermia.

In patients who did not regain consciousness, no withdrawal or withholding of any treatment was done before at least 5 days after complete stop of sedation, unless clinical signs of brain death were evident.

Neurological evaluation was performed before discharge from the ICU and after 6 months. Patients were classified according to cerebral performance category (CPC) score, as follows: 1 or 2 = no or minor neurological sequelae; 3 or 4 = severe neurological sequelae or coma; and 5 = death.

Measurement of serum markers. Serum level of NSE was assessed on the Cobas e601 analyzer by the electrochemiluminescence immunoassay method (Roche Diagnostics, Mannheim, Germany). The lower limit of quantification of the assay was 0.05 μg/l. Serum level of S100β was assessed by enzyme-linked immunosorbent assay (BioVendor, Heidelberg, Germany). The lower limit of quantification of the assay was 0.015 μg/l. S100β was measured a posteriori, with physicians obviously blinded to the results.

Bispectral index monitoring. Since 2005, all CA patients in our institution have undergone routine BIS XP monitoring with a Quatro sensor (ASPECT Medical Systems Inc., Newton, Massachusetts), integrated to standard ICU monitoring (IntelliVue, Philips, Böblingen, Germany) after admission to the ICU. BIS is an EEG monitoring method using a Fourier transformation to convert raw EEG signals into a number from 0 (flat EEG) to 100 (normal electric activity of an awake patient). In our study, only BIS values with a signal quality index above 80% and an electromyography noise signal below 40 dB were used for analyses. As all patients were paralyzed during the recording phase, muscle artefacts (high electromyography noise) could be excluded. BIS values were continuously recorded during the first 48 h after ICU admission. The lowest BIS value within this period was used for analysis.

Statistical analysis. All analyses were preceded by the Shapiro-Wilk normality test. Comparisons of normally distributed data between 2 groups were performed by *t* test. The nonparametric Mann-Whitney *U* test on ranks was used for non-normally distributed data. Categorical data were analyzed with the chi-square test and the Fisher exact test. Correlation between two variables was assessed with the Spearman test on ranks. A p value <0.05 was considered significant. Analyses were performed with SigmaPlot version 11.0 (Systat Software, Inc., Chicago, Illinois).

Prediction analyses were performed with the PredictABLE package on the R 2.14.2 statistical platform. A p value <0.05 was considered statistically significant. Multiple logistic regression models were used for the prediction of neurological outcomes, as assessed by a binary transformation of CPC (CPC 1 or 2 = 0; and CPC 3–5 = 1). Multivariable analysis using multiple logistic regression proceeding by stepwise backward elimination was used to evaluate the predictive value of selected predictors with respect to clinical indicators of prognosis. The area under the receiver-operating characteristic curve (AUC) and the risk ratios were computed to estimate predictive values. The

Table 1 Baseline Characteristics of the Study Patients

Characteristic	Good Outcome* (n = 41)	Poor Outcome† (n = 34)	p Value
Age, yrs	61 (29–82)	69 (38–83)	0.01
Sex			0.69
Male	34 (83%)	23 (68%)	
Female	7 (17%)	11 (32%)	
SAPS II	60 (43–83)	72 (48–98)	<0.001
Time to ROSC, min	20 (4–60)	30 (12–76)	0.003
Presenting rhythm			
Asystole	1 (2%)	14 (41%)	0.002
PEA	2 (5%)	7 (21%)	0.08
VF/VT	36 (88%)	13 (38%)	0.05
Other	2 (5%)	0	0.50
ICU length of stay, days	17 (6–45)	9.5 (3–96)	0.01
Time to death, days	0	7 (3–109)	—
Associated factors			
Cardiogenic shock	11 (27%)	12 (35%)	0.74
AMI	34 (83%)	18 (53%)	0.31
EEG epileptic state	1 (2%)	10 (29%)	0.01
Seizures	2 (5%)	15 (44%)	0.003
Medical history			
Tobacco	15 (37%)	8 (24%)	0.51
Alcohol abuse	4 (10%)	4 (12%)	1.00
Renal impairment	2 (5%)	4 (12%)	0.41
Hypertension	18 (44%)	19 (56%)	0.69
Heart failure	9 (22%)	12 (35%)	0.48
Coronary disease	11 (27%)	14 (41%)	0.49
Diabetes	6 (15%)	7 (21%)	0.79
CPC score at 6 months			—
1	34 (83%)	0	
2	7 (17%)	0	
3	0	2 (6%)	
4	0	3 (9%)	
5	0	29 (85%)	

Values are median (range) or n (%). *CPC 1 or 2 (no or minor neurological sequelae). †CPC 3–5 (severe neurological sequelae, coma, or death).

AMI = acute myocardial infarction; CPC = Cerebral Performance Category; EEG = electroencephalography; ICU = intensive care unit; PEA= pulseless electric activity; ROSC = return of spontaneous circulation; SAPS simplified acute physiology score; VF/VT ventricular fibrillation/ventricular tachycardia.

value of adding a variable to a model was evaluated by analysis of deviance and was tested for significance using the Wald chi-square test. Reclassification analyses and integrated discrimination improvement (IDI) were used to evaluate the capacity of new markers to improve the discrimination of patients misclassified by initial markers (18). Statistical significance was evaluated as described (19).

Survival analyses were performed using Kaplan-Meier curves and the log-rank statistic.

Results

Patients. Seventy-five CA patients treated with therapeutic hypothermia were enrolled in this study. Clinical characteristics are shown in Table 1. At 6-month follow-up, 41 patients (55%) had a good neurological outcome (CPC 1 or 2), and 34 patients had a poor outcome (CPC 3–5). Of the latter, 29 patients died during follow-up. The sex ratio was similar between patients with good and poor outcomes. Patients with poor outcomes were older. Clinical parameters potentially reflecting disease severity (simplified acute

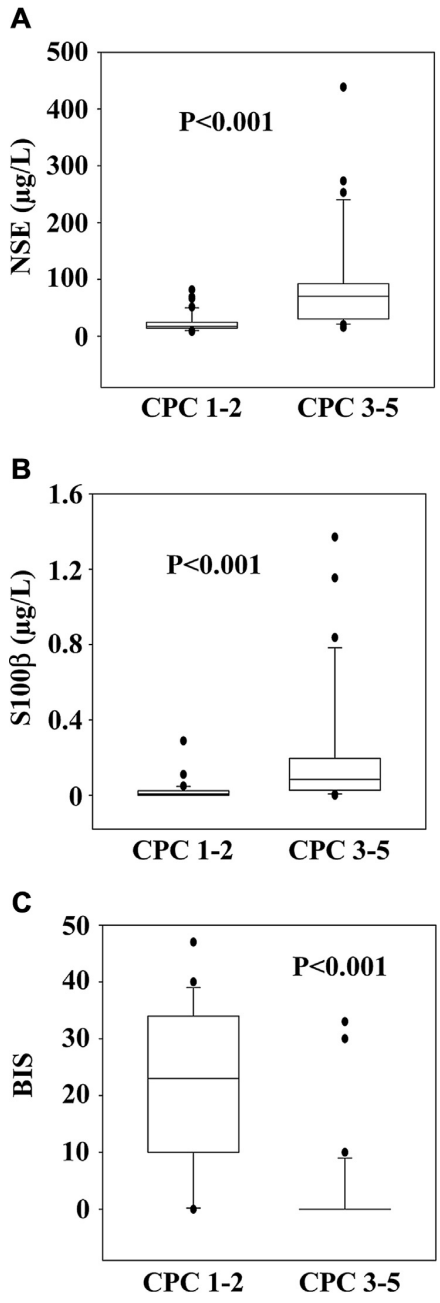

Figure 1 Biomarker Levels and BIS Monitoring According to Outcome

Serum levels of neuron-specific enolase (NSE) **(A)** and neuron-enriched S100 beta (S100β) **(B)** were determined 48 h after cardiac arrest. The lowest BIS value is presented **(C)**. BIS = bispectral index. CPC = cerebral performance category.

physiology score [SAPS II], time from CA to return of spontaneous circulation [ROSC]) were higher in the poor-outcomes group.

Serum biomarker levels and BIS monitoring. Forty-eight hours after CA, serum levels of NSE and S100β were significantly higher in patients with poor outcomes compared with those in patients with good outcomes (4-fold and 10-fold, respectively; $p < 0.001$) (Figs. 1A and 1B). Of note, serum levels of NSE and S100β were highly correlated (r: 0.61; $p = 4 \times 10^{-11}$). BIS was higher in patients with good outcomes compared with that in patients with poor outcomes (10-fold; $p < 0.001$) (Fig. 1C). The median time until the lowest BIS value was 5 h (range: 4 to 14.5 h) after CA.

Prediction of neurological outcome by serum markers and BIS monitoring. Logistic regression was used to determine the ability of serum markers NSE and S100β measured 48 h after CA, and of the lowest value of BIS to predict neurological outcomes at 6 months, as dichotomized by CPC 1 or 2 and CPC 3 to 5. S100β, NSE, and BIS had significant predictive values, with AUCs above 0.80 (Fig. 2A). Combined determination of S100β and BIS had an incremental predictive value, with an AUC of 0.95. Combination of S100β and NSE, or BIS and NSE, had lower predictive values (Fig. 2B). Adding NSE to S100β and BIS did not further improve the prediction (Fig. 2B). Analysis of deviance confirmed that the addition of S100β to the model with BIS improved the prediction (deviance: 16.3; $p = 5 \times 10^{-5}$). However, the addition of NSE to the model with S100β and BIS did not improve prediction (deviance: 2.7; $p = 0.10$).

Reclassification analyses were then performed to address the additive value of biomarkers and BIS (Table 2). First, we assessed the additive value of S100β to BIS monitoring. S100β improved the discrimination based on BIS monitoring (IDI = 0.13; $p = 0.0008$). On the other hand, BIS improved the discrimination based on S100β, with an IDI of 0.32 ($p < 10^{-5}$). NSE failed to improve the discrimination of patients misclassified by a model including BIS and S100β. BIS improved the classification of NSE (IDI = 0.14; $p = 0.0005$). A model with BIS with S100β also improved the classification of NSE (IDI = 0.20; $p < 10^{-5}$).

Therefore, together, serum level of S100β and BIS monitoring are robust predictors of neurological outcomes after CA.

Cutoffs for the prediction of neurological outcomes by S100β and BIS monitoring. We first determined the cutoffs for S100β and BIS monitoring, which provide the best compromise between sensitivity and specificity for the prediction of neurological outcomes (Figs. 3A and 3B). The cutoff for S100β was 0.03 μg/l, providing a sensitivity of 76%, a specificity of 78%, and a false positive rate of 22%. The cutoff for BIS was 5.5, providing a sensitivity of 85%, a specificity of 83%, and a false positive rate of 17%. Risk ratios of poor neurological outcome were calculated (Fig. 3C). Patients with a S100β serum level above 0.03 μg/l (n = 33) had a 3.4-fold higher risk of poor neurological outcomes (95% confidence interval [CI]: 1.88 to 6.34; $p < 0.0001$). Patients with BIS monitoring below 5.5 (n = 36) had a 6.1-fold higher risk of poor neurological outcomes

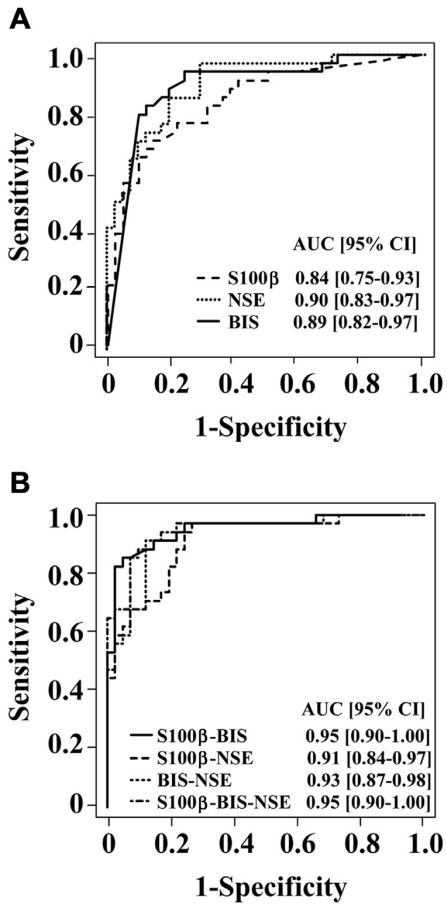

Figure 2 Prediction of Neurological Outcome by Serum Markers and BIS Monitoring

S100β and NSE measured 48 h after cardiac arrest, and BIS monitoring, were used as inputs to multiple logistic regression models for the prediction of 6-month CPC. **(A)** Receiver-operating characteristic (ROC) curves for single markers. **(B)** ROC curves for combinations of markers. The areas under the ROC curve (AUC) are indicated. CI = confidence interval; other abbreviations as in Figure 1.

Table 2 Reclassification Analyses

Initial Model	Variable Added to the Initial Model	IDI (95% CI)	p Value
BIS monitoring	S100β	0.13 (0.05 to 0.21)	0.0008
S100β	BIS monitoring	0.32 (0.22 to 0.43)	<0.00001
BIS and S100β	NSE	0.02 (–0.01 to 0.06)	0.24
NSE	BIS	0.14 (0.06 to 0.21)	0.0005
NSE	BIS and S100β	0.20 (0.11 to 0.28)	<0.00001

BIS = bispectral index; CI = confidence interval; IDI = integrated discrimination improvement; NSE = neuron-specific enolase; S100β = neuron-enriched S100 beta.

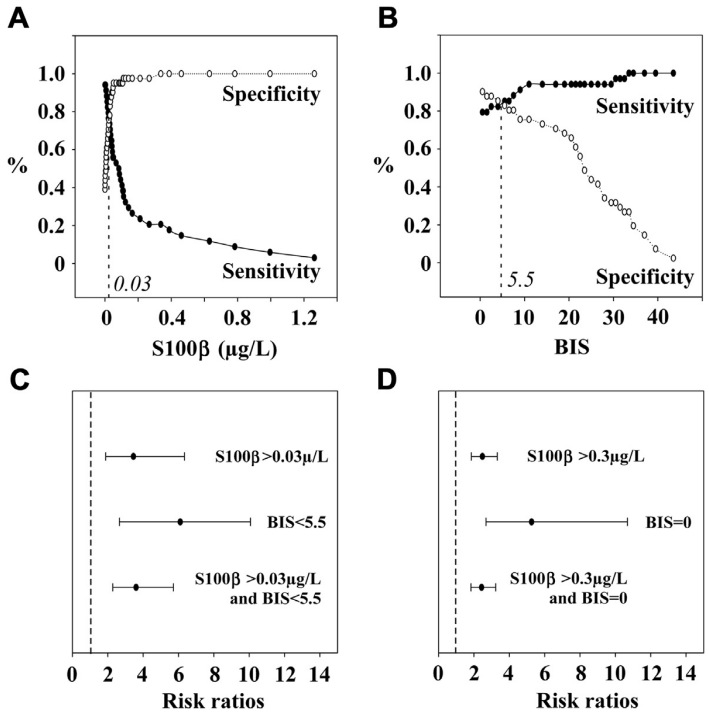

Figure 3 **Optimal Cutoffs of S100β and BIS and Risk Ratios for Prediction of Neurological Outcome**

(A and B) Cutoffs were determined by plotting sensitivity versus specificity values, and are indicated by **dotted lines** and **italicized values. (C and D)** Risk ratios of poor neurological outcome (CPC 3–5) according to cutoff values for S100β and BIS. The **dotted vertical line** indicates a risk ratio of 1. Risk ratios ± 95% CI are presented. Abbreviations as in Figures 1 and 2.

(95% CI: 2.66 to 10.07; $p < 0.0001$). Patients with both serum level of S100β above 0.03 μg/l and BIS monitoring below 5.5 ($n = 21$) had a 3.6-fold higher risk of poor neurological outcome (95% CI: 2.28 to 5.71; $p < 0.0001$).

Second, we determined the cutoff values for optimal prediction of poor outcome. A level of S100β above 0.3 μg/l predicted a poor outcome with a specificity of 100%. A BIS value of 0 predicted a poor outcome with a specificity of 90% and a false positive rate of 10%. As shown in Figure 3D, patients with a S100β level above 0.3 μg/l ($n = 7$) had a 2.5-fold higher risk of poor neurological outcome (95% CI: 1.85 to 3.32; $p = 0.003$). Patients with a BIS value of 0 ($n = 31$) had a 5.3-fold higher risk of poor neurological outcome (95% CI: 2.68 to 10.68; $p < 0.0001$). Patients with S100β level above 0.3 μg/l and a BIS value of 0 ($n = 6$) had a 2.4-fold higher risk of poor neurological outcomes (95% CI: 1.83 to 3.23; $p = 0.007$).

Prediction of mortality by S100β and BIS monitoring. Average survival time for patients who died during follow-up ($n = 29$) was 18 days (range: 3 to 107 days). Survival curves are shown in Figure 4. Patients with a serum level of S100β above the cutoff value of 0.03 μg/l were at a high risk of death during follow-up (log-rank statistic: 17.17; $p < 0.001$). Patients with a BIS value below the cutoff value of 5.5 were at high risk of death during follow-up (log-rank statistic: 35.79; $p < 0.001$). Patients with a serum level of S100β above the cutoff value of 0.03 μg/l and a BIS value below the cutoff value of 5.5 were at a high risk of death during follow-up (log-rank statistic: 50.41; $p < 0.001$). Therefore, serum level of S100β and BIS monitoring are strong predictors of mortality after CA.

Added value of S100β and BIS to traditional prognostic indicators. Finally, we evaluated the predictive value of S100β and BIS with respect to other clinical indicators of prognosis: age; sex; SAPS II; time to ROSC; presenting rhythm (asystole/pulseless electric activity vs. ventricular fibrillation/ventricular tachycardia); and associated factors such as cardiogenic shock, acute myocardial infarction, EEG epileptic state, and seizures. Multivariable analyses showed that SAPS II ($p = 0.04$), presenting rhythm ($p = 0.01$), S100β ($p = 0.01$), and BIS ($p = 0.01$) were independent predictors of neurological outcome. Reclassification analyses attested that S100β and BIS were able to improve the discrimination based on SAPS II and presenting rhythm

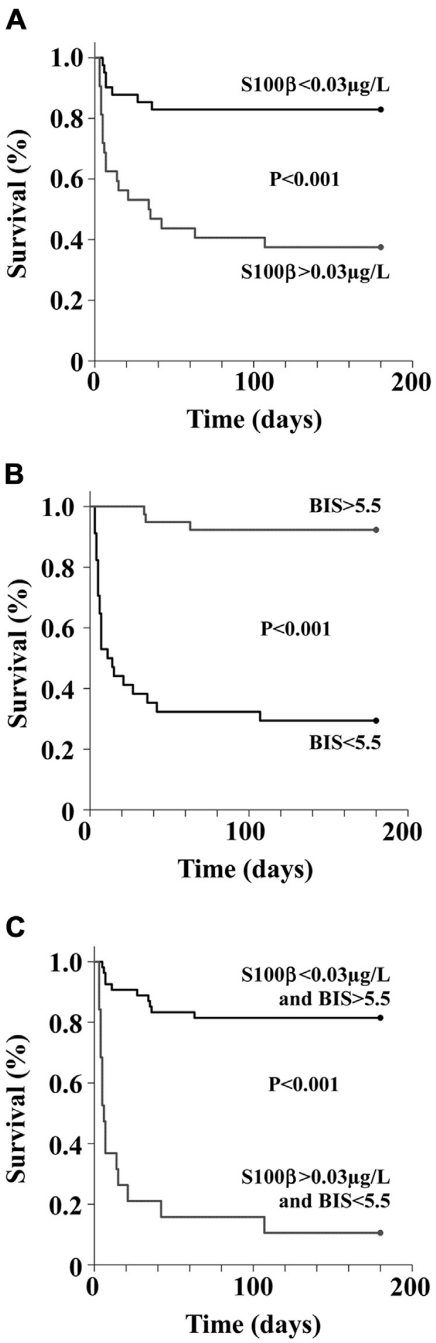

Figure 4 Survival Analyses

Kaplan-Meier curves showing the survival of patients according to their serum level of S100β **(A)**, BIS monitoring **(B)**, or both **(C)**. Abbreviations as in Figure 1.

with an IDI of 0.22 ($p = 10^{-5}$). Therefore, combined determination of S100β and BIS improves the prediction of outcome by SAPS II and presenting rhythm.

Discussion

We identified a method to accurately predict neurological outcome and survival after CA. This method relies on the measurement of serum levels of S100β and BIS monitoring. While these two markers have previously been considered as potential predictors of outcome after CA, this study is the first to report that combined determination of both markers has an incremental, and very robust, prognostic value.

The patients enrolled in this study were all resuscitated from CA and treated by therapeutic hypothermia. Due to the dichotomization of patients into good- and poor-outcomes groups, certain disease-related parameters obviously differed between groups. But, as discussed previously, those criteria alone are not sufficient to prognosticate outcome (2,20). Thus, the impact of our results should not be affected by the significant differences between the 2 groups of patients.

Booth et al. (20) identified from a review of the existing literature 4 clinical indicators that predict death or poor neurological outcome of comatose survivors of CA: absent corneal reflexes, pupillary response, withdrawal response to pain, and motor response at 24 h. These findings suggested that routine clinical examination could predict outcome. However, the studies included in this meta-analysis were performed before 2003, a time when therapeutic hypothermia was not generally performed. In addition, the authors pointed out that clinical examination alone was insufficient to predict prognosis and should be coupled with other tests or biological markers.

The calcium-binding protein S100β is enriched in astroglial cells and can cross the blood–brain barrier after hypoxic damage of the central nervous system. Its routine measurement is simple and relatively inexpensive. S100β is filtrated by the kidney (21) and has an estimated half-life of 2 h (22). Its serum level increases after CA, and its prognostic value has been studied (23–28). Because of mitigated results, its routine use has been, up to now, not recommended (16).

EEG findings during hypothermia correlate with neuronal injury post–brain anoxia (29). Amplitude-integrated EEG certainly has some potential for outcome prediction, but this technique, as well as classic EEG, generally require expertise and special training or consultant support (6). On the other hand, BIS monitoring, which is easily done, appears to be useful to predict outcome after CA (9–11,30). Interestingly, in our study, the lowest values of BIS used for outcome prediction occurred after 5 h (median value), indicating that BIS has the potential for very early prognostication.

NSE has a role in glucose metabolism. As for S100β, NSE is released from the hypoxic brain into the bloodstream, and its serum level correlates with the extent of brain injury. Also, NSE correlates with other markers of brain injury (31). NSE has a high specificity to predict adverse outcomes when measured in the few days post CA (26,32). A cutoff point of 33 µg/l is recommended (16). In our study, the specificity obtained with this cutoff was 83%. NSE levels before 24 h post CA should not be used for prognostication,

and this is consistent with our protocol, in which NSE was measured 48 h post CA. From our results, the predictive value of NSE and its potential contribution to a multimarker strategy remain uncertain. Indeed, analysis of ROC curves showed a maximal AUC for NSE of 0.90, albeit very close to the AUC of BIS (0.89). However, analysis of deviance and reclassification analyses attested that NSE did not increase the predictive value of the model with S100β and BIS. This is probably related to the fact that NSE and S100β are both originating from the brain and do not provide independent information. Consistently, a high correlation was found between serum levels of these two markers. These observations are consistent with those from the study by Einav et al. (26), in which NSE was not an independent predictor of outcome (26). Interestingly, in this same study, S100β was an independent predictor of outcome, and this is in line with our findings. The prognostic performance of NSE, alone or combined with other markers, deserves further testing.

While a cutoff value of 33 μg/l is generally accepted for NSE, there is to date no consensus on the cutoff value for S100β. In our study, a cutoff value of 0.03 μg/l of S100β was found to predict neurological outcomes with a sensitivity of 76% and a specificity of 78%. This cutoff value is lower than the cutoff values reported by Einav et al. (26), which ranged from 0.2 to 100 μg/l, depending on the presenting rhythm of the patients, their age, and the time of blood sampling. It should be noted that not all patients in the study by Einav et al. were treated with therapeutic hypothermia, which may explain the higher cutoff values obtained in that study compared to our study. In the study by Mortberg et al. (33), a cutoff value of 0.18 μg/l was retained. In the study by Rundgren et al. (13), levels of S100β above 0.51 μg/l at 24 h predicted poor outcome with a specificity of 96%. In those three studies, the cutoff values were chosen for the prediction of poor outcome, while our cutoff value of 0.03 μg/l was chosen to provide the most accurate prediction of both poor and good outcomes. In a second phase, we observed that a cutoff value of 0.3 μg/l predicted poor outcome with a specificity of 100%, meaning that all patients with a S100β level above 0.3 μg/l had a poor outcome. This cutoff, which is in line with those from previous studies (13,26,33), suggests that a single determination of S100β would be sufficient to establish a secure prognostic. However, this speculation is limited by the few patients presenting with such a high level of S100β. Overall, the cutoff values of S100β remain to be determined in larger populations, taking into account the demographic and clinical characteristics of each individual patient.

Although an accurate and reliable method to prognosticate patients with CA is still needed, several indicators of prognosis are available to the treating physician. In our group of patients, SAPS II and presenting rhythm were significant predictors of neurological outcome. Interestingly, we observed that combined determination of S100β and BIS had an incremental predictive value.

Study limitations. First, we must acknowledge that BIS is a processed EEG signal that monitors only a limited area of the brain and not the whole cortex, as standard EEG does. In order to eliminate electromyography artefacts, the patients have to be under neuromuscular blockade, at least during the first hours of BIS measurements. Furthermore, although a continuous EEG tracing is shown on the monitoring, it is not suitable for detection of any particular EEG patterns, such as burst suppression or epileptic state. Standard EEG is still required for the diagnosis and subsequent treatment of these disorders. Second, the treating physicians were not blinded to our BIS data or NSE values. However, as pointed out by our study protocol, withdrawal or withholding of treatment was done only 5 days after the cessation of sedation. Complete blinding to BIS data is complex because accurate measurements require frequent signal-quality checks to correct electrode application, for instance. Blinding of the treating physician would require a third person to check the accuracy of the BIS monitoring nearly continuously. Furthermore, the BIS data must be masked on the monitoring system and in the patient data chart, which is automatically linked to the monitoring system. This was not feasible in the current study. In contrast, treating physicians were blinded to S100β because it was a completely a posteriori analysis. Third, CPC determination can be subjective (34). However, this does not represent a major limitation of our study because 85% of patients with a poor CPC had died by the 6-month follow-up. Fourth, the distributions of S100β and BIS were left censored at 0 because 41% of patients had no EEG activity (BIS = 0) and 24% had a S100β level below the detection limit of the assay. However, this censoring does not diminish the predictive value of these markers because it does not affect *c* statistic. Fifth, the small size of the study population did not allow accurate determination of the prognostic value of each of the nine clinical predictors included in the multivariable analyses.

Conclusions

Using a biochemical and an electrophysiological marker of brain damage, outcomes after CA may be predicted. Further studies are required to confirm our findings.

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Key Words: biomarkers ■ brain injury ■ cardiac arrest ■ electroencephalogram ■ prognosis ■ survival.

9. APPENDIX I: CONTRIBUTION TO OTHER SCIENTIFIC PUBLICATIONS

1. Westhall E, Rossetti AO, van Rootselaar AF, Wesenberg Kjaer T, Horn J, Ullen S, Friberg H, Nielsen N, Rosen I, Aneman A, Erlinge D, Gasche Y, Hassager C, Hovdenes J, Kjaergaard J, Kuiper M, Pellis T, **Stammet P**, Wanscher M, Wetterslev J, Wise MP, Cronberg T, investigators TTM-trial, (2016) Standardized EEG interpretation accurately predicts prognosis after cardiac arrest. Neurology
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