ApoE 4 blood marker assay. A NEW NON-GENETIC METHOD TO EVALUATE ALZHEIMER´S DISEASE RISK USING CLINICAL CHEMISTRY PLATFORMS.

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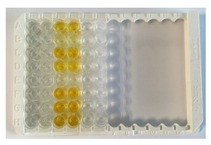
BACKGROUND

To date only the presence of one or two alleles ε4 of the *APOE* gene is accepted as a reliable biomarker and risk factor of developing late onset Alzheimer´s Disease (AD) [1, 2]. The presence of one allele ε4 of the *APOE* gene increases the risk of suffering AD by 3-5 fold, while the presence in homozygosis increases the risk by 15-20 fold [3]. Furthermore, *APOE* ε4 carriers progress faster from the preclinical stage of AD to AD than *APOE* ε4-non carriers [4]. Therefore, *APOE* ε4 carriers clearly constitute a target population where research, clinical trials and prevention strategies should be focused. Despite its clear clinical utility, apoE4 analysis is not required by neurologists in a routine basis mainly for logistic and bureaucratic reasons: It involves DNA analysis, which in Europe, requires an informed consent, and the standard analysis technique is PCR which requires additional processing of the sample to extract the DNA. Furthermore, PCR is a method of analysis more complex and expensive than the techniques normally used in a clinical laboratory setting.

DEVELOPMENT STRATEGY

Proof of concept: ELISA



ELISA microtiter plate

RESULTS

ApoE4 ISOFORM CAN BE DETECTED WITH 100 % SENSITIVITY AND 100% SPECIFICITY IN ELISA

Biocross developed a test where the ApoE4 present in plasma is specifically captured by the ELISA microplate with the aid of a *special coating agent* developed by Biocross. Then, the use of a highly specific antibody for ApoE4 - that does not recognize the rest of apolipoprotein E isoforms (E2 and E3) - allows the discrimination of *APOE* ε4 carriers (patients that carry at least one allele ε4 of the *APOE* gene) from *APOE* ε4-non carriers with 100% of sensitivity and 100% of specificity.

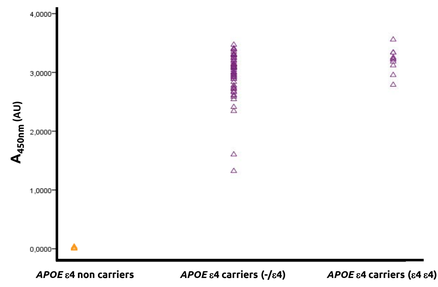


Figure 2. The presence/absence of ApoE4 was detected in 230 plasma samples from individuals previously genotyped by Real-Time PCR. A total of 157 *APOE* ε4 non carriers (e2/e3, n=16 and e3/e3, n=141) and 73 *APOE* ε4 carriers (e2/e4, n=4, e3/e4, n=59 and e4/e4, n=10) were analyzed. The method developed by Biocross revealed 100% concordance with *APOE* genotyping by Real-Time PCR [7].

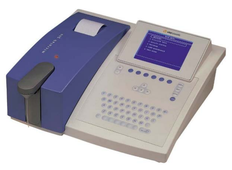
△ *APOE* ε4 non carriers (n=157)
△ *APOE* ε4 carriers (n=73)

Sensitivity: 100%
Specificity: 100%

ADAPTATION TO TURBIDIMETRY & TECHNICAL VERIFICATION

Biocross adapted the method to a turbidimetry-based kit. The kit consists of two main reagents: **R1** (which contains a highly specific anti-apoE4 antibody) and **R2** (a solution containing latex (polystyrene beads).
During the assay, the anti-apoE4 antibody induces the agglutination of latex beads in the presence of apoE4 in the sample. This agglutination is proportional to the concentration of the apoE4 and can be measured spectrophotometrically. The main studies carried out to verify the test parameters are summarized below. These studies were performed using the platform Selectra Vital Junior.

Laboratory



Selectra Vital Junior

1. Interference

One plasma sample from one *APOE* ε4 carrier (*e3/e4, purple*) and one plasma sample from one *APOE* ε4 non-carrier (*e3/e3, orange*) were tested in duplicate either in the absence (*dotted lines*) or in the presence (*solid line*) of one or several concentrations of classical interference factors in plasma (haemoglobin, bilirubin, tryglicerides or rheumatoid factor). Values are mean ± SD of duplicates. Red line represents positivity cut-off for the test (125 mAbs).

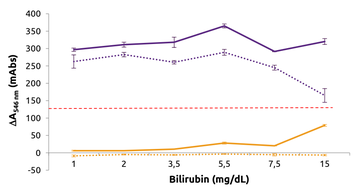


Figure 3. Bilirubin interference. Signs of positive interference were observed in both *APOE* ε4 non-carrier and *APOE* ε4 carrier samples. Nevertheless, signal from *APOE* ε4 carrier sample was above the established positivity cut point and signal from *APOE* ε4 non-carrier was below the positivity cut point, independent of the concentration of bilirubin tested. Therefore it is considered that bilirubin, at the concentrations tested, does not influence the final result of the test.

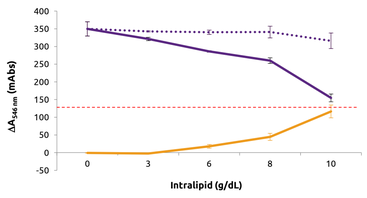


Figure 4. Triglycerides interference. Signs of positive interference for *APOE* ε4 non-carriers and signs of negative interference for *APOE* ε4 carriers were observed. Nevertheless, signal from *APOE* ε4 carrier sample was above the established positivity cut point and signal from *APOE* ε4 non-carrier was below the positivity cut point, independent of the concentration of intralipid® tested, so it is concluded that triglycerides do not interfere with the result of the test at those concentrations.

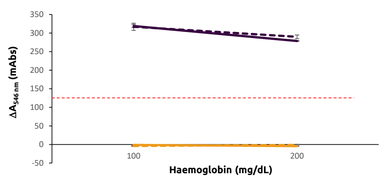


Figure 5. Haemoglobin interference. No interference were observed at the concentrations of haemoglobin tested. *APOE* ε4 non-carrier sample remained below the positive cut-off at all concentrations of haemoglobin tested. Similarly, *APOE* ε4 carrier sample remained above the positive cut-off at 100 and 200 mg/dL of haemoglobin.

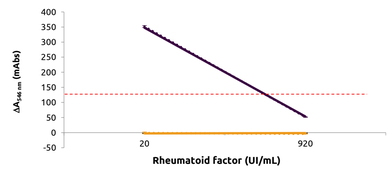
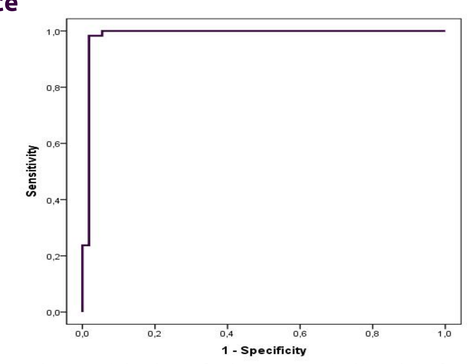


Figure 6. Rheumatoid factor interference. No interference were observed at the concentrations of rheumatoid factor tested. *APOE* ε4 non carrier sample remained below the positive cut-off at 20 and 920 UI/mL of rheumatoid factor. By contrast, *APOE* ε4 carrier sample was found to be below the positive cut-off at 920 UI/mL. However, this low value is considered to be related more to the dilution of the apoE4 signal in the plasma sample, due to the high volumes of rheumatoid factor that had to be added to reach such high concentration. Note that the same value is obtained in the *APOE* ε4 carrier sample without rheumatoid factor (where buffer, instead of rheumatoid factor, was added at the same volume).

2. Performance



AUC: 0,986 (95% CI 0,96-1,00)
Accuracy: 0,97

Figure 7. Receiver Operator characteristic (ROC) curve for the *APOE* ε4 carriership analysis of 114 plasma samples (55 *APOE* ε4 non carriers and 59 *APOE* ε4 carriers), representing sensitivity (i.e, true positive rate) versus 1-specificity (i.e, false positive rate). AUC (Area under the curve, with 95% confidence intervals) and accuracy (measured as 1-missclasification rate) values are provided. Accuracy was calculated assuming a 100% sensitivity and 95% specificity.

3. Stability

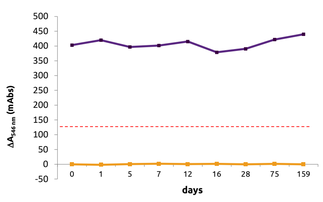


Figure 8. R1 and R2 were stored at 2-8ºC and one *APOE* ε4 carrier sample (e3/e4, purple) and one *APOE* ε4 -non carrier sample (e3/e3, orange) were analyzed on 9 different days during a period of 159 days. The results show that the assay is stable during at least 159 days if stored at 2-8ºC. Red dashed line represents positivity cut-off for the test (125 mAbs).

4. Linearity

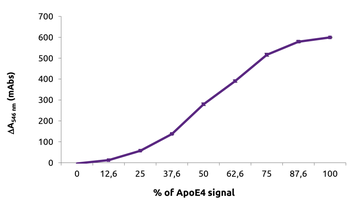


Figure 9. Nine pools were prepared combining different proportions of one *APOE* ε4 -non carrier (e3/e3) sample and one *APOE* ε4 carrier sample (e3/e4). The signal of each pool was measured in duplicate. Primary response (response vs concentration) showed a sigmoidal shape, but intermediate area of the curve had a good linearity.

ADAPTATION TO MEDIUM AND HIGH THROUGHPUT CLINICAL CHEMISTRY ANALYZERS

Hospital routine

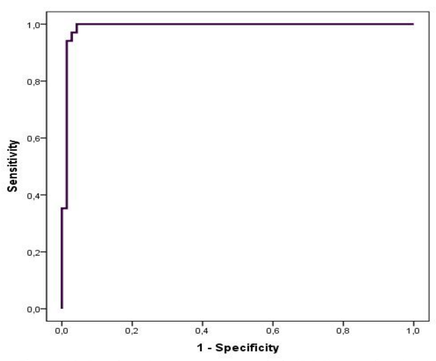
Architect c16000 (Abbott)

Figure 1. Biocross has developed a non-genetic, cost effective and highly reliable method to detect the presence of apoE4 in human blood plasma.
The method was initially developed in ELISA microtiter plates and was adapted to a turbidimetry-based assay to allow its implementation to the clinical analysis routine.

Preliminary assays were carried out to evaluate the performance of the kit in two different clinical chemistry analyzers: One medium throughput (LIDA 300, which can run a total of 300 samples/hour) and one high throughput (ARCHITECT c16000 (Abbott), which can run up to 1800 samples/hour).

The objective of these assays was to test the kit within the real environment of final customers (i.e core facilities of hospitals).

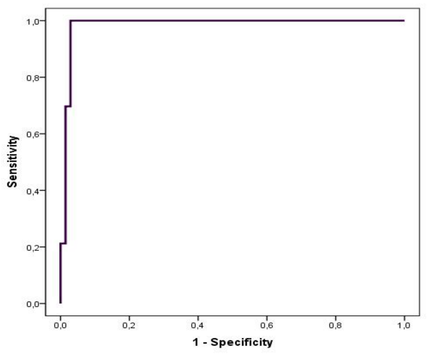
MEDIUM THROUGHPUT ANALYZER (LIDA 300)



AUC: 0,984 (95% CI 0,97-1,00)
Accuracy: 0,97

Figure 10. Receiver Operator characteristic (ROC) curve for the *APOE* ε4 carriership analysis of 105 plasma samples (71 *APOE* ε4 non carriers and 34 *APOE* ε4 carriers) representing sensitivity (i.e, true positive rate) versus 1-specificity (i.e, false positive rate). AUC (Area under the curve, with 95% confidence intervals) and accuracy (measured as 1-missclasification rate) values are provided. Accuracy was calculated assuming a 100% sensitivity and 96% specificity.

HIGH THROUGHPUT ANALYZER (ARCHITECT c16000)



AUC: 0,990 (95% CI 0,97-1,00)
Accuracy: 0,97

Figure 11. Receiver Operator characteristic (ROC) curve for the *APOE* ε4 carriership analysis of 102 plasma samples (70 *APOE* ε4 non carriers and 32 *APOE* ε4 carriers), representing sensitivity (i.e, true positive rate) versus 1-specificity (i.e, false positive rate). AUC (Area under the curve, with 95% confidence intervals) and accuracy (measured as 1-missclasification rate) values are provided. Accuracy was calculated assuming a 100% sensitivity and 96% specificity.

CLINICAL UTILITY

The ***ApoE4 blood marker assay*** could be easily incorporated into routine dementia test profiles, allowing a fast identification of *APOE* ε4 carriers in the clinic. The identification of patients with one or two alleles ε4 of the *APOE* gene would be extremely useful in the following aspects:

A) CLINICAL INVESTIGATION

Patient stratification in clinical trials

The frequency of the ε4 allele is approximately 40% in patients with AD. Furthermore, *APOE* ε4 carriers progress faster to clinical AD than *APOE* ε4 non carriers. Therefore, the presence of one or two alleles ε4 of the *APOE* gene is considered highly informative in epidemiological research and is currently being used for patient stratification in the latest clinical trials to evaluate AD treatments.

B) HEALTH MANAGEMENT

Diagnosis

The prevalence of amyloid positivity as measured by PET in AD patients that were *APOE* ε4 carriers, ranged from **97% to 90%** in patients **50 - 90 years old** respectively [3]. This data suggest that PET or CSF analysis could be spared in patients that are *APOE* ε4 carriers and with a clinical evaluation compatible with AD.

Treatments

Some studies show that the response to a treatment could be significantly different depending on *APOE* ε4 carriership [4,5], leading to the idea that *APOE* ε4 status should be determined prior to any therapeutical intervention.

Prevention

It has been suggested that reducing the prevalence of modifiable risks factor of AD (hypertension, diabetes or hypercholesterolemia would lower the risk, delay the onset and reduce the duration of AD [6]. Detection of patients at risk (i.e *APOE* ε4 carriers) would reinforce lifestyle preventive interventions that could prevent or delay the development of the disease, which in turn could represent a significant saving for health care systems.

CONCLUSION

Our results show that ***ApoE4 blood marker assay*** is a cost effective and highly reliable method to identify *APOE* ε4 carriers that can be integrated into the clinical routine setting of hospitals.

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