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Overview of Molecular Testing of Breast Cancer Use in Determining Appropriate Adjuvant Therapy

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The past 10 years have seen a proliferation of new molecular diagnostic tests in many tumor types, but most notably in the field of breast cancer (BC), where these tests have had a major clinical impact (1,2). Whereas in earlier decades, decisions regarding what adjuvant therapy to recommend were mostly guided by tumor stage alone, now it is much more nuanced based on the results of molecular profiling of tumors, especially those that are hormone receptor positive. In this article we will discuss the various commercial tests available, review the current recommendations and critical assessments of these tests made by several leading Oncology thought groups, and look to future results that are still awaited and that may change our thinking yet again.

The classic factors guiding what type of adjuvant therapy to recommend to early stage BC patients include tumor size and grade, number of axillary lymph nodes involved, patient age, hormone receptor status and Her2neu expression. However, if tumor biology is the real driver of disease behavior, then perhaps factors such as tumor size and lymph node (LN) status are less important, compared to its molecular profile. It is a given that tumors that over-express Her2neu require chemotherapy (CT) and anti-Her2 therapy, and tumors that are triple negative generally require adjuvant CT (3). Yet, the remaining majority of tumors that are hormone receptor positive still present a dilemma, thus spurring the development of other tools to help enable decision-making in this group of early breast cancer patients (4-6).

Molecular profiling has the potential to predict both the prognosis of a given tumor, and its response to CT. Yet, this type of information is still impacted by the clinical situation, such that a tumor with a favorable biology but involves multiple LNs still has a worse prognosis than the same tumor that does not involve LNs, while on the other hand, treating such a patient with CT may actually contribute little, if the molecular profile predicts a lack of response to CT. Efforts to reconcile the clinical scenario with the results of molecular profiling remains a challenge and attempts have been made to fuse the two different parameters to arrive at a unifying metric, with varying degrees of success.

There are three major commercial gene array assays available at the present time: the Oncotype Dx assay, the Mammaprint 70 gene assay and the PAM50 assay. Another test which deserves discussion is referred to as the IHC-4 score, a simpler predictive model that could theoretically be performed in most labs (7,8).

IHC-4 Score

The IHC4 score (developed by Cuzik et al and described in 2011) uses an algorithm of 4 basic histological tumor characteristics: estrogen receptor (ER), progesterone receptor (PR), Her2neu and Ki-67 status - which is a critical component of the algorithm. Analysis was based on a cohort of postmenopausal Hormone Receptor positive BC patients, of which 793 were node negative and 432 node positive. The results of the analysis showed a significant spread between the curves when comparing patients in the 25th quartile and those in the 75th quartile, with a hazard ratio (HR) of 5.7 (95% CI 3.4-9.7). Similar results were shown when excluding the Her2neu status (i.e. the IHC3 score), and in a cohort of 786 ER-positive patients treated in Nottingham, England HR= 4.1 (95% CI 2.5-6.8) (8).

When compared with the results obtained from the Oncotype Dx assay, there was little difference, with an r value of 0.72 showing significant correlation between the two tests. As an example provided by Cuzik et al, for a patient with a poorly differentiated T1 tumor on Anastrozole, the IHC4 for the 25th and 75th percentile would predict a 7.6% vs. 13.9% 9 year distant recurrence rate, while the RS predicts a 9.2% vs. 13.4% recurrence rate, respectively (8). **(Figure 1)**

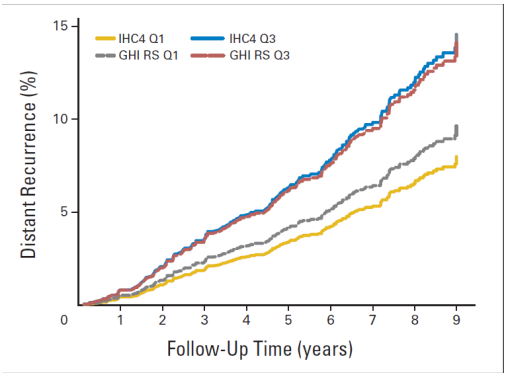

Figure 1 - IHC-4 Score Compared to RS Out to 9 years

Oncotype Dx assay

The first molecular assay to become available clinically to help predict outcome of patients with node negative HR positive BC patients was the Oncotype Dx assay, developed by Genomic Health (9,10). Genomic Health set out to analyze tumors from patients with this common variety of BC with the goal of further classifying them as to future risk of relapse. Some 250 candidate genes mentioned in the literature that were relevant to the development of breast cancer were analyzed by performing the Reverse Transcription Polymerase Chain Reaction (RT-PCR) on tissue from paraffin embedded pathology specimens from patients who participated in various clinical studies of BC. Ultimately, 16 candidate genes were chosen, where there was a 4 fold or greater variation in gene expression between specimens in the study. The 16 candidate genes were normalized with 5 genes that varied little among the specimens (i.e. control genes). The study genes comprised mainly genes involved in the ER, Her2, proliferation and invasion pathways. Next, an algorithm was developed that properly fit with the known patient outcomes from the specimens tested. This algorithm generated a score from 1-100, known as the Recurrence Score (RS).

Next the algorithm was validated in a cohort of 668 BC patients in the Tamoxifen treated arm of the NSABP B-14 study (9,11). Three groups of patients were determined based on their risk of recurrence: low risk group (RS=0-17), intermediate risk group (RS = 18-30) and a high risk group (RS of ≥31). **(Table 1)**

Table 1. Kaplan–Meier Estimates of the Rate of Distant Recurrence at 10 Years, According to Recurrence-Score Risk Categories.*

Risk Category	Percentage of Patients	Rate of Distant Recurrence at 10 Yr (95% CI)†
		percent
Low	51	6.8 (4.0–9.6)
Intermediate	22	14.3 (8.3–20.3)
High	27	30.5 (23.6–37.4)‡

The p-value for the difference between the high risk group and the low risk group was <.001. In a multivariate Cox Proportional Analysis of multiple parameters including age, grade, tumor size, differentiation and RS, only RS and poor differentiation remained statistically significant (HR= 2.81 & 3.41, respectively) (9). However, when the cohort of patients in the placebo arm of NSABP B14 was also analyzed and compared with similar patients in the Tamoxifen-treated arm, the benefit from hormonal therapy was significant in the low and intermediate risk groups only, whereas the patients in the high risk group seemed to derive no benefit (p=0.82) (12).

In addition, it is important to note that since the test was developed as a continuous variable it should be used as such – whereby there is not much difference in outcome between patients with an RS of 17 and 19, but a significant difference between patients with results of 2 and 17.

After establishing the prognostic capability of the Oncotype Dx assay, its utility in predicting benefit from CT was tested in a retrospective analysis of specimens from the NSABP B-20 trial (in which node negative hormone receptor positive BC patients were randomly assigned to receive CT+/- HT) (11,13). That study revealed a lower risk of recurrence and death (HR=0.52; p<.001, HR=0.78; p=0.63, respectively) at 12 years of follow-up in the chemotherapy arm. When tested in a subset of 651 patients from that study for whom tumor

blocks were available, the analysis revealed a significant benefit from CT in the high risk patients (HR=0.26, 95% CI 0.13-0.53) with an absolute decrease of 27.6+/- 8% in 10-year distant recurrence rate, as opposed to little or no benefit from CT in the low risk group of patients (HR=1.31; 95% CI 0.46-3.78). The intermediate RS group did not seem to benefit from CT, although a clinically significant benefit from CT could not be excluded. **(Figure 2**)

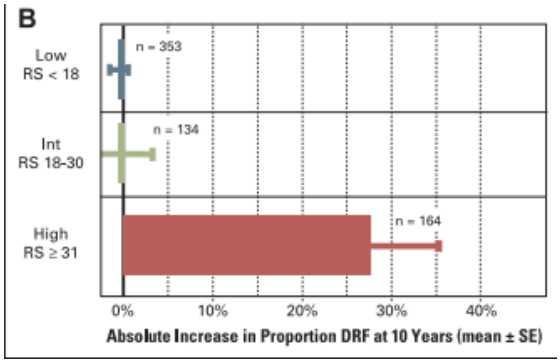

Figure 2 Benefit from Chemotherapy Based on RS group NSABP-B20

A similar analysis was performed in a cohort of patients from the Intergroup E2197 study of patients with up to 3 positive axillary nodes that compared treatment between four cycles of AC (Adriamycin and Cyclophosphamide) every 3 weeks vs. four cycles of AT (Adriamycin and Taxotere) every three weeks. That study revealed no significant difference in survival between the treatment arms. In the group of patients that was HR positive, RS was again, highly predictive of recurrence, in both node negative and node positive patients with a direct correlation between RS and 5 year recurrence risk up to a RS of 40 (p<.001) (14).

To evaluate the utility of the Oncotype Dx assay in predicting chemotherapy benefit in node positive BC patients, specimens from the SWOG-8841 trial (in which node positive hormone receptor positive postmenopausal BC patients were randomly assigned to receive CT+/- HT) were also analyzed (15). Similar to the findings in the B20 study, patients with high recurrence scores had a significant benefit from treatment with chemotherapy (HR=0.59, log rank p=0.033), while those with a low recurrence score did not benefit (HR=1.02; log rank p=0.97).

In studies looking at the clinical impact the results of RS have had in deciding what adjuvant treatment oncologists recommend, changes based on the results have occurred in from 30-40% of cases, and in general, there has been a decrease in recommendations for the use of adjuvant chemotherapy. **(Table 2)**

Summary of the Recurrence Score
International Decision Studies

Study	Patient Population	Pre-Oncotype DX Recommendation (HT; CHT)	Post-Oncotype DX Recommendation (HT; CHT)	Percent Change
Lo (US)	89 N0	52%; 47%	67%; 26%	32%
Klang (Israel)	313 N0	44%; 56%	72%; 28%	40%
Rezai (Germany)	366 N0/N+	42%; 57%	54%; 46%	33%
Albanell (Spain)	107 N0	64%; 36%	73%; 27%	32%
Holt (UK)	142 N0/N+(mic)	56%; 44%	70; 30%	33%
Yamauchi (Japan)	90 N0/N+	41%; 59%	74%; 26%	38%
de Boer (Australia)	151 N0/N+	56%; 44%	64%; 36%	24%
Hornberger (Meta-Analysis)	1154 N0/N+	42%; 58%	66%; 39%	35%

Table 2 Clinical Changes in Treatment Recommendations Based on Oncotype Dx Results

Mammaprint 70 Gene Assay

The 70 gene profile was developed by performing gene subtraction analysis, comparing untreated BC patients who developed metastases within 5 years with those who did not (16). From the entire 25,000 genes in the human genome, significant differences in approximately 5,000 genes (mainly involved in tumor growth, differentiation, angiogenesis, local invasion and extravasation) were detected between the two groups, resulting in two main gene profiles – one consistent with the cancer-free cohort of patients, and the other with a metastatic phenotype. Based on a ranking of correlation coefficients of these genes with BC prognosis, an optimal group of 70 genes was arrived at, thus the 70 gene tumor profile (17).

The assay identified two distinct prognostic groups: those having a good-prognosis signature (~85% 10-year probability of remaining free of distant metastases), and those having a poor-prognosis signature (~51% 10-year probability of remaining free of distant metastases). HR for distant recurrence in the high risk vs. the low risk group was 5.1 (95% CI 2.9-9.0; p<.001), and remained significant also when the groups were analyzed according to LN status.

Across other classification systems such as the NIH Consensus criteria, and St. Gallen classification for tumor behavior, the 70 gene profile was found to be much more accurate in predicting actual tumor behavior (18,19).

An analysis of the Mammaprint assay's ability to predict responsiveness to CT was recently performed by retrospectively reviewing patients with primary BC who had received adjuvant endocrine therapy with or without CT. From the entire group of patients, 47% were classified as low risk and 53% as high risk (20). In the low risk group, BC specific survival was 97% in the hormone alone treated group, and 99% in the hormone plus chemotherapy treated group (p=0.62). In the high risk group BC specific survival was 81% and 94%, respectively (p<.01). Multivariate analysis also revealed a statistically significant benefit for the addition of chemotherapy in the high risk group of patients (p=.02) (20).

In addition to providing standard prognostic information, Mammaprint has been shown the ability to ferret out high risk patients in otherwise "low-risk" patients, and vice-versa: a small subset of luminal type tumors were found to have a basal phenotype resulting in a high risk Mammaprint score. When analyzed further, it was discovered that this group of patients correlated with a subset of patients with the ER delta7 splice variant, in which the cell is not responsive to estrogen stimulation, which would explain resistance to hormonal therapy (21). Conversely, a subset of patients with Her2neu positive BC, were found to be at a low risk by the Mammaprint assay, and thus appear to benefit little from treatment with CT and anti-Her2 therapy. **(Figure 3)**

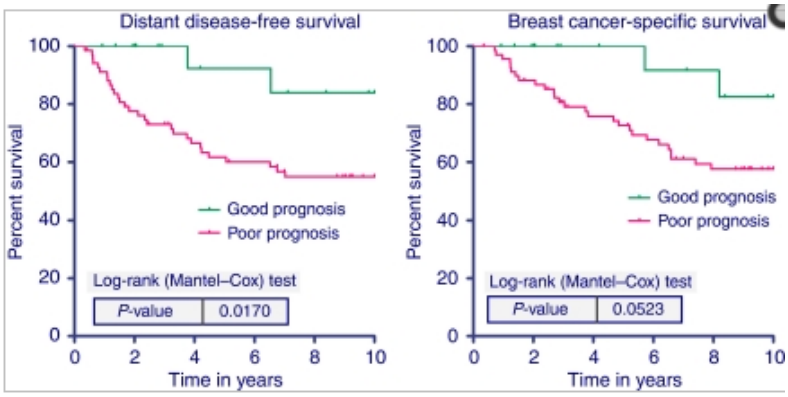

Figure 3 Good Prognosis Her2 Patient based on Mammaprint Risk Profile

The RASTER study was a prospective observational study of patients with T1-3N0 breast cancer (n=427), where the Mammaprint result was determined, and was used by the treating oncologist along with other standard clinical features, including Adjuvant Online, to determine adjuvant therapy. Of those, 124 were determined to be low risk by Mammaprint but high risk by other clinical features such as age, tumor size, grade, and hormone receptor status. Of these, only 24% received adjuvant chemotherapy while 76% did not. Nonetheless, the 5 year disease free survival was 98%. This suggests that patients with a low risk Mammaprint profile (despite a high risk clinical profile) can be safely treated without adjuvant CT (22).

PAM50 assay

The PAM50 assay was developed using microarray analysis of untreated BC specimens, and hierarchical clustering analysis to describe the 4 main subsets of the disease: luminal A, luminal B, Her2 type

and basal type. These data sets were validated to provide significant prognostic differences in a set of 761 untreated patients. The test generates a risk of recurrence score (ROR) that may also be correlated with tumor size (ROR-C). In the cohort of 626 ER-positive patients, only 76% were classified by the PAM50 assay as either luminal A or B, and 11% of ER-negative tumors were classified as luminal. When divided into three subsets based on the ROR score, only the luminal A patients were in the low risk group. **(Figure 4)** As a continuous score, the 5-year risk of relapse rose steadily with a rising ROR-C score (23).

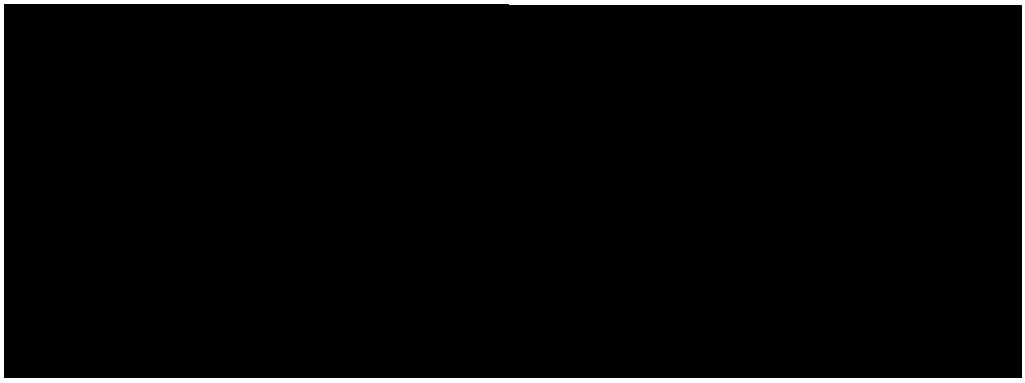

Figure 4 PAM 50 Phenotypes and Prognosis vs. PAM 50 Score

The predictive capabilities of the PAM50 were compared to the Oncotype Dx RS result, in patients from the TransATAC study (Tamoxifen vs. Anastrozole) (24). Significantly more patients were categorized as high risk than as intermediate risk by the PAM50, as compared to the RS, with a similar poor outcome in this group as the high risk patients by RS. Conversely, the patients in the intermediate ROR group had a somewhat better 10-year distant recurrence free outcome than those in the intermediate risk RS group. In a separate analysis looking at the ability of these same molecular profiles to predict 10 year recurrence rates, the PAM50 ROR maintained the strongest statistically significant predictive ability (25).

Prospective Studies: Since most of the data for which molecular profiling has been studied is retrospective, two major prospective studies were initiated in recent years to predict benefit from the addition of adjuvant CT in patients with early BC: the TAILORx study (>11,000 patients) which is based on the Oncotype Dx RS (ER positive, node negative), and the MINDACT study (6,600 patients) which is based on the use of the Mammaprint score (0-3 LN involved).

In the TAILORx study patients with RS up to 10 receive hormonal therapy, those with RS from 11-25 were randomized to receive either CT or hormonal therapy, and those with a RS above 25 receive CT in addition to hormone therapy. In MINDACT, patients were categorized by both clinical assessments using ADJUVANT ONLINE! and Mammaprint RS: those with both high risk features receive CT, those with both low risk assessments receive endocrine therapy alone, and those with discordance between the clinical and molecular assessments were randomized between CT and hormonal therapy. An additional study, the RxPonder study is looking at node positive patients (1-3) and randomizing patients with RS </= 25 between treatment with CT plus hormonal therapy or hormonal therapy alone.

The results of these studies (anticipated in 2015) should provide further evidence to support the use of molecular phenotype to guide our choice of adjuvant therapy in early breast cancer patients.

Current Guidelines and Molecular Profiles

The NCCN guidelines (v3.2013) mention the Oncotype Dx assay as an option for patients with small ER positive tumors (0.6-1.0 cm) with unfavorable features, or for LN negative hormone receptor positive tumors>1cm in conjunction with other clinical parameters, to assist in predicting prognosis and potential benefit from CT. The Mammaprint assay is also mentioned as an FDA approved test for determining risk of recurrence, but not for predicting benefit from chemotherapy, with the caveat that studies of this assay are on small numbers of patients and retrospective in nature.

The ESMO Guidelines (26) recommend the use of both the Oncotype Dx assay and the Mammaprint assay as an option in providing additional prognostic and/or predictive information regarding response to CT in patients with hormone positive early BC. However, their true clinical benefit awaits results of the above mentioned prospective trials.

Task Force statements from the IMPAKT Breast Cancer conference in December 2012 (27) concluded that both the Oncotype Dx assay and Mammaprint assay provide convincing results to determine a patient's

prognosis, but that neither were convincing as a reliable guide to determine treatment decision (chemotherapy or not).

At the St. Gallen conference in March 2013, a slim majority of panelists recommended use of a multi-gene assay in node negative, hormone receptor positive and Her-2 negative patients. The majority considered that only the Oncotye Dx assay was predictive of CT responsiveness while a minority would also endorse the PAM50 or Mammaprint for this purpose. The final recommendation therefore based on the majority vote was to recommend only the Oncotype Dx for the purpose of deciding which patients could forego CT.

Conclusions

Our ability to provide more precise prognostic information to patients based on molecular analysis of their tumors has come a long way, in association with other standard prognostic information (e.g. tumor size, lymph node status). According to one analysis, most of this added information may be largely represented by the Ki-67 expression (in association with ER, PR, and Her2 expression), and this needs to be explored in further depth. In the area of predicting response to CT, highly proliferative tumors and those with high risk genetic profiles seem to derive most of the benefit from CT, whereas those with low-risk profiles (correlated with luminal A tumors, for the most part), do not appear to benefit from the addition of systemic CT.

Prospective studies should enlighten us further. Yet, there will still remain unanswered questions, such as choosing the most appropriate cut-off values for use in the Oncotype Dx assay, and the balance between the molecular profile and pathological features like the number of the LNs involved, in guiding treatment decisions.

References

1. Zoon CK, Starker EQ, Wilson AM, Emmert-Buck MR, Libutti SK, Tangrea MA (2009) Current molecular diagnostics of breast cancer and the potential incorporation of microRNA. *Expert.Rev.Mol.Diagn.* **9**: 455-67.
2. Pusztai L, Cristofanilli M, Paik S (2007) New generation of molecular prognostic and predictive tests for breast cancer. *Semin.Oncol.* **34**: S10-S16.
3. Anonymous. NCCN Guidelines(R) Updates. J.Natl.Compr.Canc.Netw. 11[9], xxxii-xxxvi. 2013. Ref Type: Electronic Citation
4. Tuma RS (2004) A big trial for a new technology: TransBIG Project takes microarrays into clinical trials. *J.Natl.Cancer Inst.* **96**: 648-9.
5. Swain SM (2006) A step in the right direction. *J.Clin.Oncol.* **24**: 3717-8.
6. Reis-Filho JS, Westbury C, Pierga JY (2006) The impact of expression profiling on prognostic and predictive testing in breast cancer. *J.Clin.Pathol.* **59**: 225-31.
7. Zelnak AB, O'Regan RM (2013) Genomic subtypes in choosing adjuvant therapy for breast cancer. *Oncology (Williston.Park)* **27**: 204-10.
8. Cuzick J, Dowsett M, Pineda S *et al.* (2011) Prognostic value of a combined estrogen receptor, progesterone receptor, Ki-67, and human epidermal growth factor receptor 2 immunohistochemical score and comparison with the Genomic Health recurrence score in early breast cancer. *J.Clin.Oncol.* **29**: 4273-8.
9. Paik S, Shak S, Tang G *et al.* (2004) A multigene assay to predict recurrence of tamoxifen-treated, node-negative breast cancer. *N.Engl.J.Med.* **351**: 2817-26.
10. Markopoulos C (2013) Overview of the use of Oncotype DX((R)) as an additional treatment decision tool in early breast cancer. *Expert.Rev.Anticancer Ther.* **13**: 179-94.
11. Lyman GH, Cosler LE, Kuderer NM, Hornberger J (2007) Impact of a 21-gene RT-PCR assay on treatment decisions in early-stage breast cancer: an economic analysis based on prognostic and predictive validation studies. *Cancer* **109**: 1011-8.
12. Sparano JA, Paik S (2008) Development of the 21-gene assay and its application in clinical practice and clinical trials. *J.Clin.Oncol.* **26**: 721-8.
13. Mamounas EP, Tang G, Fisher B *et al.* (2010) Association between the 21-gene recurrence score assay and risk of locoregional recurrence in node-negative, estrogen receptor-positive breast cancer: results from NSABP B-14 and NSABP B-20. *J.Clin.Oncol.* **28**: 1677-83.
14. Badve SS, Baehner FL, Gray RP *et al.* (2008) Estrogen- and progesterone-receptor status in ECOG 2197: comparison of immunohistochemistry by local and central laboratories and quantitative reverse transcription polymerase chain reaction by central laboratory. *J.Clin.Oncol.* **26**: 2473-81.

15. Albain KS, Barlow WE, Shak S *et al.* (2010) Prognostic and predictive value of the 21-gene recurrence score assay in postmenopausal women with node-positive, oestrogen-receptor-positive breast cancer on chemotherapy: a retrospective analysis of a randomised trial. *Lancet Oncol.* **11**: 55-65.
16. Mook S, Van't Veer LJ, Rutgers EJ, Piccart-Gebhart MJ, Cardoso F (2007) Individualization of therapy using Mammaprint: from development to the MINDACT Trial. *Cancer Genomics Proteomics.* **4**: 147-55.
17. 't Veer LJ, Dai H, van de Vijver MJ *et al.* (2002) Gene expression profiling predicts clinical outcome of breast cancer. *Nature* **415**: 530-6.
18. van de Vijver MJ, He YD, van't Veer LJ *et al.* (2002) A gene-expression signature as a predictor of survival in breast cancer. *N.Engl.J.Med.* **347**: 1999-2009.
19. Buyse M, Loi S, van't Veer L *et al.* (2006) Validation and clinical utility of a 70-gene prognostic signature for women with node-negative breast cancer. *J.Natl.Cancer Inst.* **98**: 1183-92.
20. Knauer M, Mook S, Rutgers EJ *et al.* (2010) The predictive value of the 70-gene signature for adjuvant chemotherapy in early breast cancer. *Breast Cancer Res.Treat.* **120**: 655-61.
21. Groenendijk FH, Zwart W, Floore A, Akbari S, Bernards R (2013) Estrogen receptor splice variants as a potential source of false-positive estrogen receptor status in breast cancer diagnostics. *Breast Cancer Res.Treat.* **140**: 475-84.
22. Drukker CA, Bueno-de-Mesquita JM, Retel VP *et al.* (2013) A prospective evaluation of a breast cancer prognosis signature in the observational RASTER study. *Int.J.Cancer* **133**: 929-36.
23. Parker JS, Mullins M, Cheang MC *et al.* (2009) Supervised risk predictor of breast cancer based on intrinsic subtypes. *J.Clin.Oncol.* **27**: 1160-7.
24. Dowsett M, Sestak I, Lopez-Knowles E *et al.* (2013) Comparison of PAM50 risk of recurrence score with oncotype DX and IHC4 for predicting risk of distant recurrence after endocrine therapy. *J.Clin.Oncol.* **31**: 2783-90.
25. Sestak I, Dowsett M, Zabaglo L *et al.* (2013) Factors Predicting Late Recurrence for Estrogen Receptor-Positive Breast Cancer. *J.Natl.Cancer Inst.*
26. Senkus E, Kyriakides S, Penault-Llorca F *et al.* (2013) Primary breast cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Ann.Oncol.*
27. Azim HA, Jr., Michiels S, Zagouri F *et al.* (2013) Utility of prognostic genomic tests in breast cancer practice: The IMPAKT 2012 Working Group Consensus Statement. *Ann.Oncol.* **24**: 647-54.

Combination of markers in early detection of prostate cancer

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Abstract

Objective: Establish the combination of totalPSA, %freePSA and [-2]proPSA biomarkers and calculation of PHI in the diagnostic algorithm of early prostate cancer.

Material and Methods: We examined the serum from 76 suspected prostate cancer patients. All these patients had undergone a TRUS biopsy. We performed an assessment of total PSA and, if the interval of tPSA was between 0-30 ug/l, we also assessed the levels of freePSA and [-2]proPSA and calculated the free PSA percentage (%freePSA) and Prostate Health Index (PHI). The monitored biomarkers were measured using chemiluminescent technology on a DxI 800 (Beckman Coulter, USA). All statistical analyses were calculated using SAS version 9.2.

Results: We found a statistically significant increase in levels of [-2]proPSA and PHI in patients diagnosed with prostate cancer through prostate biopsy compared to patients with benign prostate hypertrophy ([-2]proPSA median 14 vs. 27 ng/l, PHI median 35 vs. 77). In contrast, we did not find any significant difference in tPSA and %freePSA (median tPSA 7.1 vs. 7.7 ug/l and %freePSA 16 vs.11.4%).

Conclusion: The combination of [-2]proPSA and calculation of PHI with traditional prostate cancer markers appear to be of great benefit for a more accurate differential diagnosis between benign hyperplasia and prostate cancer.

Keywords: prostate cancer, benign prostate hypertrophy, tPSA, fPSA , [-2]proPSA, PHI, prostate health index.

Introduction

Currently there are two reasons why nationwide screening for prostate cancer using PSA has virtually ceased. The first reason is that in 2011, the Congress of the American Cancer Society (ASCO 2011) stated that using PSA and free PSA cannot estimate the aggressiveness of the tumor process. This has led to the unnecessary initiation of treatment, even in cases where treatment is not feasible (overtherapy). The second reason is the low specificity of PSA. There is improvement in combination with %freePSA (most commonly used in the Czech Republic) or PSA velocity-density (used frequently abroad) or the use of age-adjusted reference values (1, 2). The low specificity of PSA leads to an excessive number of biopsies of the prostate – i.e., to "over diagnosis" (3). We are looking for new markers that can provide a more accurate differential diagnosis (4). In our pilot study, we sought to determine whether the determination of [-2] proPSA (isoforms of free PSA) is able to help solve the problem of "over diagnosis".

Material and Methods

From November 2010 to April 2012 we examined the serum from 76 urology patients with suspected prostate cancer and who had undergone a TRUS biopsy. Blood was drawn from the cubital vein into Vacuette® blood tubes (Greiner Bio-One, Austria). Serum was separated through centrifugation at 1700 × g for 10 minutes and the samples were processed either immediately or, if they could not be used within three hours, were held at -20°C. The total PSA (tPSA) of all patients was examined and if it was in the range of 0-30 ug/L, we also examined freePSA (fPSA) and [-2] proPSA. In all cases we recorded the results of a digital rectal examination, ultrasound and prostate volume, prostate biopsy and medication before and at the time of the blood sample, as in similar international literature (5, 6). All of the above markers were determined using DxI 800 chemiluminescent

technology (Beckman Coulter, USA) and we calculated %freePSA using the formula (freePSA / tPSA) x 100, and the index PHI = ([-2] proPSA / freePSA) x √ tPSA .

Patients were divided into two groups according to the results of the prostate biopsy, 50 patients with benign prostate hypertrophy (negative) and 26 patients with prostate cancer (positive). The mean age was 68 years (49-82 years). The statistical software was SAS version 9.2. A descriptive analysis was performed and because of the unequal number of patients in both groups, instead of the standard t-test method, generalized linear models (GLM) are used to compare the two groups of test statistics (F). The values of the test statistics F and the p-values are provided in the box and whisker plots (graphs referred to as Prob > F) .

Results

The box plots of markers for the benign and the malignant group – tPSA (Figure 1) [-2], proPSA (Figure 2), % freePSA (Figure 3) and PHI (Figure 4):

Figure 1: Box plot for tPSA (ug / l) in the benign and the malignant group

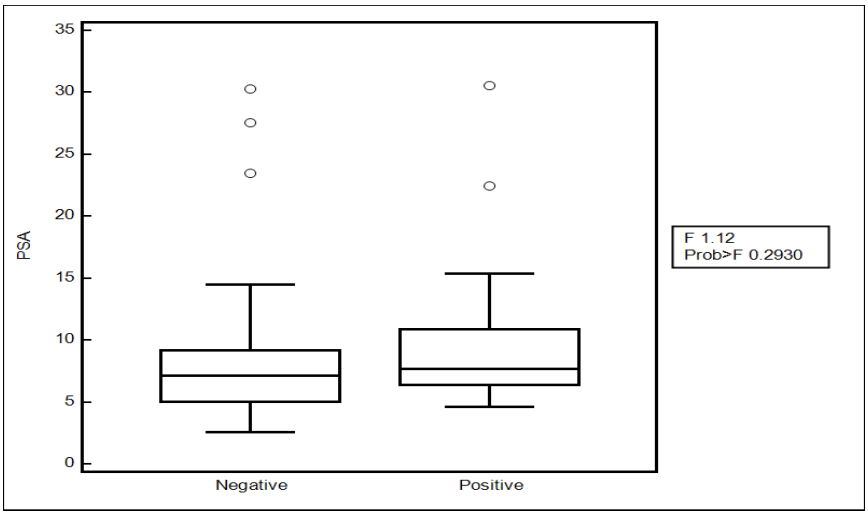


Figure 2: Box plot for [-2]proPSA (ng/l) in the benign and the malignant group

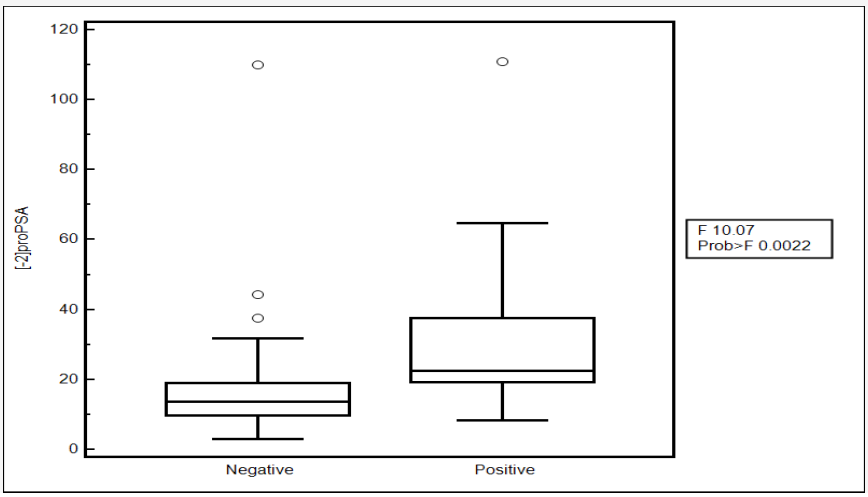


Figure 3: Box plot for %freePSA (%) in the benign and the malignant group

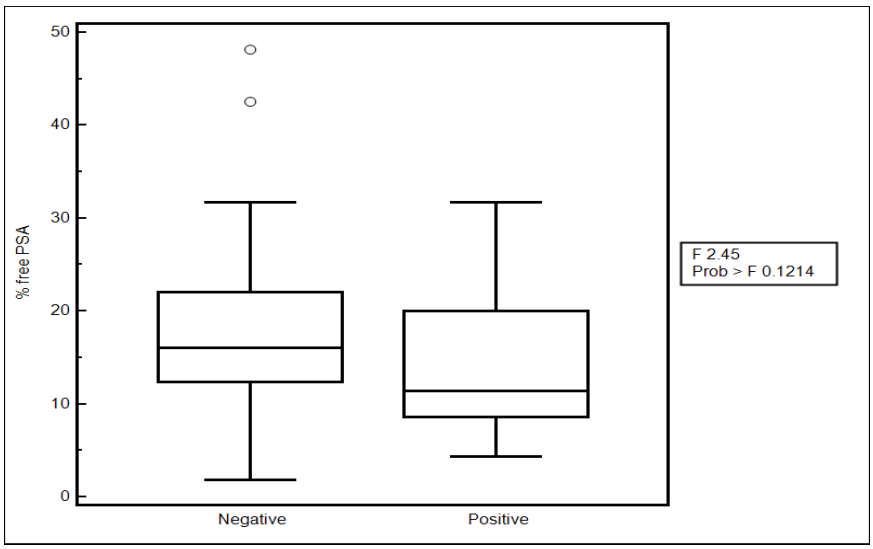
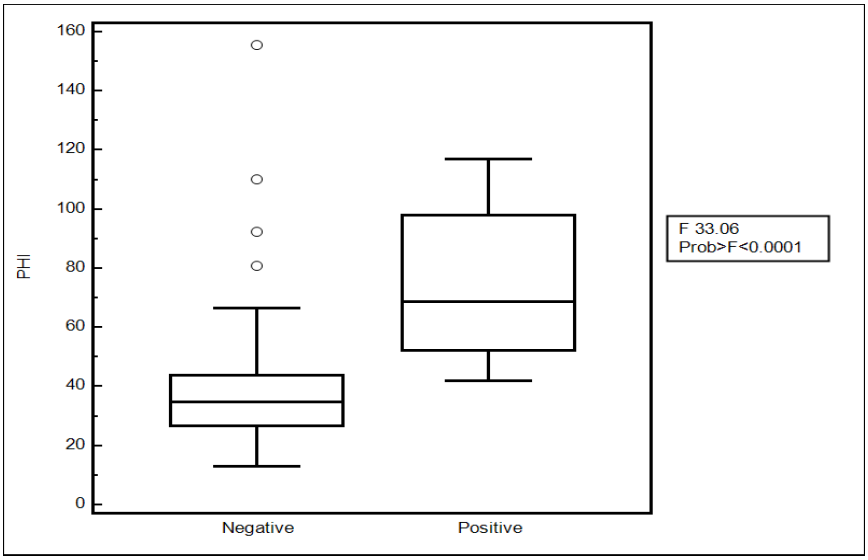


Figure 4: Box plot for PHI in the benign and the malignant group



The graphs show that we have found significant differences in levels of isoenzyme [-2] ProPSA and index PHI between the groups of benign (negative) lesions and malignant (positive) lesions ([-2] ProPSA median 14 vs. 27 ng / l, p> 0.0022, PHI median 35 vs. 77 p> 0.0001), while there were not significant differences between serum tPSA and %freePSA (tPSA median 7.1 vs. 7.7 ug / l and % freePSA 16 vs. 11.4%).

Discussion

International literature includes patients with a total PSA of 4 to 10 ug / l, which is considered to border (gray zone) tPSA values (7). But we speculate whether it would be possible to prevent repeated negative biopsies in patients with tPSA even above 10 ug / L. Based on the experience of common urological practice that patients with tPSA between 10 and 30 ug / L include patients with repeated negative biopsies, we decided to perform assay [-2] props in all patients with a total PSA to 30 ug / L. The group of benign lesions had 9 patients of 50 with a tPSA of over 10 ug / L and even three patients above 20 ug / L.

We found insignificant differences in the levels of total PSA and %fPSA between the benign and malignant group, which supports clinical experience that these markers are not sufficient due to its low specificity for differential diagnosis between BPH and prostate cancer. We therefore conducted a study of our patients according to standard parameters investigated (tPSA and %freePSA) versus PHI and evaluated whether [-2] proPSA can prevent unnecessary biopsies. We used freePSA% of 20% (over 20 = benign lesions) and PHI of 40 (above 40 = malignant lesions) as cut-off values.

Table 1: Analysis of biopsy results according to positivity of markers.

Levels of tPSA	Number of biopsies	Number of negative biopsies in the group of pathologic %freePSA	Number of negative biopsies in the group of pathologic PHI
2-4 ug/l	3	2	1
4-10 ug/l	56	26	8
10-30 ug/l	17	6	5
Total	76	34	14

Table 1 shows that a total of 34 biopsies (i.e., 45%) of the group indicated under pathological %fPSA values were negative, while negative biopsies with pathological PHI only totaled 14 (i.e., 18%). The fact that the generally indicated number of false negative first biopsies is around 25% should also be considered (8).

Table 2: Summary of number of patients with positive biopsies with marker negativity (%fPSA and PHI)

Levels of tPSA	Number of biopsies	Number of missed CaP in the group of negative %freePSA	Number of missed CaP in the group of negative PHI
2-4 ug/l	3	0	0
4-10 ug/l	56	4	0
10-30 ug/l	17	3	0
Total	76	7	0

If urologists decide to conduct biopsies on the basis of standard marker combination (tPSA and % free PSA), then there is a total of seven carcinomas (Table 2) in the %freePSA reference interval (20-32%), and conversely all diagnosed cancers had a PHI value over 40.

Compared with the literature, where the authors state more accurate biopsy indications by calculating %freePSA (9, 10), then in our group this calculation is very limited. The strong statistical significance of the absolute values of the PHI index itself and [-2] proPSA confirm that these markers could significantly help urologists in decisions to perform biopsies or rebiopsies. This was proven when considering unnecessary biopsies. Avoiding unnecessary biopsies should certainly have not only a medical and psychological, but undoubtedly also an economic impact. The cost of using these diagnostic markers is CZK 700 (about €28), while the cost of performing a biopsy amounts to about CZK 5,000 (about €200).

Conclusion

Our results suggest that the assessment of [-2] proPSA and calculation of PHI increase the reliability of the differential diagnosis of prostate cancer. At the same time, if we check our pilot experience in the extensive study, it will lead to a reduction in the number of biopsies.

References

[1] Shariat, S. F., et al. Tumor Markers in Prostate Cancer I: Blood-Based Markers. Acta Oncologica (Stockholm, Sweden). 2011, vol. 50 Suppl 1, pp. 61-75.

[2] MAKAROV, D. V., et al. Biomarkers for Prostate Cancer. Annual Review of Medicine. 2009, vol. 60, pp. 139-151.

[3] Heijnsdijk, E. A., et al. Overdetection, Overtreatment and Costs in Prostate-Specific Antigen Screening for Prostate Cancer. British Journal of Cancer. 2009, vol. 101, no. 11, pp. 1833-1838.

[4] Klecka, J. et al. Nádorové markery karcinomu prostaty. Ces Urol 2008, vol. 12, no. 3, pp. 173-185.

[5] Catalona, W. J., et al. A Multicenter Study of [-2]Pro-Prostate Specific Antigen Combined with Prostate Specific Antigen and Free Prostate Specific Antigen for Prostate Cancer Detection in the 2.0 to 10.0 ng/ml Prostate Specific Antigen Range. The Journal of Urology. 2011, vol. 185, no. 5, pp. 1650-1655.

[6] Sokoll, L. J., et al. A Prospective, Multicenter, National Cancer Institute Early Detection Research Network Study of [-2]proPSA: Improving Prostate Cancer Detection and Correlating with Cancer Aggressiveness. Cancer Epidemiology, Biomarkers & Prevention : A Publication of the American Association for Cancer Research, Cosponsored by the American Society of Preventive Oncology. 2010, vol. 19, no. 5, pp. 1193-1200.

[7] Khan, M. A., et al. Evaluation of Proprostate Specific Antigen for Early Detection of Prostate Cancer in Men with a Total Prostate Specific Antigen Range of 4.0 to 10.0 ng/ml. The Journal of Urology. 2003, vol. 170, no. 3, pp. 723-726.

[8] Roehl, K. A.; Antenor, J. A.; Catalona, W. J. Serial Biopsy Results in Prostate Cancer Screening Study. The Journal of Urology. 2002, vol. 167, no. 6, pp. 2435-2439.

[9] Catalona, W. J., et al. Use of the Percentage of Free Prostate-Specific Antigen to Enhance Differentiation of Prostate Cancer from Benign Prostatic Disease: A Prospective Multicenter Clinical Trial. JAMA : The Journal of the American Medical Association. 1998, vol. 279, no. 19, pp. 1542-1547.

[10] Mikolajczyk, S. D., et al. Free Prostate-Specific Antigen in Serum is Becoming More Complex. Urology. 2002, vol. 59, no. 6, pp. 797-802.

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Multiplex Assay and Markers of Metastatic Bone Disease

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Abstract

A multiplex panel for the measurement of serum levels, including osteoprotegerin, osteopontin, osteocalcin, parathormon and leptin, was tested in a group of cancer patients with metastatic disease, and in healthy controls using xMAP technology. Additionaly, routine serum bone markers were assessed in cancer group. Cancer cases were divided into one group with an occurrence of bone metastases and into one group without bone metastases. *Results:*In comparison to controls, both cancer groups were observed to have higher levels of osteoprotegerin and lower levels of osteocalcin. The bone metastatic group had higher levels of PIIINP and ostase than the non-bonemetastatic group. 3 of 4 patients with multiple bone metastases had values above the set normal value for both osteoprotegerin and osteopontin in comparison to all other cancer patients. *Conclusions:* Multiplex bone metastasis detection by serum test in future could help with creating of multiparametric scoring system with sufficient sensitivity and specificity for clinical practise. The panel will be tested on a larger cancer cohort and in patients with non-cancerous diseases.

Keywords: Metastatic disease, multiplex, biologic activity markers, panel.

Introduction

Bone metastases results in a number of complications in patients including bone pain, pathologic fractures, spinal cord compression, and hypercalcemia, all followed by a decrease in the quality of life. [1] The metastatic spread of cancer to the bones is observed in many malignancies but is mostly related to multiple myeloma, breast, kidney, prostate and lung cancer. The development of metastatic disease is a complex process The vicious cycle related to bone metastasis develops when factors secreted by or expressed in tumour cells (eg. Parathyroid hormone- related peptide) activate osteoblasts and osteoclasts in the bone microenviroment to produce cytokines; bone remodeling and osteolysis causes the release of growth factors , which then stimulate tumour cell growth, motility, and a release of parathyroid hormone related peptide. [2, 3] In the past, there were no therapeutic possibilities for bone metastatic disease, nowadays there is not only palliative therapy but also a curative treatment leading to a stabilized status Imaging methods, as a conventional measurement of skeletal health and treatment response in metastatic bone lesions, are imprecise and can only detect changes after the damage has occured. Simple tools are required for rapid and sensitive detection of changes in bones during cancer. The science and clinical utility of biochemical markers of bone metabolism are still evolving; therefore, they are not yet an established surrogate measurement for clinical efficacy. [4,5] The rapidly developing multiplex analytic technology opens the door to the multimarker blood monitoring of multifactorial cancer processes. One such tool can be bead-based Multi-analyte profiling technology (xMAP). [6] Presented study is focused on testing the commercially available multiplex Human Bone Panel for the measurement of the marker serum levels by xMAP technology for a detection of tumour induced bone disease (bone metastases) and on comparison to serum bone markers nowadays routinely used in the monitoring of several bone diseases.

Methods

1.1. Patient cohorts

We studied 24 cancer patients with metastatic disease. They were divided into 2 groups: group 1 – tumour disease with occurrence of bone metastases (13 patients) and group 2 tumour disease with no bone

metastases (11 patients). A control group of 20 healthy blood donors, referred to as group 0, was also studied. Study has been approved by the ethic committee of the Faculty hospital. The peripheral blood was drawn using VACUETTE® Z Serum Sep tubes (Greiner Bio-One, Austria) and allowed to clot. Sera were separated by centrifugation and all specimens were immediately aliquoted and frozen at -80°C.

1.2. **Analysis**

Serum levels of osteoprotegerin (OPG), osteopontin (OPN), osteocalcin (OC), parathormon (PTH) and leptin were measured by multiplex xMAP technology (Luminex 100 instrument) with use of Human Bone Panel A (Millipore Linco corp., USA) in the cancer and healthy groups. The multiplex analysis was run in duplicates. The following routinely used serum bone markers were assessed in the cancer groups: N-terminal propeptide of type I procollagen (PINP), C-terminal telopeptid of type I collagen (ICTP) and N-propeptide of type III procollagen (PIIINP) (all RIA, Orion Diagnostica, Finland), bone-specific alkaline phosphatase (ostase, known as well as bALP) (automated CLI, DxI UniCell 800, Immunotech - Beckman Coulter comp., USA), C-terminal cross-linking telopeptide of type I collagen (CTx) (ECLIA, Elecsys, Roche, USA) and 25-hydroxyvitamin D (vit.D) (RIA, IDS, UK). Osteocalcin levels were also measured for comparison using both routine immunoassay (RIA, CisBio International, France).and multiplex method.

1.3. Results Interpretation

The Wilcoxon test was used to compare marker levels between groups. Significance was set for P values lower than 0.05, P values between 0.1 and 0.05 were considered as border significant. For the purposes of the scoring system, the normal values for multiplex markers were set at 95percentil of the healthy group. For non-multiplex markers used cut off values (see in Table 3) were derived from cut offs valid in the routine practise of our laboratory. Each value above normal levels was given a score of 1 point. Two scoring systems for better discrimination of groups 1 and 2 were created by an empirical choice of analytes. Scoring system I – points for osteoprotegerin, osteopontin, PIIINP, ICTP and ostase were counted; in scoring system II - points for osteoprotegerin, osteopontin, PIIINP, PINP and ostase were counted. Results falling under the calibration curve ranges were stated as the value of the lowest calibration point. For a comparison of osteocalcin analytical methods Passing Bablock analysis (including Cusum test for linearity) were performed, and Spearman's coefficients of rank order correlation between methods was calculated

Results

All results for multiplexed markers are shown in Tab. 1 and markers measured by nonmultiplexed methods in Tab. 2. In comparison to the control group, significantly higher levels of osteoprotegerin and significantly lower levels of osteocalcin in both cancer groups were found. No significant differences were found between cancer groups 1 and 2 for any of the multiplexed markers. As for the non-multiplexed markers, group 1 displayed significantly higher levels of PIIINP and higher levels (with border significance) of ostase compared to group 2. Considering the nonmultiplexed markers significantly higher levels of PIIINP and ostase in group 1 compare to group 2 were observed. Interestingly, 3 of 4 patients with multiple bone metastases had values above the set normal value both for osteoprotegerin and osteopontin in comparison to all other cancer patients, where only one of these markers was positive. For cut off values for markers and percents of samples out of set reference ranges in group 1 and 2 see Tab 3. Osteocalcin serum levels obtained by multiplex measurement correlate with routine immunoassay - Spearman's coefficient: R= 0.710 with P value = 0.001. The regression equation obtained by Passing Bablock method for osteocalcin methods: Y = 0.1153 (95% CI : -1,1537 to 0,9909) + 0.2977 (95% CI : 0,2268 to 0,3962) X with no significant deviation from linearity.

Tab. 1 Medians and kvantils for all groups and differences between groups for multiplexed markers

Group			OPG (pg/mL)	OPN (pg/mL)	OC (pg/mL)	PTH (pg/mL)	Leptin (pg/mL)
0	median		262	679	7838	21	4552
	kvantil	5%	113	244	4080	11	244
		95%	639	32596	13304	46	17645
1	median		631	1978	5329	22	5254
	kvantil	5%	218	244	1017	10	369
		95%	2417	128232	22821	64	22596
2	median		504	1742	5195	24	2780
	kvantil	5%	268	260	1723	10	274
		95%	954	6918	15594	85	7426
Group difference Wilcoxon test							
0x1			P<0.0001	P>0.1	P<0.06	P>0.1	P>0.1
0x2			P<0.002	P>0.1	P<0.02	P>0.1	P>0.1
1x2			P>0.1	P>0.1	P>0.1	P>0.1	P>0.1

Tab. 2 Medians and kvantils for cancer groups and differences between groups for non-multiplexed markers

Group			PIIINP (ug/L)	ICTP (ng/mL)	VitD (nmol/l)	PINP (ug/L)	Ostase (ug/L)	CTx (ng/ml)
1	median		8.5	11.0	41.7	49.0	18.4	0.460
	kvantil	5%	3.5	3.9	17.5	35.1	5.3	0.145
		95%	73.1	75.0	74.8	250.0	121.0	1.033
2	median		4.8	8.2	42.0	47.0	8.9	0.420
	kvantil	5%	2.2	4.6	17.1	30.3	5.4	0.163
		95%	15.4	21.4	147.9	77.7	25.1	0.757
Group difference Wilcoxon test								
1x2			P<0.05	P>0.1	P>0.1	P>0.1	P<0.1	P>0.1

Tab. 3 Cut off values for markers and percents of samples out of set reference ranges in group 1 and 2

Score system	OPG (pg/mL)	OPN (pg/mL)	OC (pg/mL)		PTH (pg/mL)	Leptin (pg/mL)	PIIINP (ug/L)	ICTP (ng/mL)	VitD (nmol/l)	PINP (ug/L)	Ostase (ug/L)	CTx (ng/ml)
Cut off value	>639	>32596	>13304	<4080	>46	>17645	>6.4	>6.0	<23	>22	>100	>0.6
Group 1	46% 6/13	23% 3/13	23% 3/13	38% 3/13	8% 1/13	8% 1/13	54% 7/13	69% 9/13	31% 4/13	38% 5/13	31% 4/13	38% 5/13
Group 2	36% 4/11	0% 0/11	9% 1/11	36% 4/11	18% 2/11	0% 0/11	27% 3/11	64% 7/11	18% 2/11	9% 1/11	0% 0/11	27% 3/11

Discussion and conclusion

Our study is focused on testing the commercially available multiplex Human Bone Panel for the measurement of the serum levels of OPG, osteopontin OPN, osteocalcin OC, parathormon and leptin by xMAP technology. Our aim was to study the possibility of the detection of tumour induced bone disease using serum tests, to setup the reference serum levels for parameters included in the multiplex panel, and to compare serum bone markers nowadays routinely used in the monitoring of several bone diseases.
In accordance with sources of literature, the most promising of the multiplex markers in our study turned out to be osteoprotegerin, differing significantly in comparison to the healthy group but not between cancer groups. OPG is a soluble decoy receptor for RANKL and so prevents RANKL binding to RANK and the subsequent activation of osteoclast activity. OPG also inhibits apoptosis of tumour cells by inhibiting TRAIL (TNF-related apoptosis-inducing ligand) and resulting in improved survival.[7] Furthermore OPG has been found to stimulate angiogenesis, while RANKL is an angiogenic inhibitor. [8]. In comparison to healthy controls, increased serum OPG levels were observed in prostate cancer, bladder carcinoma, colorectal and pancreatic cancer and were reported to be associated with several other organ systems and pathologies e.g. endometriosis, periodontal disease, thyroid disease and coronary heart disease. [7,9-10] Serum OPG levels were found to be higher in patients with metastatic bone disease in prostate cancer compared with patients with organ confined disease. Increases in serum OPG may indicate disease progression/relapse in prostate cancer. [7,11-12] In contrast, when analysing sites of metastasis, Lipton et al. only observed a significant elevation of serum OPG levels in patients with liver and soft tissue metastasis and not in patients with bone or lung metastasis. [9] On the contrary, a decrease in serum levels of OPG compared to controls was reported in patients with multiple myeloma and in sarcoma patients. [7,9,13].

Lower levels of osteocalcin in cancer groups, compared to healthy controls, were found in our study, but no difference was found between the bone metastatic group and nonbone metastatic group. Serum levels were reported to correlate well with osteoblast activity. In advanced untreated metastatic bone disease, serum osteocalcin levels can be low in the presence of high serum BAP levels. The reasons for this metabolic uncoupling between two bone-formation markers are unclear; however, possibilities include the proteolytic cleavage of osteocalcin, changes in gene expression and disturbed osteoid maturation in the presence of active tumor osteopathy. [14-15]. On the contrary OC levels were found to be significantly higher in breast cancer patients with bone metastasis compared to non-metastatic or soft tissue metastasis. [16] In our opinion multiplex measurement in conjunction with multimarker data handling could, in the future, improve discrimination of bone and non-bone metastatic disease. For purposes of illustration, two scoring systems were created. For scoring system I: a score of 3 or higher was positive for 46% patients in group 1 (6/13) in comparison to 9% in group 2 (1/11);for a score of 2 or higher 61,5% patients (8/13) were positive in group 1 in comparison to 36% (4/11) in group 2. For scoring system II: a score of 3 or higher positive 38% of patients in group 1 (5/13) in comparison to 0% in group 2 (0/11); for a score of 2 or higher positive 54% of patients in group 1 (7/13) contrary to 9% (1/11) in group 2. We observed that patients with multiple bone metastases have values above set normal value for both osteoprotegerin and osteopontin. This fact could help in the discrimination of such patients according to serum tests. Inside the bone osteopontin is produced by both osteoblasts and osteoclasts and has several presumedfunctions: the attachment of osteogenic cells to the bone matrix, control of mineralization, coupling of bone formation and resorption. Elevated OPN levels were described in patients with epithelial ovarian , breast, lung and prostate cancer and were found to be associated with a shorter survival and larger numbers of metastatic sites in breast cancer. [17-19]. In our study, OPN levels were higher in cancer groups with a very high 95% kvantil in the bone metastatic group, but the differences did not reach statistical significance.

Leptin plays a role, not only in the regulation of body weight and energy balance, but also in vascular remodeling, regulating neoangiogenesis by itself and in conjunction with VEGF and fibroblast growth factor, acts as a mitogen transforming factor and supresses apoptosis. No significant differences among groups were

observed in our study and reported studies on serum leptin levels in cancer have produced ambiguous results, for prostate cancer, higher leptin levels were associated with more advanvanced tumors [20-21].

PTH, the last member of the multiplex panel, is thought to promote the growth and invasiveness of cancer in bone thanks to the increased expression of the PTH receptor in cancer metastases [22], but did not differ between groups in our study.

With regards to the nonmultiplexed markers, significantly higher levels of PIIINP and ostase were observed in bone meatastatic group in comparison to the non-bone metastatic group. Only these two markers discriminate the bone and non-bone metastatic group. No significant differences between bone metastatic and non-bone metastatic patients in any of bone resorption marker (CTx and ICTP) levels were found. Serum levels of ostase were reported to correlate closely with osteoblast differentiation and activity. In most cases of advanced metastatic bone disease ostase levels in serum are elevated, reflecting either a strong osteoblastic component or, in lytic lesions, active repair. [14]

According to Demers et al. the best markers for assessing the presence of progression of skeletal metastases appear to be the collagen breakdown products of type I bone collagen [23]. In our study we have shown that interest should be focused as well on the propeptide of type III procollagen, which is considered to be a marker of connective tissue metabolism [15] and a marker of liver fibrosis. In our study we have observed elevation of the P3NP in bone-metastatic group in comparison to non-bone metastatic group and OPG in bone metastatic group in comparison to healthy group. Because Lipton et al. observed elevation of serum OPG levels only in patients with liver and soft tissue metastasis and not related to bone disease [9], observations could be influenced by the liver metastatic status of the patients. That is why, in future studies, liver metastases should be precisely considered in larger cohort studies of bone metastatic disease.

The bone metastatic development and spread is a consequence of broken balance among cytokines and other proteins regulating osteoblastic and osteoclastic activity that is why the absolute level of one marker is not so important as a ratios among regulation proteins. The only way how to monitor this net regulation is multiplex analysis which enables to measure all markers under the same conditions. This “simulation” of pathofysiological process cannot be sufficiently performed by measurements of markers one by one but only by a multiplex solution.

Studied cohort of patients is quite small and doubtless investigation on larger cohort is necessary but it seems that would be very useful for oncology to incorporate other bone markers e.g. ostase into the multiplex panel. According to literature we conclude that further incorporating of at least RANKL and TGFbeta [3] in future studies is necessary for complex point of view. The treatment monitoring and tailoring is one of the most desired future function of markers in bone metastatic disease management. For example Lester et al. have evaluated a bone marker directed schedule of treatment with zolendronic acid based on levels of the bone resorption marker, urinary N-telopeptide of type I collagen. [24] Nowadays the necessity of proper patient choice for treatment by novel targeted antibodies drugs is coming up. The trouble is that the bone markers are mainly studies separately, e.g. NTx levels in BISMARK study [4] or in a group of 2 to 3 markers. The assessment of a panel of bone markers should be a part of clinical trials of newly introduced drugs, which have a multicentric manner and precisely defined patient cohorts.

Multiplex bone metastasis detection by serum test in future would have advantages of easiness of venous blood sampling, no radiation exposure of patients in comparison to imaging methods, the possibility of regularly monitoring of the therapy efficacy, the monitoring of whole body bone remodelling not only the imaged area, better cost-benefit ratio than single analytical methods and hopefully it can help with creating of multiparametric scoring system with sufficient sensitivity and specificity for clinical practise.

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References

[1] Selvaggi G, Scagliotti GV: Management of bone metastases in cancer: a review. (2005) Crit Rev Oncol Hematol. 56(3): pp.365-78,

[2] Eccles SA, Welch DR: Metastasis: recent discoveries and novel treatment strategies. (2007) Lancet 369(9574): pp. 1742-57

[3] Virk MS, Lieberman JR: Tumor metastasis to bone. Arthritis Res Ther 9 Suppl 1: S5, 2007;

[4] Coleman R, Brown J, Terpos E, Lipton A, Smith MR, Cook R, Major P Bone markers and their prognostic value in metastatic bone disease: clinical evidence and future directions. (2008) Cancer Treat Rev. 34(7): pp. 629-39
[5] Joerger M, Huober J. Diagnostic and prognostic use of bone turnover markers. (2012) Recent Results Cancer Res. 192: pp.197-223
[6] Kellar KL, Iannone MA: Multiplexed microsphere-based flow cytometric assays. (2002) Experimental Hematology 30(11): pp.1227-37
[7] Holen I, Shipman CM: Role of osteoprotegerin (OPG) in cancer. (2006) Clin Sci . 110(3):279-91
[8] McGonigle JS, Giachelli CM, Scatena M. Osteoprotegerin and RANKLdifferentially regulate angiogenesis and endothelial cell function. (2009) Angiogenesis.;12(1): pp. 35-46
[9] Lipton A, Ali SM, Leitzel K, Chinchilli V, Witters L, Engle L, Holloway D, Bekker P, Dunstan CR: Serum osteoprotegerin levels in healthy controls and cancer patients. (2002) Clin Cancer Res. 8(7): pp. 2306-10
[10] Narita N, Yuasa T, Tsuchiya N, Kumazawa T, Narita S, Inoue T, Ma Z, Saito M, Horikawa Y, Satoh S, Ogawa O, Habuchi T: A genetic polymorphism of the osteoprotegerin gene is associated with an increased risk of advanced prostate cancer. (2008) BMC Cancer.8: pp. 224
[11] Jung K, Lein M, von Hösslin K, Brux B, Schnorr D, Loening SA and Sinha P: Osteoprotegerin in serum as a novel marker of bone metastatic spread in prostate cancer. (2001) Clin Chem. 47(11): pp. 2061-3
[12] Jung K, Lein M, Stephan C, Von Hösslin K, Semjonow A, Sinha P, Loening SA and Schnorr D: Comparison of 10 serum bone turnover markers in prostate carcinoma patients with bone metastatic spread: diagnostic and prognostic implications. (2004) Int J Cancer. 111(5): pp. 783-9,
[13] Terpos E, Szydlo R, Apperley JF, Hatjiharissi E, Politou M, Meletis J, Viniou N, Yataganas X, Goldman JM, Rahemtulla A: Soluble receptor activator of nuclear factor kappaB ligand-osteoprotegerin ratio predicts survival in multiple myeloma: proposal for a novel prognostic index. (2003) Blood 102(3): pp. 1064-9,
[14] Seibel MJ: Clinical use of markers of bone turnover in metastatic bone disease. (2005) Nat Clin Pract Oncol 2(10): pp. 504-17
[15] Fohr B, Dunstan CR, Seibel MJ: Clinical review 165: Markers of bone remodeling in metastatic bone disease. (2003) J Clin Endocrinol Metab 88(11): pp. 5059-75
[16] Salem AM, Zohny SF, Abd El-Wahab MM and Hamdy R: Predictive value of osteocalcin and beta-CrossLaps in metastatic breast cancer. (2007) Clin Biochem 40(16-17): pp. 1201-8,
[17] Kim JH, Skates SJ, Uede T, Wong KK, Schorge JO, Feltmate CM, Berkowitz RS, Cramer DW, Mok SC: Osteopontin as a potential diagnostic biomarker for ovarian cancer. (2002) JAMA 287(13): pp. 1671-9,
[18] Fedarko NS, Jain A, Karadag A, Van Eman MR, Fisher LW: Elevated serum bone sialoprotein and osteopontin in colon, breast, prostate, and lung cancer. (2001) Clin Cancer Res 7(12): pp. 4060-6
[19] Rodrigues LR, Teixeira JA, Schmitt FL, Paulsson M, Lindmark-Mänsson H: The role of osteopontin in tumor progression and metastasis in breast cancer. (2007) Cancer Epidemiol Biomarkers Prev 16(6): pp. 1087-97
[20] Somasundar P, McFadden DW, Hileman SM, Vona-Davis L: Leptin is a growth factor in cancer.(2004) J Surg Res 116(2): pp. 337-49,
[21] Garofalo C, Surmacz E: Leptin and cancer. (2006) J Cell Physiol 207(1): pp. 12-22,
[22] Schwartz GG: Prostate cancer, serum parathyroid hormone, and the progression of skeletal metastases. (2008) Cancer Epidemiol Biomarkers Prev 17(3): pp. 478-83,
[23] Demers LM: Bone markers in the management of patients with skeletal metastases. (2003) Cancer 1;97(3 Suppl): pp. 874-9,
[24] Lester J, Horsman J, Purohit OP, Brown JE and Coleman RE: Bone resorption marker directed therapy for metastatic bone disease. (2006) Bone 38 (3) Suppl.: 79, (Bone Workshops Davos 2006 - Abstracts from the Workshops FRONTIERS OF SKELETAL BIOLOGY and WHAT IS NEW IN BISPHOSPHONATES?)

Integrating MRI Multivariate Markers with Cognitive Neuropsychological Scores for an Optimal Decisional Space in Predicting Alzheimer's Disease

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Summary

This study proposes a statistics-based multidimensional approach to classify Alzheimer's disease (AD) and its prodromal stages using regional measures (cortical volume, cortical thickness and surface area) obtained from MRI scans and a neuropsychological test (MMSE). Normalization effect of different approaches on these measures is also studied and validated on 314 subjects. Results indicate neuropsychological test enhances classification and when combined with selected subcortical volumes yield a high classification accuracy of 92.3% for AD classification, 72.4% for amnestic mild cognitive impairment (aMCI) and 75.1% for non-aMCI, based on 2-fold cross validation using support vector machine (SVM) classifier. Also, normalization approaches and hierarchal models do not enhance performance significantly.

Introduction

Characterized as a neurodegenerative disease that progresses with time, AD is thought to be the cause of the majority of dementia cases (Duchesne et al., 2008). Early and reliable diagnosis of AD and its prodromal stage mild cognitive impairment (MCI) through imaging and volumetric calculations is not only challenging, but remains essential in search of prospective treatments, especially when longitudinal studies become more meaningful in light of this optimal multidimensional decisional space.

MCI is the transitional stage between age-related memory decline and AD, which has two subtypes: non-amnestic and amnestic, of which aMCI is frequently seen as a prodromal stage of AD. The most noticeable problem of aMCI is memory and roughly 10% to 15% of those defined as such convert to AD (Grundman et al., 2004). Non-aMCI population generally displays impairments of cognition, such as impairments in language, visuospatial awareness, and attention. These patients convert to Alzheimer's disease less often than aMCI based on a 30 months follow up study done by Fischer et al., showing that non-aMCI has a conversion rate of 26.8% compared with 48.7% for aMCI subjects (Fischer et al., 2007).

In AD research, multiple modalities of biomarkers identifying AD and MCI have been found to be effective, including structural MRI (Westman et al., 2012, Walhovd et al., 2010, Vemuri et al., 2009), functional imaging modalities like Single-Photon Emission Computed Tomography (SPECT) (Johnson et al., 1998), Positron Emission Tomography (PET) (Walhovd et al., 2010), as well as Central Spinal Fluid (CSF) (Westman et al., 2012, and Vemuri et al., 2009). These biomarkers have been widely used to guide clinicians in delineating AD from cognitively normal controls (CN).

This study aims to deduce the statistically significant MRI measures for AD classification and explore appropriate normalization schemes to enhance the classification performance.

Materials and Methods

A total of 189 subjects with 129 cognitively normal controls (CN), 69 aMCI, 56 non-aMCI and 60 AD patients are included in this study. Table 1 provides the patient demographics. All participants are from the Wien Center for Alzheimer's Disease and Memory Disorders with the Mount Sinai Medical Center, Miami Beach, FL,

USA. All subjects have taken the Folstein Mini-Mental State Examination (Folstein et al., 1983) with a minimum score of 15 out of 30.

Table 1: Patient Demographics

Group	Number of Subjects	Age	Female/ Male	MMSE
CN	129	72.9 ± 6.4	92 / 37	28.7 ± 1.4
AD	60	79.5 ± 6.9	34 / 26	22.6 ± 3.4
Non-aMCI	56	74.1 ± 6.5	36 / 20	26.9 ± 2.3
aMCI	69	75.2 ± 6.8	37 / 32	26.5 ± 2.5

Freesurfer pipeline version 5.1.0 is widely used to generate regional measures from MRI scans (Cuingnet et al., 2011, Ewers et al., 2012, Zhou et al., 2013). The number of dimensions in the classifier is determined by an incremental error analysis, which in turn defines and ranks variables on their statistical significance to be used as input to an SVM-based classification process. Classification of MCI is also conducted to study how well the proposed method can detect early stages of AD.

Results and Discussion

3.1. AD and MCI classification

Proposed approach of multivariate classification based on selected features was performed for all groups of AD, aMCI and non-aMCI separately. Table 2 provides optimal classification accuracies found in the study using the top-ranked statistically significant regions.

Table 2: Classification Accuracy

Classification Accuracy	
aMCI vs. CN	72.4 %
non - aMCI vs. CN	75.1%
AD vs. CN	92.3 %

Figure 1 shows the top two significant regions of the sub-cortex which are deemed the most significant towards the classification of the different sub-groups together with the neurophysiological score (MMSE). This demonstrates the clustering of the subjects within a 3D decisional space comprised of the most significant variables. It is seen that the significant features used in the decisional spaces vary for different classification categories indicating shifts in regional atrophy with progression of AD. The proposed method classifies AD from CN very efficiently as both groups form more compact clusters.

3.2. Normalization effect on AD classification

To study the normalization effect on the combination of different measures for AD classification, single-measure models and hierarchical models with and without normalization are both examined to find the optimal model. Single measure models include one of the regional MRI measures (subcortical volume, cortical thickness and surface area) or MMSE. A hierarchical model combines two or more of the single-measure models to examine if the interaction augments the classification process.

Table 3: Performance of Single and Hierarchical models for AD Classification with and without Normalization

	(Accuracy ,Sensitivity, Specificity)		
Model	**RAW**	**ICV**	**MT/TSA***
MMSE	(88.3, 81.0, 91.6)	-	-
Cortical volume (CV)	(83.1 ,77.9, 85.6)	(83.5, 74.4, 87.7)	-
Cortical thickness (CT)	(77.7, 74.8, 79.0)	(79.0, 78.8, 79.2)	(90.3, 78.4, 79.2)
Surface area (SA)	(71.4, 58.7, 77.2)	(88.3,42.6, 86.1)	(88.6, 61.2, 77.9)
Hierarchical Models			
MMSE + CV	(92.3, 88.2, 94.2)	(91.7, 85.8, 94.5)	-
MMSE + CT	(91.4, 85.3, 94.2)	(91.5, 86.9, 93.6)	(90.3, 90.8, 90.1)
MMSE + SA	(88.6, 76.3, 94.3)	(88.3, 80.9, 91.7)	(88.6, 80.9, 94.2)
CT + CV*	(83.1, 77.9 ,85.6)	(83.1, 75.8, 86.5)	-
SA + CT + CV*	(83.1, 77.9, 85.6)	(83.4, 78.0, 85.9)	-
MMSE + SA + CT + SV**	(92.3, 88.2, 94.2)	(91.7, 86.0, 94.4)	-

* ICV column represents where all measures are normalized with ICV
* MT/TSA column represents where cortical thickness is normalized by mean cortical thickness and surface area is normalized by total surface area

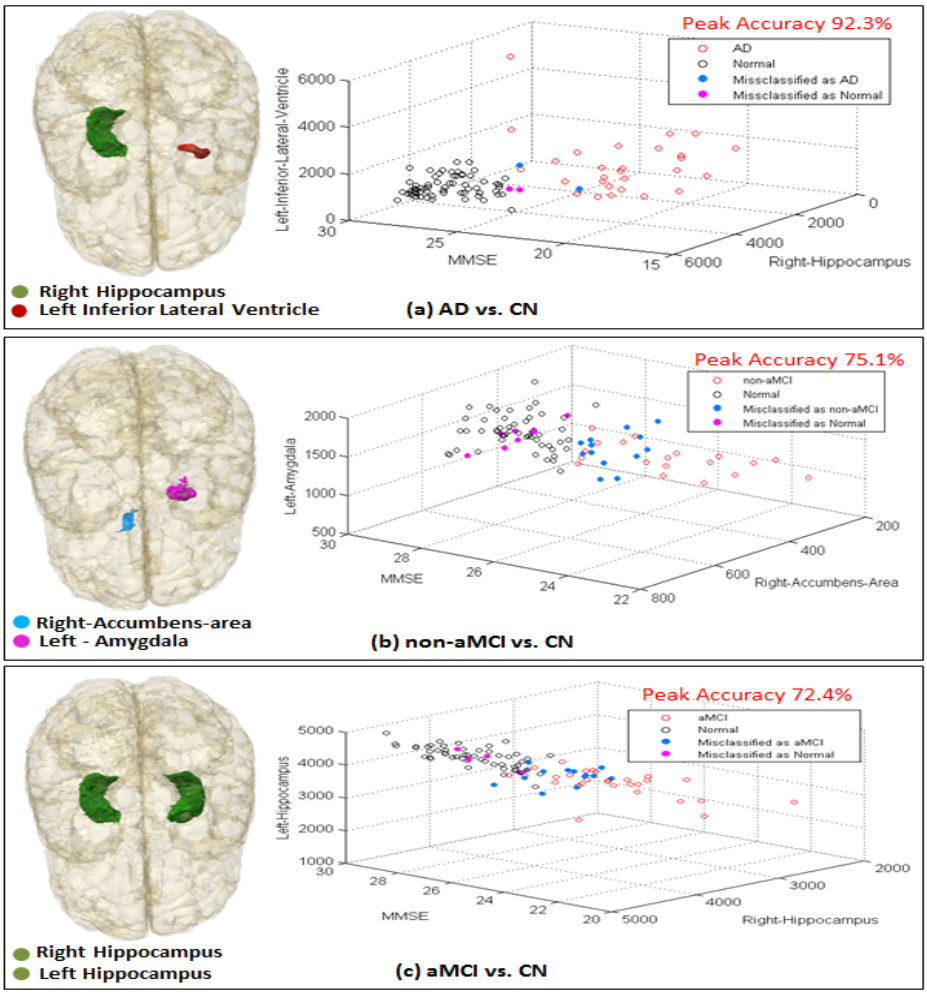

Figure 1: Top significant variables for classification of (a) AD and CN, (b) non-aMCI and CN, (c) aMCI and CN in an optimal 3D decisional feature space

The best classification (92.3 %) is obtained using hierarchal models which include sub-cortical volumes with MMSE, which is seen to have considerable effect on the classification performance with an average improvement of greater than 10%. Furthermore, the results show that cortical thickness should be normalized by either the mean thickness of all the regions or ICV, while subcortical volumes should not be normalized by ICV. However normalization doesn't enhance classification significantly if any. For some cases it even has a negative

impact. This could be explained by that current normalization approaches do not efficiently remove nuisance factors while preserving the atrophy patterns among the sub-classes.

Acknowledgment

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References

CUINGNET, R., et al. 2011. Automatic classification of patients with Alzheimer's disease from structural MRI: A comparison of ten methods using the ADNI database. *Neuroimage,* 56**,** 766-781.

DUCHESNE, S., et al. 2008. MRI-based automated computer classification of probable AD versus normal controls. *Ieee Transactions on Medical Imaging,* 27**,** 509-520.

EWERS, M., et al. 2012. Prediction of conversion from mild cognitive impairment to Alzheimer's disease dementia based upon biomarkers and neuropsychological test performance. *Neurobiology of Aging,* 33**,** 1203

FISCHER, P., et al. 2007. Conversion from subtypes of mild cognitive impairment to Alzheimer dementia. *Neurology,* 68**,** 288-291.

FOLSTEIN, M. F., et al. 1983. The Mini-Mental State Examination. *Arch Gen Psychiatry,* 40**,** 812.

GRUNDMAN, M., et al. 2004. Mild cognitive impairment can be distinguished from Alzheimer disease and normal aging for clinical trials. *Archives of Neurology,* 61**,** 59-66.

JOHNSON, K. A., et al. 1998. Preclinical prediction of Alzheimer's disease using SPECT. *Neurology,* 50**,** 1563-1571.

VEMURI, P., et al. 2009. MRI and CSF biomarkers in normal, MCI, and AD subjects: predicting future clinical change. *Neurology,* 73**,** 294-301.

WALHOVD, K. B., et al. 2010. Combining MR imaging, positron-emission tomography, and CSF biomarkers in the diagnosis and prognosis of Alzheimer disease. *AJNR Am J Neuroradiol,* 31**,** 347-54.

WESTMAN, E., et al. 2012. Combining MRI and CSF measures for classification of Alzheimer's disease and prediction of mild cognitive impairment conversion. *Neuroimage,* 62**,** 229-238.

ZHOU, Q., et al. M. 2013. Combining Anatomical Biomarkers With Neuropsychological Data For Multidimensional Classification Of Alzheimer's Disease", The 17th International Conference on Image Processing, Computer Vision, & Pattern Recognition, IPCV-2013, Vol. 1, pp. 255-261, Las Vegas, USA.

The Parameters of Erythrocytes in the Diagnosis of different Pathogenic Variants of Stroke

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Summary

Total of 292 men (45.7±5.2 years old) with stroke, mainly in the subacute and residual periods. Erythrocyte parameters were studied by dielectrophoresis, chromatography, ^{31}P NMR spectroscopy. We revealed different hemorheological variants of stroke: 193 patients had "hard" erythrocytes on the background of metabolic syndrome (the 1st group) and 99 patients had "fragile" cells on the background of connective tissue dysplasia, viral infections without traditional risk factors (the 2nd group). Differences in erythrocyte electrical, viscoelastic parameters correlated with the levels of intracellular macroergic compounds and composition of erythrocyte membranes. We used a variety of therapeutic approaches in these groups and had a positive effect as the descrease of ischemic attacks. Identification of the various pathogenetic variants of stroke can develop new approaches to therapy and prevention of cerebrovascular disease.

Introduction

Evaluation of different pathogenetic types of stroke remains to be one of the key issues in verification of the diagnosis in patients with cerebrovascular pathology as this evaluation predetermines prognosis and administration of adequate therapy. The methods applied in evaluation of formed elements of blood are known to have some advantages that are significant for studies of biological objects. First of all, their high sensitivity rate when measuring low concentrations of bioorganic compounds in solutions or when applying these solutions to hard substrates is very crucial. Second of all, they do not destroy biological objects, numerous of which being of a complicated structure. Third of all, these methods enjoy high operational efficiency of measurements which do not require any special conditions.

The aim of the work was to investigate the electrical and viscoelastic characteristics of erythrocytes (Er) in comparison with the structural changes of their membranes, levels of macroergic compounds to patients with stroke to identify different pathogenetic variants of the disease.

Materials and Methods.

Total 292 males (45.7±5.2 years old) with re-bare ischemic stroke (238 patients) and hemorrhagic (54 patients) who gave their written consent for involvement in the clinical trials were included into the study. 74 patients were examined in the dynamics of the therapy. Control comprised 35 men aged 35-60 years old in whom biochemical and instrumental studies revealed no clinically manifested chronical diseases of inner organs and whose biochemical and hematological blood findings were normal.

The patients with verified diagnosis and males of control were examined their electric as well as viscoelastic parameters of erythrocytes by method of dielectrophoresis (DEPh) in nonhomogeneous alternating electric field (UAEF) with the help of an automated specialized plant applying an electro-optical system of cells detection [1]. The following parameters were evaluated: conductivity of membranes, indices of aggregation and destruction of erythrocytes, cellular membrane capacity, velocity of erythrocyte motion to electrodes, amplitude of deformation of erythrocytes, polarizability of cells, summarized rigidity and viscosity, values of induced dipole moment and charge. The *CELLFIND* original software package was applied in computer processing of the data obtained.

The structural changes of erythrocyte membranes, the level of macroergic compounds were studied by the methods of chromatography, ^{31}P NMR spectroscopy (NMR spectrometer DRX 500 company Bruker (Germany) [2]. The typical ^{31}P NMR spectrum of erythrocyte suspensions is shown in Figure 1.

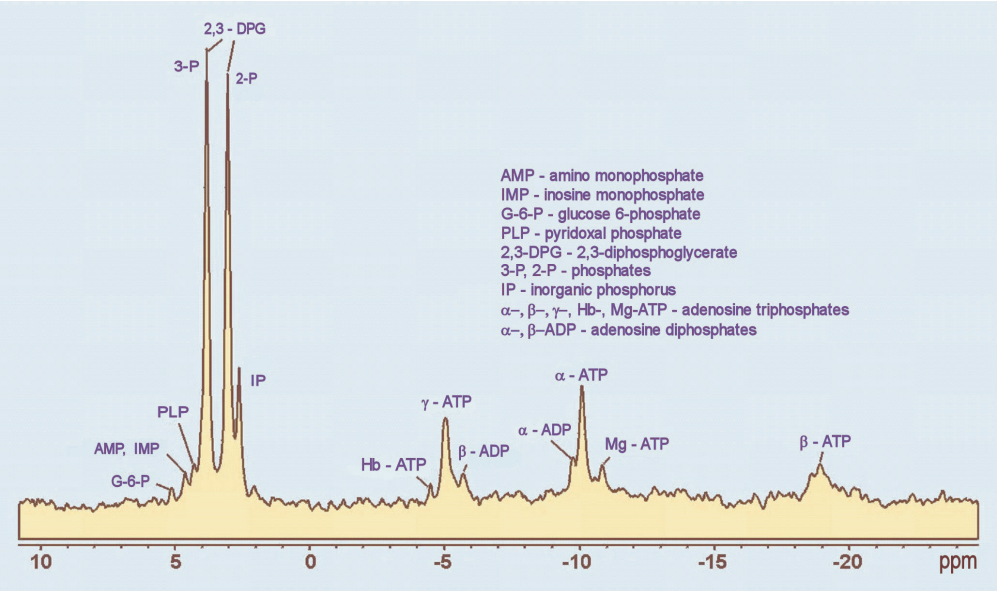

Figure 1. Typical ^{31}P NMR spectrum of erythrocyte suspensions.

Results

Evaluation of parameters of erythrocytes by means of electrooptic systems revealed that at high frequencies ($5 \cdot 10^5$ Hz and 10^6 Hz) erythrocytes of healthy subjects were moving to the closest electrode with high forward velocity (positive dielectrophoresis) and displayed a clearly marked ability to deform under the influence of non-uniform alternating electric field (NUAEF). No deformation of cells was registered at low frequencies ($5 \cdot 10^4$ Hz, 10^5 Hz). Erythrocytes would push off the electrodes (negative dielectrophoresis), some single cells being destroyed by the field (Figure 2a).

Interaction of erythrocytes with NUAEF in patients with stroke tended to differ greatly, i.e. at high frequencies the amplitude of deformation (at the background of summarized indices of viscosity and rigidity), the value of dipole moment, velocity of the forward motion of cells against the electrodes tended to be significantly lower as compared to control ($p<0.001$-0.03).

Two groups of patients with differences in the red blood cells were revealed.

The patients with the manifestations of the metabolic syndrome (the 1st group -193 people with ischemic stroke) had marked disturbances of the erythrocyte deformability (Figure 2b), high electrical conductivity, leading to the development of microcirculatory disorders [3] and tissue hypoxia with expressed deficit of intracellular macroergs. These patients noted the presence of manifestations of metabolic syndrome (hypertension, obesity, hyperlipidemia, predominantly type IIB ($p<0,05$).

The intensities of ^{31}P NMR peaks, reflecting the signals of 2,3-diphosphoglycerate, inorganic phosphate, the summarized rigidity and viscosity, the relative polarizability were significantly above, and, in contrast, the average cell diameter, capacity of cell membranes, velocity of erythrocyte motion to electrodes, the level of induced dipole moment, the macroergastic resonance intensities□(□-,□-□□-,Mg-ATP, □- и □-ADP) were lower than those in the controls and the 2nd group without evidence of metabolic syndrome ($p<0,0001$-$0,05$) (Figure 3).

We observed high levels of cholesterol fraction, the index of cholesterol/phospholipids (PHL) and low levels of total lipid fraction, easily oxidable PHL, omega-3 index in erythrocyte membranes in 1st group than those in the patients without the metabolic syndrome ($p < 0,0001$ - $0,03$). There were correlations between the level of glycosylated hemoglobin and summarized rigidity ($r=+0.72$, $p <0.01$), the erythrocyte plasticity ($r=-0.69$, $p <0.03$), between microalbuminuria and the velocity of erythrocyte motion to the electrodes ($r=-0.71$, $p<0.03$), the dipole moment ($r=-0.69$, $p<0.01$), between the amplitude of erythrocyte deformation and HOMA-IR index ($r=-0.64$, $p<0.02$).

Patients without traditional risk factors (the 2nd group - 45 people with ischemic stroke and with 54 hemorrhagic one) had erythrocytes with moderately reduced plasticity, energy shortages (the levels of □□-,□-□□-,Mg-ATP), but pronounced decrease of polarizability at all frequencies, the susceptibility to cell destruction, aggregation ($p<0,001$-$0,02$) (Figure 2 c, d). Intravascular thrombus formation is likely to play a major role in the pathogenesis of stroke in these patients. Most of these patients showed signs of dysplasia of connective tissue, markers of viral infections, liver function abnormalities ($p<0,05$).

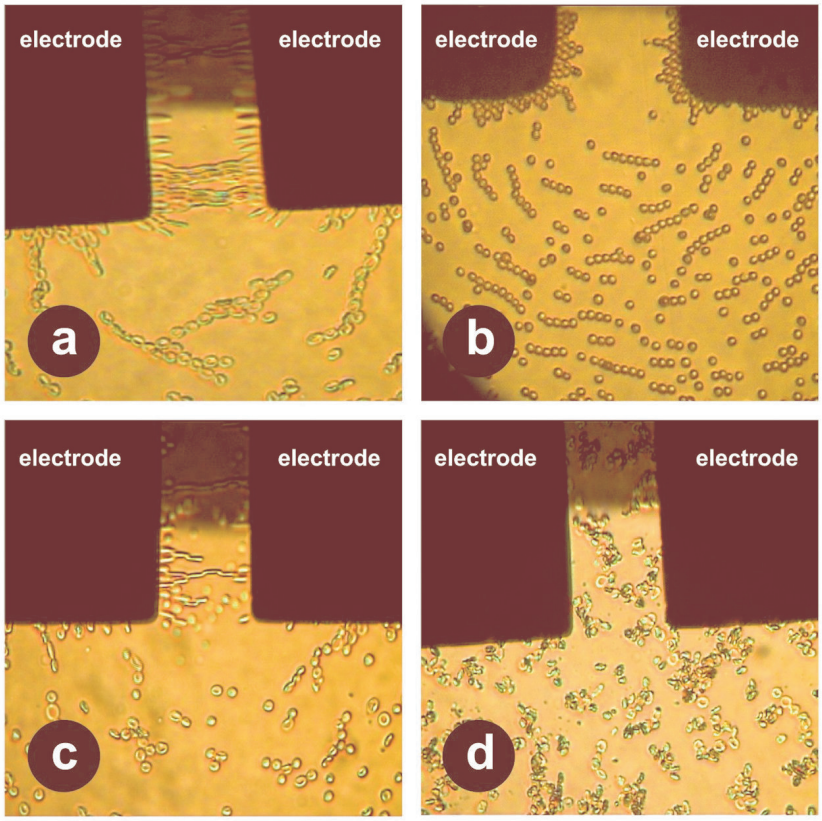

Figure 2. Division and deformation of erythrocytes influenced by nonhomogeneous alternating electric field at frequency of 1 MHz: a) high deformation amplitude in the controls; b) low deformation amplitude in patients with metabolic syndrome; c) increased hemolysis of erythrocytes in patients of 2nd group; d) increased development of aggregation in patients of 2nd group.

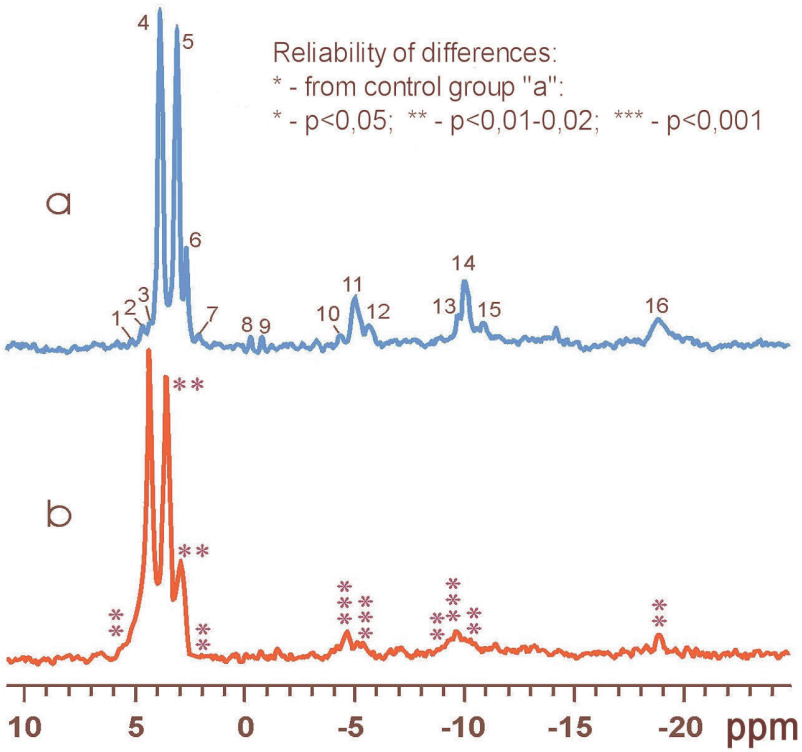

Figure 3. ^{31}P NMR spectra of erythrocytes in patients with stroke (b) and those in control group (a).

We revealed the deficit of easily oxidized phospholipid fractions, triglycerides and cholesterol esters in erythrocyte membranes of the 2nd group patients (p<0,01-0,05).

So, we used a variety of therapeutic approaches in these groups in accordance with different pathogenetic variants of stroke.

In the 1st group patients, we assigned essential phospholipids, antioxidants and drugs increasing the level of intracellular macroergic phosphates along with the set of standard antithrombotic, thrombolytic therapy [4]. Positive dynamics of the viscoelastic erythrocyte parameters was reflected in significant increase of erythrocyte amplitude deformation, capacity, speed of erythrocyte motion to the electrodes, the dipole moment levels, polarizability at high frequencies and significant decrease in conductivity, erythrocyte viscosity and rigidity (p <0,001-0,05).

Membrane stabilizing drugs, Mg-, S-containing drugs, 3,5,7,3 ', 4'-pentaoxiflavon, antiaggregants were additionally assigned in the 2nd group patients with "fragile" erythrocytes. Positive dynamics was accompanied by the increased polarizability at all frequencies, reducing the relative polarizability, aggregation and destruction indices while maintaining sufficient cell plasticity (p <0,0001-0,03).

Positive changes in the erythrocyte parameters were correlated with the "+" dynamic on MRT and CT (r = +0,88, p <0,01), a significant reduction in neurological symptoms (r = +0,72, p <0.03), positive changes in the parameters of hemostasis (r = +0,69, p <0,02).

Lack of positive or negative dynamics of erythrocyte parameters (low erythrocyte deformability, high aggregation and destruction indices, low membrane capacity, dipole moment, polarizability) in combination with high hematocrit, low activity of platelet aggregation, protein-C-deficiency and high D-dimer, soluble fibrin monomer complex in the monitoring of patients (14 patients of the 1st group, 11– of the 2nd one) correlated with the recurrence of ischemic attacks, increased of hypoxia areas on MRT and increased neurological symptoms (p <0,0001-0,02).

Most of these patients (68%) had hetero- or homozygous mutations in the genes of the hemostasis system (mutations in the protrombin, factor V Leiden, antitrombin III, proteins C, *S,* tissue plasminogen activator, enzymes of homocysteine metabolism genes).

These patients are likely to have associated mutations of the erythrocyte enzyme system (including nitric oxide synthase, glucose 6-phosphate dehydrogenase, Na,K-ATPase), erythrocyte membrane scaffolding proteins, which didn't allow one to achieve the effect of therapy in terms of supervision and/or required the use of other drugs.

Conclusion

We identified different pathogenetic variants of stroke which required different approaches to the therapy. Hemorheological parameters were found to be the predictors of recurrent stroke.

References

1. Generalov V. M., Kruchinina M. V., Durymanov A. G., Medvedev A. A., Safatov A. S., Sergeev A. N., Buryk G. A, Kurilovich. S. A. and Gromov A. A. Dielektroforez v diagnostike infektsionnykh I neinfektsionnykh zabolevaniy (Dielectrophoresis in diagnosis of infectious and noninfectious diseases) // Novosibirsk: ZERIS. – 2011. – 172p.
2. Jardetsky O. NMR in molecular biology. / O. Jardetsky, G.C.K.Roberts // London: Academic Press. – 1981. – P.27, 152-153.
3. Cote R., Wolfson C., Solymoss S. et al. Hemostatic markers in patients at risk of cerebral ischemia // Stroke. 2000. – V.31(8). – P.1856-1862.
4. Adams H.P., del Zoppo G.J., von Kummer R. Management of stroke: A practical guide for the prevention, evaluation and treatment of acute stroke/ Publ. By Professional Communication, Inc., 2002

The Cardiopulmonary Exercise Test as a tool for Antihypertensive Treatment Evaluation and Possible Personal Treatment Adjustment

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Summary

63 patients (pts), 42 males and 21 females, were studied: 16 with lone hypertension treated only with vasodilator agents, 26 with lone hypertension treated only with beta-blocking agents, and 21 hypertensive pts with LV dysfunction treated with beta-blockers along with other medications. A cardiopulmonary exercise test (CPET) was performed in all pts while taking their medications, including beta-blockers, in which the indices: HR, BP, O2-consumption (VO2), O2-pulse (O2P), Ventilatory anaerobic threshold (VAT), and Respiratory exchange ratio (RER) were measured. Peak values of the cardiopulmonary indices were compared among the three groups, for each index separately. The results showed significant differences of the peak-VO2 and VAT values among all the three groups, and of the O2P between groups A or B and group C. A clear tendency of lower values was demonstrated with the beta-blockers. All groups have reached the target value of RER (>1.15), in spite the differences in peak-HR. As the CPET showed a significant physiological disadvantage in the treatment of beta-blockers compared to vasodilators in pts with lone hypertension, and it is even worser in the LV dysfunction group, we conclude that this very test might serve as a tool for personalized medical adjustment in hypertension, as in our study.

Introduction

The use of beta-blockers as first-line therapy for hypertension has been, and still is, widely conceptual. However, debates have been raised about it in the last few years (1-3). Another study showed that even the risk reduction effect of stroke by beta-blockers was only marginal (4). On the other hand, these drugs were shown to be associated with new-onset of diabetes mellitus (5). The functional effect of these antihypertensive medications, compared to others like vasodilators, and as expressed by CPET, is rarely investigated. Such an assessment might allow us to prescribe personally the optimal antihypertensive treatment and to follow-up its short and long-term effects. The aim of this study was to evaluate this very physiological effect of medical treatment in hypertensive pts with and without LV dysfunction by CPET.

Methods

63 pts, 42 males and 21 females, were divided into three groups:
A)16 lone hypertensive pts treated only with vasodilator agents.
B)26 lone hypertensive pts treated only with beta-blocking agents.
C)21 hypertensive pts with LV dysfunction treated with beta-blockers along with other medications.
A CPET was performed in all the pts while taking their medications, including beta-blockers.

Cardiopulmonary exercise test

An upright symptom-limited test was performed on an electronically braked cycle ergometer (Ergoline 800). Exercise was initiated after a 3-minute rest and 2 minutes of free pedaling at a rate of 60 rpm. The effort was then progressively increased by 10-20 Watt/min until the predefined end-point was reached, namely, symptoms, volitional fatigue, or attainment of the target HR. Cardiopulmonary data were collected by an online metabolic unit (ZAN-GPI-3.00, Cardiopulmonary function, Germany). Pts breathed through a low-resistance, 2-way valve, connected to the expiratory limb. The breath-by-breath signals were integrated by a computer to yield 30-sec and averages of HR, minute ventilation (VE), VO2, VCO2, and O2P (VO2/HR). VAT was defined as the point at which the ventilator equivalent of O2 (VE/VO2) increased in the absence of CO2 (VE/VCO2). BP was

measured at rest, every 2 minutes, at peak exercise and during recovery. For exercise completion, we tried to reach a peak-RER (Respiratory exchange ratio) of at least 1.15. Peak values of the cardiopulmonary indices were compared among the three groups, for each index separately, and P values less than 0.05 were considered statistically significant.

Results

The following table summarized the results:

Group	N	age	peak-HR*	peak-VO2*	peak-O2P*	VAT(%VO2-max)	peak-RER
A	16	58+/-13	90+/-8#	96+/-9#	108+/-13#	55+/-8#	1.17+/-0.12
B	26	59+/-10	69+/-12&	69+/-11&	102+/-33#	43+/-9&	1.17+/-0.1
C	21	53+/-8	72+/-8&	57+/-10$	79+/-14&	34+/-5$	1.18+/-0.09

*Expressed by % related to normal predicted values. Statistically significant (referred to each column separately; $p<0.05$): # vs & or $; & vs $. The results showed significant differences of the peak-VO2 and VAT values among all the three groups, and of the O2P between groups A or B and group C. The clear tendency was of lower values with the beta-blockers. All groups have reached the target value of RER (>1.15), in spite of the differences in peak-HR.

Conclusions

A significant physiological disadvantage was shown in the treatment of beta-blockers compared to vasodilators in pts with lone hypertension. The physiological function in the LV dysfunction group is even worser, but it is still to determine the benefit compared to the disadvantage of beta-blockers in these patients. We conclude that the CPET might serve as a tool for personalized medical adjustment in hypertensive pts, as shown in the present study.

References

1. BANGALORE S, MESSERLI FH, KOSTIS JB, PEPINE CJ. Cardiovascular protection using beta-blockers: A critical review of evidence. J Am Coll Cardiology 50: 563-572, 2007.
2. LINDHOLM LH, CARLBERG B, SAMUELSSON O. Should beta-blockers remain first choice in the treatment of primary hypertension? A meta-analysis. Lancet 366: 1545-1553, 2005.
3. MESSERLI FH, GROSSMAN E, GOLDBOURT U. Are beta-blockers efficacious as first-line therapy for hypertension in the elderly? A systemic review. JAMA 279: 1903-1907, 1998.
4. BRADLY HA, WIYSONGE CS, VOLMINK JA, MAYOSI BM, OPIE LH. How strong is the evidence for use of beta-blockers as first-line therapy for hypertension? Systemic review and meta-analysis. J Hypertens 24: 2131-2141, 2006.
5. BANGALORE S, PARKER S, GROSSMAN E, MESSERLI FH. A meta-analysis of 94,492 pts with hypertension treated with beta-blockers to determine the risk of new-onset diabetes mellitus. Am J Cardiology 100:1254-1262, 2007.

Personalised Medicine – Pre- and Postgraduate Education in the Czech Republic

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Abstract

The main goal of personalized medicine is the individualized approach to the patient´s treatment. It can be achieved only by the integration of the complexity of novel findings in diverse "omics" disciplines, new methods of medical imaging as well as implementation of various biomarkers into the medical care. The implementation of personalized medicine principles into the clinical practice is dependent on the adaptation of pre-graduate and postgraduate medical education to these principles. The current situation in the education of personalized medicine in the Czech Republic is described together with novel educational tools that are at present established in our country.

Key-words: Personalized Medicine – Pregraduate Education – Postgraduate Education – Biomarkers – Questionnaire

Introduction

Personalized medicine (PM) is the novel model of individual patient´s medical care [1, 2]. PM combines information from various "omics" disciplines (genomics, metabolomics, transcriptomics, pharmacogenomics or proteomics) with innovative preventive and therapeutic strategies that are more efficient, safe and cost-effective [3–5]. This philosophy of PM has become a reality with the sequencing of human genome and the development of novel technologies including laboratory diagnostics, advances in genetics, new methods of medical imaging and implementation of various biomarkers into the medical care [6–9]. The main goal of PM is the shift from the concept of "one medicine fits to all patients with the same disease" to individual treatment of each patient – "the right treatment to the right patient at the right time in a right dose" [10–12]. This novel integrative approach evidently requires the complex effort directed toward the better education in PM [13, 14].

Concept of personalized medicine education

The medical pre-graduate and postgraduate educational system in the Czech Republic has very long history. During the past decade the system changed due to the step by step reveal of new findings mentioned above. The implementation of PM principles into the common medical education as well as into the clinical practice is essential.

Survey of PM awareness among medical students

The implementation of PM principles into the medical studies started with the findings of the real situation - how intense was the knowledge of PM among medical students. The "Personalized Medicine Questionnaire" was prepared and addressed to medical students at the Faculty of Medicine in Pilsen. Students had to answer 9 nominal questions about the PM. Each question had the response range from 1 to 5 (from "the least" up to "the most"). There were 84 responders mainly students from the fourth year of medical school. The summary of the means of responses for each question is included in Tab. 1.

Tab. 1. The summary of the means of responses for each question in the PM questionnaire

Question in PM questionnaire	The mean of responses (1-5)
Have you ever heard the term "personalized medicine?"	2,26
Would you be able to explain, what does the "pesonalized medicine" mean?	2,36
Do you consider "personalized medicine" to be important?	4,16
Do you think "personalized medicine" should be studied as an independent discipline or through the various disciplines separately?	3,16
Do you consider the role of "personalized medicine" will increase with the progress of knowledge?	4,2
Is tuition in "personalized medicine" suficient in pregraduate education in medical school?	1,85
Do you think "personalized medicine" should be implemented into the pregraduate education of medicine for all students?	3,66
Do you think "personalized medicine" should be implemented into the pregraduate education of medicine as credit course?	4,14
Will you choose the course of "personalized medicine"?	3,84

As the results from the PM questionnaire show, the awareness about PM among students was quite weak (more than 39% have not even heard the term "personalised medicine"), although most students (more than 75%) recognised the importance of PM and would welcome its implementation into the pre-graduate education.

Educational activities in PM

Several educational activities were realized in our institution (workshops, conferences, seminars, new optional courses) addressed essentially to medical students and young physicians.

Since 2009, lectures concerning PM and its applications have been included in the scientific programme of the Immunoanalytical Days – the congress with the international participation, organised every year by the Czech Society of Nuclear Medicine. Since 2011, PM topics have had their independent section at this congress.

In 2011, a two-day conference „Personalised medicine – from bench to bed" was held at the Faculty of Medicine in Pilsen. The topics of presentations were varying from strategic ones (Horizon 2020 presented by Dr. Patrick Kollar from the European Commission, DG Research and Innovation, Head of Unit Personalized Medicine) through PM overviews from the USA experts, to the examples of research topics and case-studies from cardiology, oncology, neurology and so on, presented by Czech physicians and researchers.

Since 2012, the optional course „Personalised medicine – fiction or future for medicine?" is included into the education scheme at the Faculty of Medicine in Pilsen. This course aims to introduce PM principles to pre-graduate students in a rather popularised way so that they pick up the idea and do not get overwhelmed by the amount of information.

Perspectives

One of the main activities of the working group of professionals in the personalized medicine domain at the Faculty of Medicine in Pilsen and in Faculty Hospital in Pilsen is the organization of the first course of the "Summer School of Personalized Medicine in Pilsen 2014", the first event in the Czech Republic on this topic.

At present there are three web sites targeted to PM and related topics; their content is built up and updated by medical professionals in the Czech Republic. These are: web site oriented to the immunoanalytical laboratory methods (version for professionals and students), and web pages focused on molecular biology methods and their application in clinical routine (www.molekgen.eu). The web pages are in Czech language, however their English version is being prepared. The goal of these web sites is to contribute to the awareness of both medical professionals and general population as regards current development and perspectives of PM.

Summary

The concept of personalized medicine is one of the most perspective trends in medicine at present. PM assures the individual approach to each patient with tailored therapy and distinctive medical care. The education in this field is the keystone for understanding and application of PM principles. The educational activities of the working group of professionals in the PM at the Faculty of Medicine in Pilsen, Charles University in Prague and in Faculty Hospital in Pilsen are unique through the country and will be extended among pre-graduate and postgraduate medical students at other medical faculties in the Czech Republic.

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References

[1] Miles A, Loughlin M, Polychronis A (2008) Evidence-based healthcare, clinical knowledge and the rise of personalised medicine. J Eval Clin Pract 14:621–649.
[2] Blay J-Y, Lacombe D, Meunier F, Stupp R (2012) Personalised medicine in oncology: questions for the next 20 years. Lancet Oncol 13:448–449.
[3] Scott SA (2011) Personalizing medicine with clinical pharmacogenetics. Genet Med Off J Am Coll Med Genet 13:987–995.
[4] Howland RH (2012) Where are we today with personalized medicine? J Psychosoc Nurs Ment Health Serv 50:11–13.
[5] Tremblay J, Hamet P (2013) Role of genomics on the path to personalized medicine. Metabolism 62 Suppl 1:S2–5.
[6] Harvey A, Brand A, Holgate ST, et al. (2012) The future of technologies for personalised medicine. New Biotechnol 29:625–633.
[7] Dunn G, Emsley R, Liu H, Landau S (2013) Integrating biomarker information within trials to evaluate treatment mechanisms and efficacy for personalised medicine. Clin Trials Lond Engl.
[8] Cancer Genome Atlas Research Network, Genome Characterization Center, Chang K, et al. (2013) The Cancer Genome Atlas Pan-Cancer analysis project. Nat Genet 45:1113–1120.
[9] European Society of Radiology (2011) Medical imaging in personalised medicine: a white paper of the research committee of the European Society of Radiology (ESR). Insights Imaging 2:621–630.
[10] Samani NJ, Tomaszewski M, Schunkert H (2010) The personal genome--the future of personalised medicine? Lancet 375:1497–1498.
[11] Golubnitschaja O, Costigliola V (2011) European strategies in predictive, preventive and personalised medicine: highlights of the EPMA World Congress 2011. EPMA J 2:315–332.
[12] Hu R, Wang X, Zhan X (2013) Multi-parameter systematic strategies for predictive, preventive and personalised medicine in cancer. EPMA J 4:2.
[13] Golubnitschaja O, Costigliola V, EPMA (2012) General report & recommendations in predictive, preventive and personalised medicine 2012: white paper of the European Association for Predictive, Preventive and Personalised Medicine. EPMA J 3:14.
[14] Bonter K, Desjardins C, Currier N, et al. (2011) Personalised medicine in Canada: a survey of adoption and practice in oncology, cardiology and family medicine. BMJ Open 1:e000110.

Network Integration Approach for Personalized Medicine

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Abstract

The purpose of this paper is to develop analytic methods using graph theory that can be used to create *in silico* models that can help gain insights into the combined effects of genomic, environmental, and clinical factors on human pathophysiology. We describe various topological properties of biological networks to characterize such networks that can be used to compare and contrast the networks corresponding to diseased and healthy tissues. Further, we describe a perturbation model for such networks and demonstrate how small but selective perturbations can have drastic impacts on such networks. Our proposed methods have been applied to CIDeR, a multi-modal and multi-dimensional database that was created by manual curation of information on neurological and metabolic diseases, obtained from published research from authoritative sources.

Keywords: personalized medicine, interactions, genome, data analytics, graphs

1. Introduction

Over the last decade, since the completion of the Human Genome Project in 2003, a great amount of progress has been made in translational bioinformatics. It has been recognized that combining bioinformatics with health informatics can provide deep insights in the study of complex diseases and their treatments [7]. The emergence of P4 (preventive, predictive, personalized, and participatory) medicine [8], widely regarded as the future of medicine, essentially extends the concept of translational bioinformatics to encompass many other factors such as the environment, disease comorbidities, drugs and chemical compounds, and biological processes to study the causes and effects of human pathophysiology. Rapid technological growth in whole genome sequencing has drastically reduced the cost of identifying genetic variants in individuals. These have implications in the treatment of diseases, identification of genetic predispositions of individuals to certain diseases, and pharmacogenomics. However, genetic variations such as single nucleotide polymorphisms (SNPs) can explain only a small percentage of the risk of diseases and the predictive power may vary between diseases [10]. It is increasingly being realized that the determinants of diseases include a myriad of other factors including interactions among intra-cellular and inter-cellular components, as well as those of social and environmental factors [8,9]. Thus, one way to model diseases as well as develop potential prevention and treatment strategies is through the study of the interactions of a large number of multi-scale objects that govern the human pathophysiology. These networks are often multi-modal, multidimensional, and time varying or dynamic in nature. In this paper we describe a graph-theoretic approach for analyzing various aspects of biological networks. We hope that such analysis methods can be useful to molecular biologists and clinicians through *in silico* models [2].

The rest of the paper is organized as follows. In Section 2 we describe how network properties can be used to characterize biological networks. In section 3 we describe a methodology for identifying important nodes in biological networks using link analysis. In Section 4 we describe an *in silico* perturbation model for biological networks. Finally, in Section 5 we discuss the limitations of our work and plans for future studies.

2. Topological Properties

The human biology is an interconnected system of various networks such as genetic networks, metabolic networks, disease networks, molecular networks, and cellular networks in which interactions occur between nodes both within and between these networks. Each of these networks consists of a homogeneous set of nodes, such as genes, SNPs, and metabolites, and a set of edges that describe the interactions among the nodes. In order to study the system holistically we prefer to combine each of the component networks into a large network, sometimes referred to as the "interactome". Such a network is inherently multi-modal where each mode refers to a specific class of objects such as genes, SNPs, and metabolites that make up the component sub-networks. Such a network is also multi-dimensional because multiple types of edges may exist between the objects. For example, an object can activate or inhibit the functions of another object. Edges can be directed such as in the case of transcription factors in regulatory networks, or undirected such as in protein-protein interaction networks [3,4].

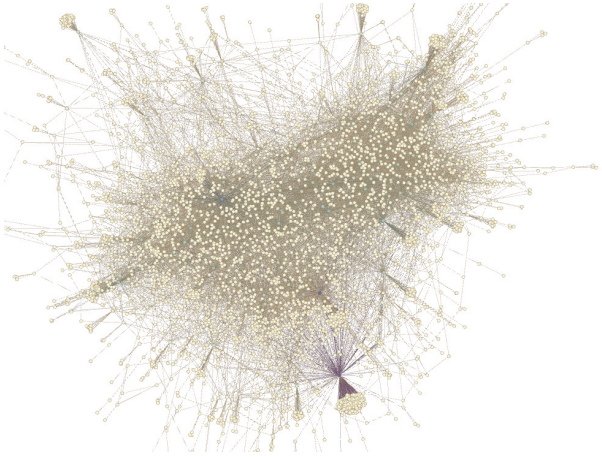

Figure 0. Graphical Depiction of CIDeR

In this paper we use the CIDeR (Curated Information of Disease Related Interactions) database [12], which is a manually curated database of neurological and metabolic diseases. The CIDeR database has been generated from published articles obtained from authoritative resources such as the PubMed. CIDeR provides web-based functionalities to selectively search for and visualize information related to nodes and subgraphs. However, it does not offer any analytic capabilities of the networks.

In this section we describe some of the topological properties of the interactions among various objects in the CIDeR database and discuss their implications. Figure 1 shows a graphical display of the interactions of the objects in the entire CIDeR database. The degrees of the nodes are color-coded with where high-degree nodes are shown with darker colors and low-degree nodes shown in lighter colors. The CIDeR database contains more than a dozen different types of nodes including SNPs, cellular components, genes, diseases, biological processed, drugs/chemical compounds, and environments, with a total of 5168 nodes and 14410 edges. The diameter of the network is 16, which indicates that the farthest pair of nodes can be connected with sixteen links. The network has 51 connected components, or sub-networks that are not connected with each other. However the nodes within each connected component are either directly or indirectly (through other nodes) connected with each other. The average path length (APL) of the network is 4.46, which is the average number of links required to connect any pair of nodes. Lastly, the average degree is 2.8, which is the average number of immediate neighbors for a given node. The average clustering coefficient is 0.064, which is the ratio of the actual number of connections between neighboring nodes to the maximum number of possible connections if every node were connected with all of its neighbors. These measures indicate that the network is very sparse, with most nodes having a small number of immediate neighbors. The entire network can be affected very quickly due to its capacity to spread influence very rapidly as indicated by the short APL. The topological properties also indicate that the network has a small number of high-influence nodes and a large number of low-influence nodes. The last property is indicative of a scale free network [1,6], which can be demonstrated by its degree distribution,

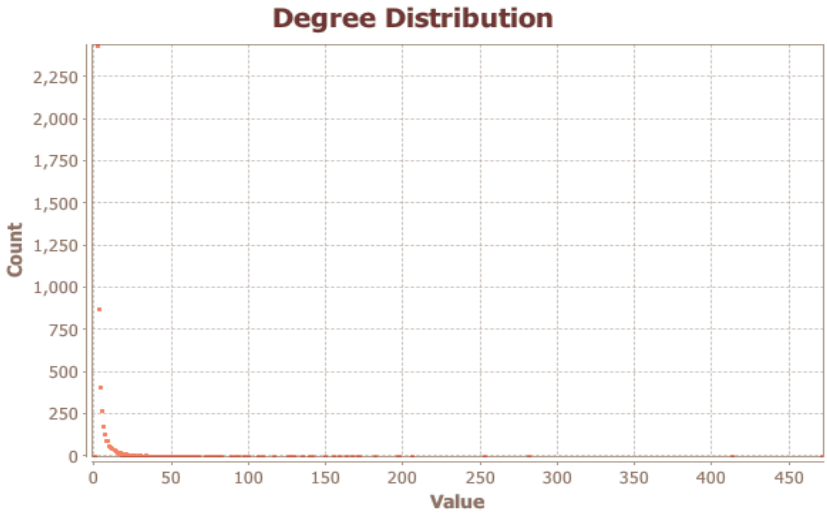

Figure 0. Degree Distribution

shown in Figure 2. The degree distribution plots the number of nodes (y-axis) for different degrees (x-axis). As can be seen from Figure 2, there are a very small number of nodes that have high degrees (greater than 100), with a large number of nodes with very small degrees (1-3). In a random network the degree distribution follows a Gaussian distribution where the degrees of most nodes are centered near the mean. The scale-free nature of the network implies that it is very resilient to random perturbations, but very susceptible to targeted perturbations.

3. Identifying Important Nodes

Our next objective is to find nodes that are highly influential, i.e., perturbing these nodes will result in drastic changes in the network. It is important to identify influential nodes from biological standpoint. For example, it has been shown that new nodes entering these networks have a higher tendency to attach themselves to influential nodes rather than to nodes with lesser influence. This analysis may also help in drug development that can potentially target nodes based on their influence scores. A node with a high receptor score is one that is influenced by a large number of other nodes, whereas a node with a

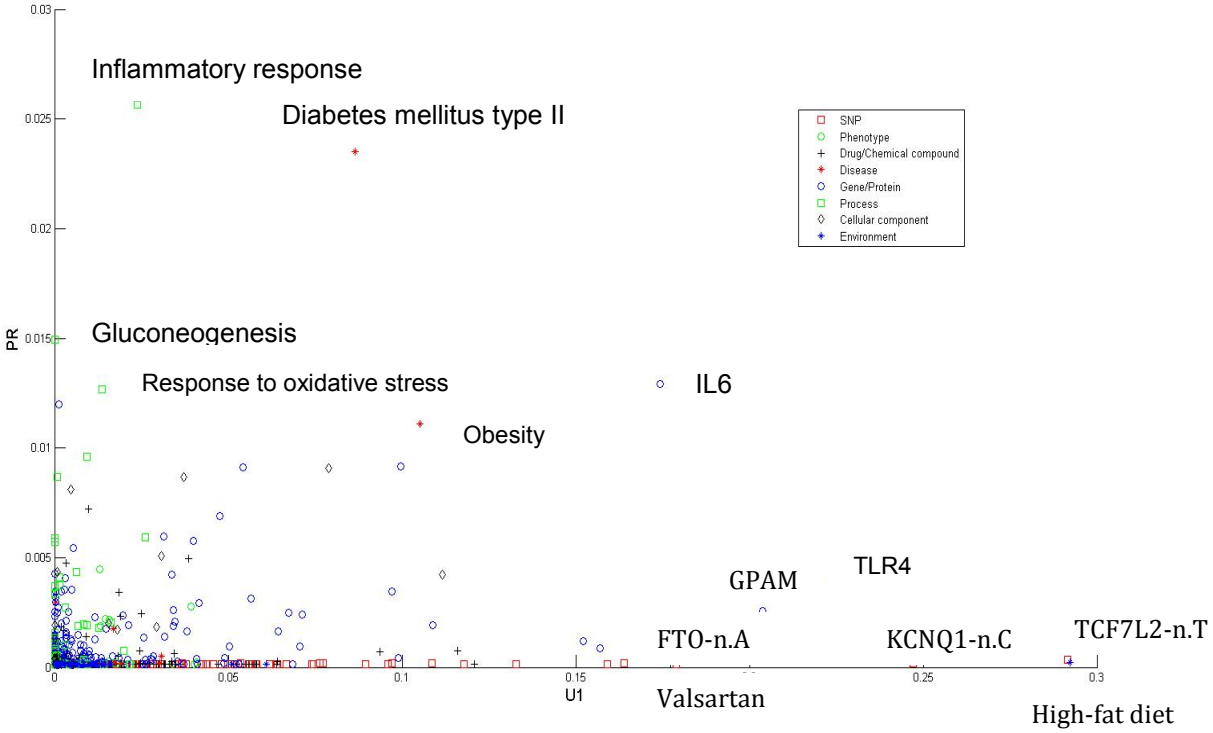

Figure 1. Receptor versus Effector Scores

high effector score has high influence on a large number of other nodes. The receptor and effector scores correspond to hub and authority respectively [11]. The receptor score can be computed using the PageRank algorithm [5]. We analyzed the *Diabetes mellitus type II* sub-network on CIDeR using the receptor and effector scores of the nodes. The result of this analysis is shown in Figure 3, where the receptor scores (y-axis) are plotted against the effector scores (x-axis). The plot shows that most of the objects are clustered near the origin with very low receptor and effector scores. These are low-influence nodes that are also impacted by a small number of potentially high-influence nodes. However, there are three other categories of nodes: those with low receptor and high effector scores (α), those with high receptor and low effector scores (β), and those with high receptor and high effector scores (γ). The α nodes are influencers that are not influenced by others, and appear at the bottom-right corner of the plot. Examples of these nodes include SNPs such as TCF7L2-n.T, KCNQ1-n.C and FTO-n.A, genes such as GPAM and TLR4, and environmental factors such as *high-fat diet*. β nodes are those with little influence on others but are susceptible to the influence of a large number of nodes. The β nodes appear on the top-left corner of the plot and include processes such as *inflammatory response*, *gluconeogenesis*, and *response to oxidative stress*. The γ nodes are those that both influence and are influenced by a large number of nodes. Examples of γ nodes include the gene *IL6* and diseases including *Diabetes mellitus, type II* and *obesity*. Although the receptor and effector scores of all nodes are calculated only a small number of these nodes are labeled in Figure 3 due to space limitations.

4. Perturbation Models

The network model described above can be used in a systems approach to diseases. A disease can be seen as a consequence of the perturbation of one or more biological networks. The CIDeR network provides a comprehensive model generated from heterogeneous data types such as SNPs, genes/proteins, metabolites, and comorbidities. Our objective in this paper is to find out the effect of perturbations on the connectivity of the network. There are multiple ways to perturb a biological network, such as removing nodes, removing edges, inserting nodes and edges, and various combinations of the above. Significant differences in the network structure and connectivity have been observed between diseased and healthy tissues [8]. In this section we describe a perturbation study on the *Diabetes mellitus type II* sub-network of CIDeR. Our approach is to selectively remove high-influence nodes and measure the effect on the connectivity of the network, which would drastically impact the flow of information, mass, and energy within the network [6].

The influence of a node is captured by several centrality measures including the hub score, out-degree centrality, betweenness centrality, and closeness centrality. We have already explained hub scores in a previous section. The out-degree centrality of a node is the total number of out-links of that node. It is expected that nodes with high out-degree centrality measures are highly influential because of the influence they have on their immediate neighbors. The closeness centrality of a node is measured by the average distance of that node from all other nodes in the network. The betweenness centrality of a node is the percentage of the number of shortest paths between any pair of nodes that go through that node. Nodes with high betweenness centrality measures might be "bridge nodes" that connect a large number of sub-networks. The result of our perturbation studies is

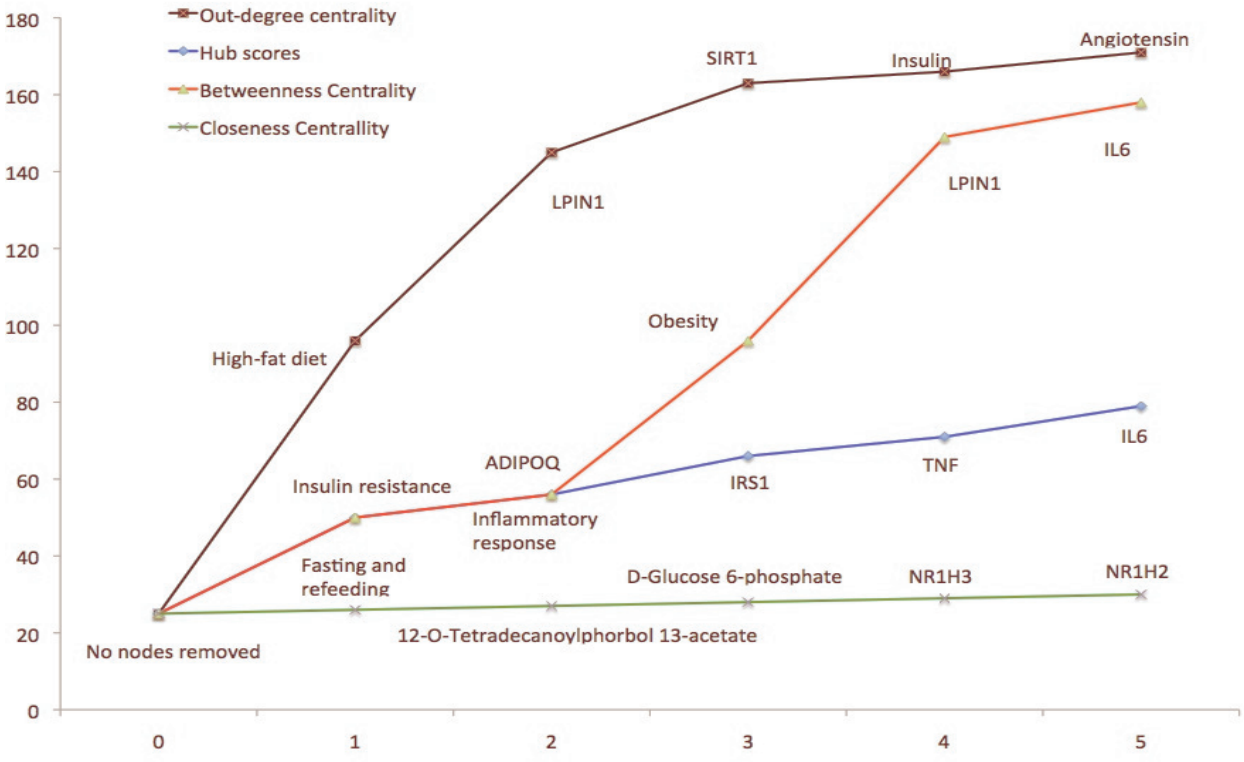

Figure 1. Perturbation Analysis

shown in Figure 4, where the number of connected components is shown on the y-axis and the number of nodes removed is shown on the x-axis. The data point at the bottom-left corner of the graph corresponds to the case where no nodes have been removed, or the original network that had 25 connected components. Next the nodes are selected for removal based on the values of their centrality measures: the node with the highest centrality measure is removed first and so on. The effect of all of the centrality measured discussed above are plotted.

As can be seen from Figure 4, the out-degree centrality measure seems to have the highest impact in terms of perturbation by node removal followed by betweenness centrality, hub score, and closeness centrality. Another important fact to note is the rate at which the network becomes disconnected with the removal of selected nodes. The network consists of 1856 nodes. Removing the node with the highest out-degree centrality results in 96 connected components, which is nearly 4 times as disconnected as the original network. Selective removal of the five nodes with the highest out-degree centrality results in close to 171 connected components,

6.8 times that of the original network. In other words, removing only 0.27% of selected nodes can result in a drastic reduction in the connectivity of the network.

5. Conclusion and Future work

In this paper we described a method for analyzing biological networks. Our proposed methods were applied to the CIDeR database, which is a multimodal and multi-dimensional database consisting of various types of multi-scale objects such as SNPs, genes, proteins, cellular components, diseases, biological process, and phenotypes. The objective of this research is to illustrate how such networks can be analyzed to gain insights into the various network characteristics and relate them to human pathophysiology, drug development, disease modeling, and clinical treatment strategies. We have demonstrated that such networks exhibit many of the same characteristics as real-work networks, such as the scale-free property and the small world effect. We further developed a method for characterizing the nodes in terms of the influence they have on other nodes (effector score) and the influence that other nodes have on them (receptor score). In addition we described a perturbation model that identifies the impact of removing the most influential nodes on the connectivity of the network.

This work is limited to one specific database, namely CIDeR. Hence, further studies are needed to demonstrate the efficacy of the proposed methods in more general settings. The results obtained from this research also need to be validated and the clinical implications need to be ascertained by delving into the realms of molecular biology and the practice of medicine. The drastic impact of removal of a small number of nodes on the connectivity of the network need to be further investigated in terms of its clinical and biological implications.

References

[1] Albert, R. (2005). Scale-free Networks in cell Biology. Journal of Cell Science. 118, pp. 4947-4957.

[2] Alon, U. (2007). Network motifs: theory and experimental approaches. Nature Reviews Genetics 8, pp. 450-461.

[3] Bader, D. A and Madduri, K. (2007). A Graph-Theoretic Analysis of the Human Protein-Interaction Network using Multicore Parallel Algorithms. *Sixth IEEE International Workshop on High Performance Computational Biology* (HiCOMB), Long Beach, CA.

[4] Barabási, A-L., Gulbache, N. and Loscalzo, J. (2011). Network Medicine: a Network-based Approach to Human Disease. Nature Review Genetics, 12(1): pp. 56-68.

[5] Brin, S. and Page, L. (1998). The Anatomy of a Large-Scale Hypertexual Web Search Engine. Computers Networks and ISDN Systems, pp. 107-117.

[6] Chakrabarti, D. (2005). Tools for Large Graph Mining. PhD dissertation, Carnegie Melon University.

[7] Feero, W. G., Guttmacher, A. E., Collins, F. S. (2010). Genomic Medicine — An Updated Primer. The New England Journal of Medicine; 362, pp. 2001-2011.

[8] Hood, L., Flores, M. A., Brogaard, K. R., and Price, N. D. (2013). Systems Medicine and the Emergence of Proactive P4 Medicine: Predictive, Preventive, Personalized, and Participatory. Handbook of Systems Biology Concepts and Insights, Elsevier, pp. 445-467.

[9] Hoyt, R. E. and Sarkar, I. (2012). Bioinformatics. Health Informatics Practical Guide for Healthcare and Information Technology Professionals (5th Editon), pp. 409-423.

[10] Jeong H., Tombor B., Albert R., Oltvai Z.N., and Barabási A.-L. (2000). The large-scale organization of metabolic networks. Nature 407, pp. 651-654.

[11] Kleinberg, J. M. (1999). Authoritative Sources in a Hyperlinked Environment. Journal of the Association of Computing Machinery 46(5), pp. 604-632.

[12] Lechner, M., Höhn, V., Brauner, B., Dunger, I., Frishman, G., Montrone, C., Kastenmüller, G., Waegele, B., and Ruepp. A. (2012. CIDeR: Multifactorisl Interaction Networks in Human Diseases. Genome Biology, 13: R62.

Establishing infrastructure in Personalized Medicine: The University of Maryland Program for Personalized and Genomic Medicine

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Abstract

In 2011, the University of Maryland established the Program for Personalized and Genomic Medicine (PPGM) to accelerate the pace of discovery and implementation of evidence-based personalized and genomic medicine. Establishing such a program required a multi-pronged approach that addressed education, translational medicine, and discovery initiatives. We describe the efforts adopted to create a culture of innovation and early adopters of pharmacogenomics within our educational and clinical care systems. As the evidence base grows, we expect to expand our efforts to realize fully the promise of personalized medicine.

Key words: Pharmacogenomics, individualized medicine, personalized medicine, precision medicine translational research, implementation science, CYP2C19, clopidogrel, anti-platelet pharmacogenetics

Introduction

Within the last 10 years, there has been an explosion in our knowledge of sequence variation in the human genome and its relationship to health and disease. The genetic causes of more than 3,000 monogenic diseases and phenotypes are now known (http://www.omim.org/statistics/geneMap). Through genome-wide association analysis, common variants at thousands of loci have been associated with common polygenic diseases and traits. Candidate gene studies and other approaches have identified genetic variants that influence response to drugs; now more than 100 medications contain pharmacogenomic information in their labeling (http://www.fda.gov/drugs/scienceresearch/-researchareas/pharmacogenetics/ucm083378.htm). New knowledge of the human genome has unveiled new insights into human biology, pathophysiology, and novel drug targets for cancer, cardiovascular disease, diabetes, and other disorders. _ENREF_1The goal of personalized medicine is to apply this genomic knowledge to optimize patient care for more effective treatment and prevention of disease.

The University of Maryland Program for Personalized and Genomic Medicine

Despite these remarkable advances in human genomics, translation into clinical practice has been slow. There are multiple barriers to implementation of genomics into health care [1, 2]. To identify and overcome these barriers, in 2011 the University of Maryland established the Program for Personalized and Genomic Medicine (PPGM). Supported by institutional funds from the University of Maryland School of Medicine (UMSOM) and University of Maryland Medical Center (UMMC), the mission of PPGM is to advance discovery in genomics and other "omic" sciences; to accelerate translational research and implementation of these discoveries into more effective and safe individualized health care; and to enhance the training and education of current and future generations of physicians and scientists through a personalized and genomic medicine driven curriculum. The impetus for major investment in personalized medicine throughout our medical system is the promise of enhanced quality of care for patients, improved patient safety, and elevated reputation, which in turn are expected to increase patient encounter volumes and reduce costs by enhancing efficiencies (e.g., decreased length of stay, reduced hospital readmissions) [3].

Through a series of strategic meetings, we developed a pathway to establish the personnel and infrastructure to enable a variety of projects across the spectrum of our mission, from basic discovery to translational research and clinical trials, to implementation of evidence-based personalized and genomic medicine (Figure 1). We first established a multidisciplinary core team of faculty and staff (program manager, genetic counselor, clinical research coordinators, and administrative assistant). We convened an executive advisory board and several

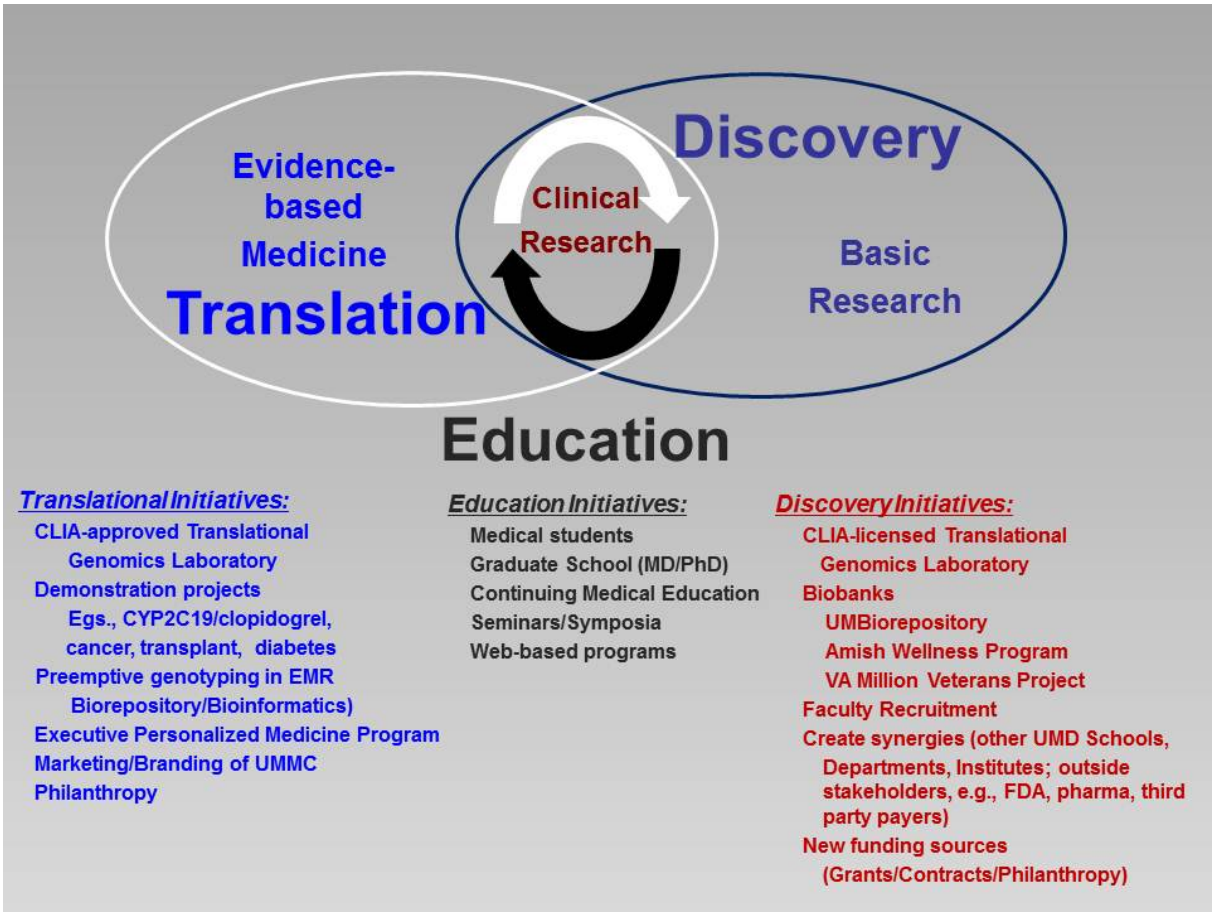

Figure 1. Schematic of the University of Maryland Program for Personalized and Genomic Medicine. Its mission is to advance discovery in genomics and other "omic" sciences; to accelerate translational research and implementation of these discoveries into more effective and safe individualized health care; and to enhance the training and education of future generations of physicians and scientists in personalized and genomic medicine. Illustrated are several initiatives currently underway.

multi-disciplinary working groups charged with developing priorities for the new program and obtaining committed institutional support for program initiatives. In addition, we established a

Pharmacogenomics Subcommittee to the UMMC Pharmacy and Therapeutics Committee, which consists of UMMC clinical pharmacists, School of Pharmacy faculty, physicians, geneticists, and the director of the PPGM (*ex officio)*. This new subcommittee's tasks are reviewing the evidence base, approving the implementation of drug-gene pairs into patient care at UMMC, and providing oversight to the implementation process. This subcommittee is a critical component of the institutional support infrastructure for evaluation and implementation of new genetic tests for drug-gene pairs into clinical care. Finally, strategic faculty recruitment continues to build a critical mass of researchers with focused interest in molecular diagnostics, genomic medicine, biomedical informatics, and population genomics.

Central to the overall implementation strategy was formation of the Translational Genomics Laboratory (TGL), a joint effort between PPGM and the Department of Pathology. The TGL provides centralized genotyping and sequencing services for both clinical and translational research, as well as routine patient care. CLIA certification for the TGL has been pivotal to a number of genomic medicine implementation projects. We developed reports that meet the requirements of a regulated laboratory, while giving physicians enough information to interpret results in the context of how to treat their patients most effectively. We established a process for physicians to order genomic tests and subsequently receive results in a timely manner, all through the EHR. We are now moving toward CAP accreditation and developing a process by which the TGL can bill third-party payers for genomic testing. Another high-priority infrastructure building initiative was the establishment of the University of Maryland Biobank (UMBiorepository). Organized around a new semi-automated robotics storage system (Hamilton) with a capacity of >1 million samples, patient samples amassed from UMMC and outpatient clinics of the UMSOM physician practice will advance future discovery in clinical and translational research efforts. Informatics under development through the Clinical and Translational Research Institute (CTSI) Research HARBOR (Helping Advance Research by Organizing Resources) initiative will allow faculty to query inventory and make sample requests (http://medschool.umaryland.edu/ctsi/harbor.asp). Linkage of samples with the electronic health record (EHR) will provide rich prospective clinical information. A subset of patients participating in the UMBiorepository will give consent to allow reporting of actionable genomic results into their EHR, providing the opportunity to study the clinical and economic impact of implementation of genomic medicine into patient care. A unique resource at our institution is the Amish Biorepository, which now includes DNA, serum and plasma from over 6,000 well-phenotyped Old Order Amish research participants. As all Amish can be linked into a single 14-generation pedigree, the Amish Biorepository provides unprecedented opportunities for genomic discovery for complex diseases and traits, which are applicable to the general population_ENREF_4 [4-6]. Finally, through its affiliation with the Baltimore Veterans Affairs Medical Center (BVAMC), the University of Maryland is one of forty sites for the U.S. Department of Veteran Affairs Million Veteran Program (MVP). The MVP will be among the largest and most comprehensive EHR-linked biobanks in the world (http://www.research.va.gov/MVP/).

Promoting a culture of innovators and informed early adopters

In order to promote research and the early adoption of evidence-based personalized and genomic medicine into clinical practice, PPGM orchestrated a multi-faceted education and outreach program for medical and graduate students, residents, clinical and research post-doctoral fellows, and faculty. These education programs actively solicit engagement and utilization of PPGM resources and infrastructure. As part of the Maryland Center of Excellence in Regulatory Science Initiative, PPGM co-sponsored a one-day symposium with the U.S. Food and Drug Administration (FDA), entitled "Regulatory Issues in NextGen Medicine and Pharmacogenomics" held September 2013 at the UMSOM. Open house symposiums along with a monthly PPGM seminar series augment these education initiatives.

Perhaps the most valuable PPGM-related educational activity is co-sponsoring Grand Rounds in clinical departments and research seminars in basic science departments, for which we tailor presentations to the specific research and clinical interests of the respective audience. Presentations embedded in routine weekly departmental didactic forums have been especially effective in beginning to transform the culture and establish an ongoing dialogue through which clinicians and researchers brainstorm exciting new ideas and projects. These forums stimulate new evidence-based personalized and genomic medicine implementation projects to address clinically important issues.

The future of discovery, clinical and translational research, and implementation of personalized and genomic medicine lies with the next generations of physicians and health care providers. For the past two summers, a multi-disciplinary team conducted a four-week medical school elective entitled "Role of Personal Genomes in Medicine" (see Supplemental Information). As an educational exercise, students enrolled in the course had the opportunity to use their own DNA for pharmacogenomic testing using the Affymetrix Drug Metabolism, Excretion and Transport (DMET™) test. This experience proved very successful as a learning tool

and in demonstrating to students the utility of pharmacogenomics. In the fall of 2013, we offered DMET™ testing to pharmacy students in the "Advanced Pharmacogenomics" elective. In 2014 all rising second year medical students at the UMSOM receive DMET™ results, either from their own samples or from a de-identified control, that will be used as a learning tool throughout their second year curriculum. Our goal is that, through the investigation of these genomes, students will gain new knowledge and champion the application of pharmacogenomics during their third and fourth year clinical rotations and beyond.

Implementation of personalized medicine and pharmacogenomics into clinical practice

In the clinical arena, the PPGM provides support to physicians through our clinical genetics and genetic counseling services in UMMC's Center for Genetics and Genomic Medicine. This clinic serves adults with personal or family histories of a variety conditions that have both known or unknown genetic etiologies. In less than three years, patient volume in this clinic has grown by 50%. Another initiative, the UMMC Executive Health Program is a new clinical service that now offers one-on-one meetings with a genetic counselor for all patients. This service includes assessment of detailed family medical history and a discussion of its implications. Soon the program will offer preemptive pharmacogenomic and other genomic testing, available through the UMSOM TGL.

Additionally, the PPGM engaged physician-champions from a number of disciplines to develop innovative evidence-based genomic medicine implementation projects to close gaps in conventional patient care practices. For example, monogenic forms of diabetes are estimated to constitute 1 - 2% of all diabetes cases, yet recent studies suggest that 94% of monogenic diabetes cases are misdiagnosed as type 1 or type 2 diabetes [7]. The Personalized Diabetes Medicine Program at the UMMC Center for Diabetes and Endocrinology uses clinical and laboratory information to screen patients for monogenic forms of diabetes and offers a 40-gene diabetes sequencing panel for patients whose clinical and laboratory data suggest an atypical form of diabetes. As another example, the oncology group has begun to evaluate DNA and RNA sequencing data from normal and cancerous tissue to help guide therapeutic choices. Other projects employing exome and whole genome sequencing in patient care and research are in various stages of development.

Perhaps the field of personalized medicine most ready for evidence-based implementation is pharmacogenomics. The University of Maryland is one of eight implementation sites of the Translational Pharmacogenomics Program (TPP) funded by the National Institutes of Health Pharmacogenomics Research Network [8]. The goal of the TPP is to implement into patient care drug-gene pairs published in the Clinical Pharmacogenetics Implementation Consortium (CPIC) guidelines [9], and to identify barriers to implementation and develop and disseminate real-world solutions.

In March 2013, we implemented *CYP2C19* genetic testing in the cardiac catheterization laboratory for individualized anti-platelet therapy for patients undergoing percutaneous coronary intervention (PCI) procedures. In this research study, we offer patients the *CYP2C19* genetic test, which predicts metabolism of clopidogrel. The TGL performs the genotyping with a turnaround time of approximately five hours. The prescribing physicians receive the results and may then use this genetic information in conjunction with other clinical information to individualize their patients' anti-platelet therapy.

While the program has been quite successful, leading to a change from clopidogrel to an alternative anti-platelet therapy in more than half of PCI patients who are *CYP2C19* intermediate or poor metabolizers, the implementation process has been challenging. We have learned a number of lessons that will be useful to other institutions contemplating similar pharmacogenetic implementation programs. First, engagement of many parties within the healthcare system especially "clinician champions" is essential, along with strong institutional support. There is a need for active clinical decision support that interactively interprets genetic data and guides providers through prescription options. Recurrent education/in-service programs are essential. Finally, monitoring implementation metrics such as uptake of pharmacogenomic testing and resulting genotype-tailored prescriptions is valuable in identifying implementation barriers that need addressing.

Concluding remarks

Discoveries of genetic variation that contributes to disease and predicts drug response are just the first steps in realizing the promises of personalized and genomic medicine. Despite a growing evidence base, barriers to implementation of genomic medicine into clinical practice are many. Early adopter institutions and physicians must lead the way in finding real-life solutions to these barriers to accelerate the implementation process. Dissemination of best practices will facilitate widespread adoption, thus ushering in the age of genomic medicine.

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Conflict of Interest Disclosure

Dr. Shuldiner receives support from NIH for anti-platelet pharmacogenomics research, and is a consultant to United States Diagnostic Standards, Inc. and Merck, Inc. Other authors have no conflicts of interest to declare.

Supplemental Information

Table 1. Role of Personal Genomes in Medicine course topics.

Session	Topic
1	Intro to course & genomics
2	Genetic Counseling and Resources
3	Genetics & Legal Issues
4	Overview of ethics
5	Informed consent
6	Student presentations re: ethics
7	Advanced topics in Genetics and Genomics
8	Advanced topics, DMET™/Pharm chips. Lab session: DNA extraction
9	Communicating Test Results
10	Genomics and Pathology
11	Electronic Health Record/system-wide approach
12	Protecting Health Information
13	Personal genome sequencing: What it can and cannot tell you about your health, and how it may change the future of healthcare
14-18	Lab sessions: CYP2C19 Genotyping
	TGL to run DMET™ chips
19	Drug Metabolism
20	Cancer Genomics
21	Multifactoral disorders
22	Interpretation of data: DMET™
23	Day trip to Amish Research Clinic
24	Microbiomes
25	Student Presentations
26	Student Presentations

References

[1] Manolio, T. A., Chisholm, R. L., Ozenberger, B., Roden, D. M., Williams, M. S., Wilson, R., Bick, D., Bottinger, E. P., Brilliant, M. H., Eng, C., Frazer, K. A., Korf, B., Ledbetter, D. H., Lupski, J. R., Marsh, C., Mrazek, D., Murray, M. F., O'Donnell, P. H., Rader, D. J., Relling, M. V., Shuldiner, A. R., Valle, D., Weinshilboum, R., Green, E. D., and Ginsburg, G. S. (2013) Implementing Genomic Medicine in the Clinic: The Future Is Here. Genet Med 15(4), pp. 258-267.

[2] Cohen, M. J., Ginsburg, G. S., Abrahams, E., Bitterman, H., and Karnieli, E. (2013) Overcoming Barriers in the Implementation of Personalized Medicine into Clinical Practice. Isr Med Assoc J 15(10), pp. 599-601.

[3] Depta, J. P., and Bhatt, D. L. (2012) Antiplatelet Therapy and Proton Pump Inhibition: Cause for Concern? Curr Opin Cardiol 27(6), pp. 642-650.

[4] Pollin, T. I., Damcott, C. M., Shen, H., Ott, S. H., Shelton, J., Horenstein, R. B., Post, W., McLenithan, J. C., Bielak, L. F., Peyser, P. A., Mitchell, B. D., Miller, M., O'Connell, J. R., and Shuldiner, A. R. (2008) A Null Mutation in Human Apoc3 Confers a Favorable Plasma Lipid Profile and Apparent Cardioprotection. Science 322(5908), pp. 1702-1705.

[5] Rampersaud, E., Damcott, C. M., Fu, M., Shen, H., McArdle, P., Shi, X., Shelton, J., Yin, J., Chang, Y. P., Ott, S. H., Zhang, L., Zhao, Y., Mitchell, B. D., O'Connell, J., and Shuldiner, A. R. (2007) Identification of Novel Candidate Genes for Type 2 Diabetes from a Genome-Wide Association Scan in the Old Order Amish: Evidence for Replication from Diabetes-Related Quantitative Traits and from Independent Populations. Diabetes 56(12), pp. 3053-3062.

[6] Shuldiner, A. R., O'Connell, J. R., Bliden, K. P., Gandhi, A., Ryan, K., Horenstein, R. B., Damcott, C. M., Pakyz, R., Tantry, U. S., Gibson, Q., Pollin, T. I., Post, W., Parsa, A., Mitchell, B. D., Faraday, N., Herzog, W., and Gurbel, P. A. (2009) Association of Cytochrome P450 2c19 Genotype with the Antiplatelet Effect and Clinical Efficacy of Clopidogrel Therapy. JAMA 302(8), pp. 849-857.

[7] Pihoker, C., Gilliam, L. K., Ellard, S., Dabelea, D., Davis, C., Dolan, L. M., Greenbaum, C. J., Imperatore, G., Lawrence, J. M., Marcovina, S. M., Mayer-Davis, E., Rodriguez, B. L., Steck, A. K., Williams, D. E., and Hattersley, A. T. (2013) Prevalence, Characteristics and Clinical Diagnosis of Maturity Onset Diabetes of the Young Due to Mutations in Hnf1a, Hnf4a, and Glucokinase: Results from the Search for Diabetes in Youth. J Clin Endocrinol Metab 98(10), pp. 4055-4062.

[8] Shuldiner, A. R., Relling, M. V., Peterson, J. F., Hicks, J. K., Freimuth, R. R., Sadee, W., Pereira, N. L., Roden, D. M., Johnson, J. A., Klein, T. E., Vesely, M., Robinson, S. W., Ambulos, N., Jr., Stass, S. A., Kelemen, M. D., Brown, L. A., Pollin, T. I., Beitelshees, A. L., Zhao, R. Y., Pakyz, R. E., Palmer, K., Alestock, T., O'Neill, C., Maloney, K., Branham, A., Sewell, D., Crews, K., Hoffman, J., Cross, S., Haidar, C., Baker, D., Bell, G., Greeson, F., Gaur, A., Reiss, U., Huettel, A., Cheng, C., Gajjar, A., Pappo, A., Howard, S., Hudson, M., Pui, C. H., Jeha, S., Evans, W. E., Broeckel, U., Altman, R. B., Gong, L., Whirl-Carrillo, M., Manickam, K., Sweet, K. M., Embi, P. J., Roden, D., Peterson, J., Denny, J., Schildcrout, J., Bowton, E., Pulley, J., Beller, M., Mitchell, J., Danciu, I., Price, L., Weinshilboum, R., Wang, L., Nelson, D., Clare-Salzler, M., Elsey, A., Burkley, B., Langaee, T., Liu, F., Nessl, D., Dong, H. J., Lesko, L., and Chute, C. G. (2013) The Pharmacogenomics Research Network Translational Pharmacogenetics Program: Overcoming Challenges of Real-World Implementation. Clin Pharmacol Ther 94(2), pp. 207-210.

[9] Relling, M. V., and Klein, T. E. (2011) Cpic: Clinical Pharmacogenetics Implementation Consortium of the Pharmacogenomics Research Network. Clin Pharmacol Ther 89(3), pp. 464-467.

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