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**Biomarkers for pancreatic cancer: Recent achievements in proteomics and genomics through classical and multivariate statistical methods**

Marengo E *et al.* Statistical biomarkers identification in pancreatic cancer

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**Abstract**

Pancreatic cancer (PC) is one of the most aggressive and lethal neoplastic diseases. A valid alternative to usual invasive diagnostic tools would certainly be the determination of biomarkers in peripheral fluids to provide less invasive tools for early diagnosis. Nowadays, biomarkers are in general investigated mainly in peripheral blood and tissues through high-throughput–omics techniques comparing control versus pathological samples. The results can be evaluated by two main strategies: (1) classical methods in which the identification of significant biomarkers is accomplished by monovariate statistical tests where each biomarker is considered as independent from the others; and (2) multivariate methods, taking into consideration the correlations existing among the biomarkers themselves. This last approach is very powerful since it allows the identification of pools of biomarkers with diagnostic and prognostic performances which are superior to single markers in terms of sensitivity, specificity and robustness. Multivariate techniques are usually applied with variable selection procedures to provide a restricted set of biomarkers with the best predictive ability; however, standard selection methods are usually aimed to the identification of the smallest set of variables with the best predictive ability and exhaustivity is usually neglected. The exhaustive search for biomarkers is instead an important alternative to standard variable selection since it can provide information about the etiology of the pathology by producing a comprehensive set of markers. In this review, the most recent applications of the –omics techniques (proteomics, genomics and metabolomics) to the identification of exploratory biomarkers for PC will be presented with particular regard to the statistical methods adopted for their identification. The basic theory related to classical and multivariate methods for biomarkers identification is presented; then, the most recent applications in this field are discussed.

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**Key words:** Pancreatic cancer; Biomarker identification; Multivariate analysis; Principal component analysis; Ranking-principal component analysis

**Core tip:** Biomarkers are statistically identified as significant by: (1) classical statistical tests where each biomarker is independent from the others; and (2) multivariate methods that take into consideration the correlation among the biomarkers. This last approach provides pools of biomarkers with superior diagnostic and prognostic performances. Multivariate techniques are often applied with variable selection procedures to provide the smallest set of biomarkers with the best predictive ability. The exhaustive identification is instead a valid alternative since it can provide comprehensive information about the etiology of the pathology.The most recent applications of the –omics approaches to the identification of biomarkers for PC are presented with particular regard to the statistical methods adopted.

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**INTRODUCTION**

Pancreatic cancer (PC) is one of the most aggressive and lethal neoplastic diseases: its early detection is therefore fundamental since surgery at an early disease stage is the preferred and most promising therapy. About 20% of patients could be operated at time of diagnosis; the 5-year survival rate for not-operable patients is about 1%, while the 5-year survival after surgery is about 20% without an adjuvant therapy and about 25%-30% with the therapy[1-3]. The lack of early symptoms and the high aggressiveness are the main causes of late diagnosis and high mortality of this disease. To this respect, the search for biomarkers of early diagnosis would be highly recommended to improve the early diagnostic rate thus improving patients’ prognosis.

Diagnosis is usually based on invasive techniques [ultrasound endoscopy (EUS), explorative laparoscopy or laparotomy], or on methods that can be at least inconvenient for patients [computed tomography (CT), magnetic resonance imaging (MRI), endoscopic retrograde or magnetic resonance cholangiopancreatography (ERCP and MRCP)][2].

A valid alternative would certainly be the determination of specific biomarkers in peripheral fluids in order to provide less invasive tools for early diagnosis: in this direction are directed the most recent efforts in the field of biomarker identification for PC. A wide range of serum markers for PC has been reported[2,4] but few of them are exploited in clinical routine since they show in general low sensitivity and/or specificity. Bunger *et al*[2] reviewed about 43 serum biomarkers for PC divided into four main groups: carbohydrates (CA19-9, CA 50, CA 125, CA195, CA 72-4), carcinoembryonic antigens, other markers and the combination of different markers.

Together to diagnostic markers, great efforts have recently been made to identify predictive and prognostic biomarkers for PC. Tissue biomarkers for the prognosis of Pancreatic Ductal Adenocarcinoma (PDAC) have recently been reviewed by Jamieson *et al*[4]. The considerations driven for diagnostic biomarkers apply also for predictive and prognostic ones: also in this case, the identification of markers from peripheral blood should be the best alternative to provide prognostic methods less invasive for patients’. Nowadays, both diagnostic and prognostic/predictive biomarkers are in general investigated mainly in peripheral blood or tissues through high-throuput –omics techniques. The results can be evaluated by two different strategies, namely by: (1) classical statistical methods consisting in the use/identification of significant biomarkers by monovariate statistical tests where each biomarker is considered as independent from the others; and (2) multivariate methods, able to take into consideration the multivariate structure of the data and the correlations existing among the potential biomarkers. This last approach is very powerful since it allows the identification of pools of biomarkers with diagnostic and/or prognostic performances superior to single markers in terms of sensitivity, specificity and robustness.

It is important to point out that usually biostatistical methods can be defined as multivariate (several endpoints and several predictors) or multivariable (one endpoint, several predictors): in this specific context, the authors will generally apply the term multivariate to identify methods that allow the evaluation of the correlations existing between the variables, i.e. their synergisms and/or antagonisms.

Multivariate techniques are usually applied with variable selection procedures[5,6] to provide a set of candidate biomarkers with the best predictive ability; however, standard selection tools are aimed to the identification of the smallest set of variables with the best predictive ability. It is the authors’ opinion that also exhaustivity should be addressed to[7]. Biomarkers are in facts useful not only for diagnostic/prognostic purposes but also to better understand the etiology of pancreatic cancer: from this point of view, the exploitation of high-throughput methods provides a lot of information that should not be neglected. The exhaustive identification of all possible biomarkers, showing large correlations, could provide information about the overall mechanism of action of the disease thus opening the way towards new therapeutic strategies.

In this review, the most recent applications of the –omics approaches (proteomics, genomics and metabolomics) to the identification of biomarkers for pancreatic cancer will be presented with particular regard to the statistical methods adopted for their identification and focusing especially on exploratory biomarkers. High-throughput techniques will probably constitute the future in the field of exploratory biomarker search due to the great amount of information they convey; moreover, the possibility of combining the results emerging from proteomic, genomic and metabolomic studies with clinical information can provide exhaustive panels of markers, thus improving their predictive performance with better sensitivity and specificity.

First, the theory of the classical and multivariate methods for biomarkers identification will be presented; then, the most recent applications in this field will be presented.

**STATISTICAL METHODS**

The statistical methods presented here can be divided into four main groups: (1) classical methods for biomarkers identification based on monovariate approaches; (2) tools for biomarkers search based on multivariate approaches; (3) methods for the analysis of survival outcomes; and (4) other methods. Only the tools recently applied to the specific case of pancreatic cancer will be briefly presented, an exhaustive treatment of all multivariate procedures in the field of biomarkers search being out of the scope of the present review.

**CLASSICAL MONOVARIATE METHODS FOR BIOMARKERS IDENTIFICATION**

The classical approach to the identification of markers of a specific disease consists in the evaluation of which variables show a different behavior between two groups of samples (control *vs* pathological, control *vs* drug-treated, *etc*). The easiest statistical way to solve this problem is the application of classical statistical tests to each biomarker candidate separately and the calculation of the I type error that can be accomplished comparisonwise (for each hypothesis independently) or experimentwise (testing all hypotheses together). The second alternative is to be preferred since the type I error probability increases as the number of tests increases. The identification of significant markers is therefore accomplished by the Student’s *t*-test, for each variable independently and by applying a correction taking into account the number of multiple tests available: Bonferroni’s method with subsequent modifications[8] or the corrections proposed by Dunn and Sikak and by Dunnet[9-11]. This approach is incorrectly defined multivariate since it does not take into consideration the correlations eventually existing between the variables. The same approach can also be applied to non-parametric tests, to be exploited when the number of samples is too small or when the assumptions at the basis of parametric tests are not verified. Among them, the most widespread in the biomedical field is the Mann-Withney test[8].

An alternative is the exploitation of global tests aimed to demonstrate a global hypothesis (*e.g.,* the effect of a therapy) considering all the tests simultaneously: an example is the T2 Hotelling test[8].

Classical procedures also comprise the approach based on the analysis of variance both in its two-way (ANOVA) or multi-way (MANOVA) versions[8]. In this case it is possible to compare also more than two groups of samples.

An alternative to classical hypothesis testing is the use of the Bayes factors[12], providing a more robust approach This factor, for comparing two hypotheses H1 and H2 may be approximated as the ratio of the marginal likelihood of the data under the two hypotheses.

can be interpreted as follows: B ≤ 0.1: strongly against H1; 0.1 < B ≤ 1: against H1; 1 ≤ B < 3: barely worth mentioning for H1; 3 ≤ B << 10: substantially for H1; B > 10: strongly for H1.

**MULTIVARIATE METHODS FOR BIOMARKERS IDENTIFICATION**

Methods that can be properly defined *multivariate* are based on the comparison of two or more groups of samples taking into account the relationships existing between the variables, rather than considering them as independent. This approach is certainly more effective since it is fundamental to consider the synergic or antagonistic effects of different factors, i.e. their interactions; moreover, when independent tests are performed on several factors and a high correlation exists between them, the outcome of the test can be completely wrong[13]. The methods here presented belong to two approaches: (1) unsupervised methods (pattern recognition methods), in which no *a priori* information is assumed, and the evaluation of the existence of groups of samples is suggested by the statistical method itself; (2) supervised methods (classification tools), in which the *a priori* information is provided in terms of membership of each sample to a specific class: the statistical method is therefore aimed to the identification of the variables responsible for the separation of the samples in the different classes.

Here, only the methods most recently applied in literature to the case of pancreatic cancer will be briefly discussed. The methods presented are listed in Table 1.

**UNSUPERVISED AND PATTERN RECOGNITION METHODS**

***Principal component analysis***

Principal Component Analysis (PCA)[14,15] represents the objects in a new reference system characterised by new variables called Principal Components. The first principal component accounts for the maximum variance contained in the original dataset, while subsequent components account for the maximum residual variance: they are calculated hierarchically, so that systematic variations (*i.e.,* information) are explained in the first components while experimental noise and random variations are contained in the last ones. The components are linear combinations of the original variables and are orthogonal to each other, thus containing independent sources of information. They are often used for dimensionality reduction, by considering a smaller number of significant components, containing only relevant information.

The graphical representation of the *scores* (the co-ordinates of the samples in the new reference system) in the space of the principal components, allows the identification of groups of samples showing a similar behaviour (samples close one to the other in the graph) or different characteristics (samples far from each other). The corresponding loading plot (representing the *loadings*, i.e. the coefficients of the linear combination describing each principal component) identifies the variables that are responsible for the analogies or the differences detected for the samples in the score plot.

***Cluster analysis***

Cluster analysis techniques[14-16] allow the identification of groups of samples or of variables in a dataset by investigating the relationships between the samples or the descriptors. Agglomerative hierarchical methods[14,15] are the most widespread, grouping the samples on the basis of their similarity. The most similar samples or groups are linked first. The final result is a graph, called dendrogram, where the samples are represented on the X axis and are connected at decreasing levels of similarity along the Y axis. The groups can be identified by applying a horizontal cut of the dendrogram and identifying the number of vertical lines crossed by the horizontal cut. Figure 1 reports a dendrogram where cutting at level 4000 produces only two clusters (the 2 different tumor cell lines), while cutting at level 1500 produces 4 clusters (PACA and T2M4 cell lines, treated and untreated with Trichostatin-A). The results of hierarchical clustering strongly depend on the measure of similarity and on the linking method adopted. Clustering techniques can be applied both to the original variables or to the relevant principal components[16].

***Multidimensional scaling***

Multidimensional scaling (MDS)[17,18] is aimed to dimensionality reduction and graphical representation of the data. Given a set of *n* objects and a measure of their similarity, MDS searches for a low dimensional space in which the objects are represented by points in the space and such that the distances between the points match as much as possible with their original similarities[17]. There are several different approaches to MDS depending on the measure of the similarities matching, on the metrics, on the method used to compute the similarities and on the way the samples configuration is obtained[19,20]. Shepard[21,22] and Kruskal[23] provided an extension of classical MDS to the study of nonparametric similarities.

**SUPERVISED CLASSIFICATION METHODS**

Classification tools are supervised methods able to separate the objects in the classes present (known *a priori*, *e.g.,* control *vs* pathological) and providing the variables most responsible for their belonging to different classes (candidate biomarkers). The final aim of their application in the biomedical field is both the development of diagnostic tools and the identification of the differences existing between the classes, to shed light on the etiology of a disease or the effect of a new drug. Here, only the methods already applied to the identification of biomarkers for PC will be described.

***Soft-independent model of class- analogy***

The Soft-independent model of class- analogy (SIMCA)method[24-27] is based on the independent modelling of each class by means of PCA. Each class is described by its relevant principal components. The samples belonging to each class are contained in the so-called SIMCA boxes, defined by the relevant components of each class. The classification of each sample with SIMCA is not affected by experimental uncertainty and random variations since each class is modelled only by its relevant components. This method is also useful when more variables than objects are available since it performs a substantial dimensionality reduction.

The identification of the candidate biomarkers by SIMCA can be accomplished by the analysis of the Discrimination Power (DP), a measure of the ability of each variable to discriminate between two classes at a time. The greater the DP, the more a variable weights on the classification of an object in one of the two classes compared.

***Ranking-PCA***

Ranking-PCA is a ranking method proposed by Marengo, Robotti *et al*[7,28,29] based on the description of the original data by means of Principal Components. The use of PCA in the field of biomarker identification in the -omics sciences is particularly effective since it allows to take into consideration the relationships between the variables, providing sets of correlated biomarkers with a similar function, and the possibility of solving problems where the number of variables is larger than the number of samples.

PCA is used here to describe the data coupled to a ranking procedure of the candidate biomarkers in forward search: one variable is added at each cycle. The first variable selected is the one providing the best separation between the classes on the first principal component. The addition of another discriminating variable further improves the distance between the two classes on the first principal component. If successively a non-discriminating variable is added, instead, the two classes will not be further separated on the same componentSometimes, more than one component could be necessary for class separation: in this case different independent sources of information related to the class structure are present in the data! In this case, the subsequent principal component accounting for class separation will be included in the model.

The proposed method allows the ranking of the variables according to their discrimination ability, thus assuring the exhaustiveness of the results. The result can be presented in graphical form (Figure 2) where the classification performance or the class distance are reported as a function of the variables added to the model.

***Orthogonal partial least squares***

PLS[14,15,30] establishes a relationship between one or more dependent variables (Y) and a group of descriptors (X). X and Y variables are modeled simultaneously, to find the latent variables (LVs) in X that will predict the LVs in Y. These LVs are calculated hierarchically, as for PCA. PLS was originally set up to model continuous responses but it can be applied even for classification purposes (PLS-DA) by establishing an appropriate Y related to the association of each sample to a class. The regression is then carried out between X-block variables and the Y just established. Orthogonal Partial Least Squares (OPLS)[31] is a modification of PLS developed for highly decorrelated datasets.

***Classification and regression tree***

Classification trees[24] are built by subsequent divisions (splits) of subgroups of the original data D in two descending subgroups with the aim of classifying the data in homogeneous groups as much as possible different one from the others. It is possible to derive a tree diagram where, starting from the root node (where the data D are not separated), a series of nodes and branches separate; each node h represents a subgroup of D. Nodes not undergoing a further split are called terminal nodes: to each terminal node a mode for y is associated.

Starting from the root node h1, data are separated in a series of splits: in each node the split giving the most homogeneous division of the data in the two descendent nodes is selected.

***Random forests***

Random Forests[32] is an extension of classification trees and is structured to grow many classification trees. To classify a new object, the new object is first classified by each independent tree in the forest. The forest chooses the most recurrent classification (over all the trees in the forest).

The error rate depends on: the *correlation* between any two trees in the forest (increasing the correlation increases the forest error rate); the *strength* of each individual tree in the forest (inversely correlated to the tree error rate).

**METHODS FOR THE ANALYSIS OF SURVIVAL OUTCOMES**

In clinical trials it is usually important to evaluate the time until the participants present a particular event (endpoint), *i.e.,* a clinical outcome (death, recurrence of a disease, remission *etc*). All participants are followed from a certain starting point (operation, starting of a therapy, diagnosis, *etc.*) up to the moment when the event occurs (the time requested is recorded). However, often the outcome of some participants can be unknown: when the study ends before all participants have presented the event; when some participants withdraw from the study. In these cases (censored data) the time of follow-up is recorded.

***Kaplan-Meier estimates of survival functions***

The Kaplan-Meier method[33] is used to provide survival functions and it can be effectively applied also to censored data. It provides a survival curve where time is reported on the x-axis, while the cumulative survival probability is on the y-axis. The time corresponding to the point where the curve crosses 50% survival is the estimate of median survival. Kaplan-Meier curves can be compared across groups by applying mainly two non-parametric tests (the log rank test [34] and the generalized Wilcoxon test[35]). The generalized Wilcoxon test [35] is a weighted alternative where early time points weight more than late ones: this is to be preferred when the effect of an experimental condition vanishes with time. The log rank test instead is to be preferred to detect differences during all the follow-up period.

***Cox regression***

Cox regression[36] is applied to evaluate the effect of several risk factors on survival, defining the hazard as the probability of the endpoint. The hazard is modeled as:

$$ln\left(\frac{H\left(t\right)}{H\_{0}\left(t\right)}\right)=b\_{1}X\_{1}+b\_{2}X\_{2}+…+b\_{k}X\_{k}$$

where H(t)/H0(t) is the so-called hazard ratio, X1 ... Xk are predictor variables and H0(t) is the baseline hazard at time. In general, the hazard ratio may assume only positive values: if it equals 1 the groups show a not statistically different survival; if it is smaller than 1 a subject with a higher value for X has lower risk than a subject with a lower value for X; the opposite behavior is obtained if the hazard ratio estimate is larger than 1.

The Cox model works under the so-called proportional hazards assumption: the ratio between the hazards of two patient groups remains constant over the complete follow-up period. Since in Cox regression a hazard ratio is calculated, this estimate should apply to all death times: this simplification is only justified if the group difference remains constant over the whole range of follow-up time.

If the monovariate Cox regression is extended to include more than one X variable (multivariate Cox regression), the effect of the interaction between different factors can be evaluated.

**OTHER METHODS**

***Prediction analysis for microarrays***

Prediction analysis for microarrays (PAM) performs sample classification from gene expression data and survival outcomes, exploiting the nearest shrunken centroid approach[37]. A standardized centroid for each class is computed. The nearest centroid classification compares the gene expression profile of a new sample to each class centroid: the class whose centroid is closest, in squared distance, is the predicted class for the new sample.

Nearest shrunken centroid classification[37] is a modification of this approach that When PAM is applied to survival outcomes, supervised principal components analysis[38] is performed, where, instead of using all the genes to perform PCA, only a subset of genes is used, i.e. those highly correlated with survival.

***Metropolis algorithm and monte carlo simulation***

Monte Carlomethods[39] are computational algorithms that rely on repeated random sampling to obtain numerical results: simulations are run several times to calculate probabilities. They are useful for simulating systems with several degrees of freedom and modeling systems characterized by large uncertainty in inputs

The Metropolisalgorithm[40] is used to generate a series of numbersx1, x2, .., xn with a distribution fixed *a priori*. The method is based on the generation of numbers that are accepted or rejected to obtain the selected type of distribution.

The Metropolis algorithm can be implemented in Monte Carlo simulations to perform random sampling. The Monte Carlo optimization can be used in biomarkers search, to determine the coefficients of model containing the relevant biomarkers.

**STUDIES FOR THE IDENTIFICATION OF BIOMARKERS**

***Studies based on serum and tissue biomarkers determined by non-omics techniques***

The studies based on serum and tissue biomarkers determined by non-omics techniques usually exploit monovariate and multivariate Cox regression to evaluate the effect played by different factors on time to progression (TTP) and overall survival (OS). They will be presented here divided in prognostic/predictive biomarkers and diagnostic biomarkers.

***Prognostic and predictive biomarkers***

Recent studies about prognostic and/or predictive biomarkers (Table 2) determined in serum or plasma regard the determination of glycoproteins, both alone or associated to other markers, and the determination of circulating factors of the insulin-like growth factor. Three recent studies have been published based on CA19-9, one of the most debated biomarkers for PC. The first study, by Boeck *et al*[41], includes 115 patients with histologically confirmed advanced PC treated with first-line therapy. The novelty of this study is the modelling of the effect of CA 19-9 kinetics by treating it as a time-varying covariate. For CA 19-9 kinetics during chemotherapy, data from 69 patients (TTP) and 84 patients (OS) were available. The proposed approach allowed the modeling of the effect of log(CA 19-9) measured during therapy on the event (TTP or OS) rather than modeling the effect of the pretreatment value of log(CA 19-9).

In the second study, Haas *et al*[42] pooled pre-treatment data on CA 19-9, CEA, CRP, LDH and bilirubin from two multicenter randomized phase II trials and prospective patient data. Marker levels were assessed before the start of palliative first-line therapy for advanced PC and during treatment (for CA 19-9 only).

In the third study, Boeck *et al*[43] evaluated pre-treatment (palliative first-line chemotherapy) values and weekly values of Cytokeratin 19-fragments (CYFRA 21-1), CA 19-9 and CEA in blood samples from patients with PC. CYFRA 21-1 are biomarkers for different epithelial diseases but their role in PC have not yet been investigated.

Boeck *et al*[41] pre-treatment log(CA19-9) proved to be significantly associated with TTP and OS. Moreover, log (CA 19-9) kinetics after start of treatment was found to be a significant predictor for both TTP and OS. Similar results were found Haas *et al*[42] where pre-treatment CA 19-9, LDH and CEA levels were significantly associated with TTP. Regarding OS, baseline CA 19-9, LDH, CRP and bilirubin were significant. Boeck *et al*[43], CYFRA 21-1 and CA 19-9 showed a high correlation with TTP and OS, while in multivariate analysis, only CYFRA 21-1 and performance status were independent predictors for OS.

For what regards baseline circulating factors of the insulin-like growth factor (IGF) axis, McCaffery *et al*[44] assessed their predictive nature on the treatment effect of ganitumab plus gemcitabine in metastatic PDAC. Baseline levels of IGFs/IGF binding proteins were analysed in serum or plasma while mutations and gene expression were analysed in archival samples. Ganitumab was associated with improved OS versus placebo. The treatment effect on improved OS was larger in patients with higher levels of IGF-1, IGF-2 or IGFBP-3, or lower levels of IGFBP-2. Interaction between treatment and IGFs/IGFBPs showed predictive potential for IGF-2 and IGFBP-2.

The studies about prognostic and/or predictive biomarkers determined in tissue samples, usually by immunohistochemistry or tissue microarray analysis (TMA), include instead the determination of glycoproteins, other proteins and enzymes.

Fong *et al*[45] investigated the expression of TROP2 (human trophoblast cell-surface) antigen, a glycoprotein found to be strongly expressed in a variety of human epithelial cancers, and the expression of junctional adhesion molecule A (JAM-A) antigen[46], a type I transmembrane glycoprotein, which has been recently shown to affect the prognosis of several malignancies. The two studies involved 197 and 186 patients with PDAC respectively. TROP2 overexpression was observed in 55% of patients, while low expression of JAM-A was observed in 42% of samples; both markers were significantly associated with decreased OS. They both were correlated with lymph node metastasis and tumour grade. In the subgroup of patients surgically treated with curative intent, TROP2 and low expression of JAM-A correlated with poor progression-free survival.

In the study by Zong *et al*[47], the expression of the T-box transcription factor 4 (TBX4) was investigated in 77 stage II PDAC tumours. 62.3% of cases expressed TBX4 at a high level. Significant correlation was only detected between TBX4 expression and tumor grade and liver metastasis recurrence. The survival with TBX4-high expression was significantly longer.

Applying tissue microarray (TMA) analysis, Schafer *et al*[48] correlated heat shock protein 27 (HSP27) expression status with clinic-pathologic parameters in PDAC from 86 patients. HSP27 expression was found in 49% of tumour samples. A significant correlation was found with OS. The authors also assessed the impact of HSP27 on chemo- and radio-sensitivity directly in PC cells. HSP27 expression emerged as an independent prognostic factor and correlated inversely with nuclear p53 accumulation, indicating protein interactions between HSP27 and p53 or TP53 mutation-dependent HSP27-regulation. HSP27 overexpression rendered HSP27 low-expressing PL5 PC cells more susceptible towards treatment with gemcitabine, while HSP27 protein depletion in HSP27 high-expressing AsPC-1 cells caused increased gemcitabine resistance.

Marechal *et al*[49] identified Deoxycitidine Kinase (dCK), a recombinant enzyme, as associated with prolonged survival after adjuvant Gemcitabine administration for resected PDAC. The study involved 45 patients. The lymph node (LN) ratio and dCK protein expression were significant predictors of DFS and OS in univariate analysis. On multivariate analysis, a step-down procedure based on the likelihood ratio test, dCK protein expression was the only independent prognostic factor.

Having previously reported that Notch3 activation appeared to be associated with more aggressive PC disease, Mann *et al*[50] examined components of this pathway (Notch1, Notch3, Notch4, HES-1, HEY-1) in resectable and non-resectable tumours compared to uninvolved pancreas. All three Notch family members were significantly increased in tumour tissue with expression maintained within matched lymph node metastases. Significantly higher nuclear expression of Notch1, -3 and -4, HES-1, and HEY-1 was noted in locally advanced and metastatic tumours compared to resectable cancers. Nuclear Notch3 and HEY-1 expression were significantly associated with reduced OS and DFS following tumour resection.

***Diagnostic biomarkers***

For what regards diagnostic biomarkers (Table 2), the most recent studies are based on the determination of proteins panels in serum or plasma exploiting ELISA or proximity ligation assay (PLA) for their determination. PLA is a highly sensitive technique for multiplex detection of biomarkers in plasma with little interfering background signal. Some of the studies proposed regard the determination of both diagnostic and prognostic markers. All studies here presented involve CA19-9 as a potential biomarker in association with other markers.

In the first study, Chang *et al*[51] applied PLA to the identification of plasma levels of 21 biomarkers in 52 locally advanced PDAC cases and 43 age-matched controls. The optimal diagnostic biomarker panel was computed using a combination of the PAM algorithm and logistic regression modeling.

In the second study, Brand *et al*[52] investigated 83 circulating proteins in sera of patients diagnosed with PDAC, benign pancreatic conditions and healthy controls. Samples from each group were split randomly into training and blinded validation sets prior to analysis. A Metropolis algorithm with Monte Carlo simulation (MMC) was used to identify discriminatory biomarker panels in the training set. Identified panels were evaluated in the validation set and in patients diagnosed with colon, lung and breast cancers.

In the third study, Schultz *et al*[53] tested the hypothesis that high plasma YKL-40 and IL-6 associate with PC and short OS. 559 patients with PC from prospective biomarker studies were studied. Plasma YKL-40 and IL-6 were determined by ELISA and serum CA 19.9 by chemiluminescent immunoassay.

Chang *et al*[51] three markers (CA19-9, OPN and CHI3L1) were found to have superior sensitivity for PC *vs* CA19-9 alone (93% *vs* 80%) and two markers (CEA and CA125) proved to have a prognostic significance for survival of PC (*P* < 0.003) when measured simultaneously. Brand *et al*[52] the panel of CA 19–9, CEA, and TIMP-1 discriminated PDAC patients from benign subjects with an SN/SP of 76/90% in the training set and of 71/89% in the validation set. The CA19–9, ICAM-1, OPG panel is selective for PDAC and does not recognize breast (SP 100%), lung (SP 97%), or colon (SP 97%) cancer. Schultz *et al*[53] instead, high preoperative IL-6 and CA 19.9 were independently associated with short OS. High pre-treatment levels of each biomarker were independently associated with short OS in non-operable patients.

**PROTEOMIC BASED STUDIES**

The most recent studies on biomarkers identification in proteomics (Table 3) mainly regard the determination of diagnostic markers. The studies are presented separated in those carried out from serum or tissue samples and those carried out on cell lines or animal models. The studies here presented are mainly devoted to identify exploratory biomarkers.

The studies based on proteomic approaches show a great potential for the identification of PC biomarkers: the panels of markers identified by these techniques are characterized by good performance for what regards both sensitivity and specificity, showing results at least equal to or in some cases better than classical approaches. It is the authors’ opinion that in future the information provided by such high-throughput techniques should be coupled to clinical information to provide exhaustive sets of biomarkers with better predictive ability.

***Diagnostic biomarkers in tissue and serum samples***

Tissue and serum biomarkers in proteomics are usually determined by: SDS-PAGE followed by LC-MS for identifying the most up- or down-regulated proteins; matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry; surface-enhanced laser desorption/ionization time-of-flight (SELDI-TOF) mass spectrometry. The exploitation of instrumental techniques providing a high amount of information makes it necessary the application of multivariate methods in order to detect panels of biomarkers with the best predictive ability.

In the first study, by McKinney *et al*[54], matched pairs of tumor and non-tumor pancreas from patients undergoing tumor resection were treated to obtain cytosol, membrane, nucleus and cytoskeleton cellular protein fractions. The fractions were analysed by SDS-PAGE followed by LC-MS/MS to identify 2393 unique proteins. The spectral count data were compared using a power law global error model (PLGEM) to identify statistically significant protein changes between non-tumor and tumor samples[55,56]. Among the 104 proteins significantly changed in cancers, four (Biglycan (BGN), Pigment Epithelium-derived Factor (PEDF) Thrombospondin-2 (THBS-2) and TGF-β induced protein ig-h3 precursor (βIGH3)) were further validated and proved to be up-regulated in cancer and potential for development as minimally-invasive diagnostic markers.

Kojima *et al*[57] differentiated pancreatic neoplasia from non-neoplastic pancreatic disease. Samples from 50 patients (15 healthy (H), 24 cancer (Ca), 11 chronic pancreatitis (CP)) were collected. A high-throughput method was applied, using high-affinity solid lipophilic extraction resins, enriched low molecular weight proteins for extraction with a high-speed MALDI-MS. Multivariate analysis was carried out by MDS as in Mobley *et al*[58]. Using eight serum features, Ca were differentiated from H (SN 88%, SP 93%), Ca from CP (SN 88%, SP 30%), and Ca from both H and CP combined (SN 88%, SP 66%). In addition, nine features obtained from urine differentiated Ca from both H and CP combined with high efficiency (SN 90%, SP 90%). Interestingly, the plasma samples did not show significant differences.

Ehmann *et al*[59] and Hauskrecht *et al*[60] groups applied instead SELDI-TOF mass spectrometry. In the first study[59], 96 serum samples from patients undergoing cancer surgery were compared with 96 controls. Samples were fractionated by anion exchange chromatography. Data analysis, involving Mann-Withney test and Classification and Regression Tree (CART) analysis, identified 24 differentially expressed protein peaks, 21 of which were under-expressed in cancer samples. The best single marker can predict 92% of controls and 89% of cancer samples. The best model including a set of 3 markers showed a sensitivity of 100% and a specificity of 98% for the training data and a sensitivity of 83% and a specificity of 77% for test data. Apolipoprotein A-II, transthyretin and apolipoprotein A-I were identified as markers (decreased in cancer sera). Hauskrecht *et al*[60] instead proposed a feature selection method that extracts useful feature panels from high-throughput spectra. 57 PC samples were compared to 59 controls. The results clearly show the improved classification performances when the method is compared to standard strategies.

The last study makes use of protein microarrays to explore whether a humoral response to PC-specific tumor antigens has utility as a biomarker of PC. To determine if such arrays can be used to identify novel autoantibodies in the sera from PC patients, Patwa *et al*[61] resolved proteins from a PDAC cell line (MIAPACA) by 2-D liquid-based separations, and then arrayed them on nitrocellulose slides. The slides were probed with sera from a set of patients diagnosed with PC and compared with age- and sex-matched normal subjects. To account for patient-to-patient variability, a non-parametric Wilcoxon rank-sum test was used, in which protein biomarkers were identified. Classification, by the PAM algorithm, showed 86.7% accuracy, with a SN and SP of 93.3% and 80%, respectively. The identified candidate autoantibody biomarkers were validated using an independent sample set of 238 samples. Phosphoglycerate kinase-1 and histone H4 were noted to elicit a significant differential humoral response in cancer sera.

***Diagnostic biomarkers from cell lines or animal model samples***

Diagnostic biomarkers in proteomics have also been recently determined from cell lines (PACA44, T3M4) or on animal models. The most exploited analytical techniques in this case are SDS-PAGE followed by LC-MS, 2D-PAGE or 2D-LC approaches.

Marengo, Robotti *et al*[62] identified the regulatory proteins in human PC treated with Trichostatin A by 2D-PAGE maps and multivariate analysis. PCA was applied to a spot quantity dataset comprising 435 spots detected in 18 samples belonging to two different cell lines (Paca44 and T3M4) of control and drug-treated PDAC cells. PCA allowed the identification of the groups of samples present in the dataset: the loadings analysis allowed the identification of the differentially expressed spots, which characterize each group of samples. The treatment of both the cell lines with Trichostatin A showed an evident effect on the proteomic pattern of the treated samples. Identification of some of the most relevant spots was also performed by MS analysis. The same authors applied to the same set of data different multivariate statistical tools to provide sets of candidate biomarkers: the first application regards the exploitation of SIMCA classification to evaluate the biomarkers characterized by a significant discriminant power[25], while the second application regards the development of Ranking-PCA[28]. This second application is particularly interesting to overcome the limitations of the methods usually adopted as variable selection tools to identify only significant biomarkers: they are usually aimed to the selection of the smallest set of variables (spots) providing the best performances in prediction. This approach seems not to be the best choice in the identification of potential biomarkers since all the possible candidate biomarkers have to be identified to provide a general picture of the ‘‘pathological state’’: exhaustivity has to be preferred to provide a complete understanding of the mechanisms underlying the pathology. Ranking-PCA allowed the exhaustive identification of a complete set of candidate biomarkers.

Paulo *et al*[63] compared differentially expressed proteins in rat in activated and serum-starved non-proliferating Pancreatic stellate cells (PaSC), emerging key mediators in chronic pancreatitis and PC pathogenesis. About 1500 proteins were identified after SDS-PAGE and LC-MS/MS. Qualitative and quantitative proteomic analysis revealed several hundred proteins as differentially abundant between the two cell states. Significance analysis was performed using QSPEC, a recently published algorithm for determining the statistical significance of differences in spectral counting data from two sample sets[64]. This algorithm exploits the Bayes Factor, instead of the p-value, as a measure of statistical significance[65,66]. Proteins of greater abundance in activated PaSC included isoforms of actin (*e.g.,* smooth muscle actin) and ribosomal proteins. Proteins more abundant in non-proliferating PaSC than in activated PaSC included signaling proteins MAP kinase 3 and Ras-related proteins. The molecular functions and biological pathways for these proteins were also determined by Gene Ontology analysis and KEGG pathway.

***Other studies based on a proteomic approach***

Paulo *et al*[67] also evaluated the endoscopic pancreatic function test (ePFT) as a method able to safely obtain pancreatic fluid for MS analysis from patients during upper endoscopy and reproducibly identify pancreas-specific proteins. The ePFT-collected pancreatic fluid from 3 individuals without evidence of chronic pancreatitis was analysed by SDS-PAGE and GeLC-MS/MS. The SDS-PAGE analysis revealed no significant variation in protein concentration during the 1-hour collection. The GeLC-MS/MS analysis identified pancreas-specific proteins previously described from endoscopic retrograde cholangiopancreatography and surgical collection methods. Gene ontology further revealed that most of the proteins identified have a molecular function of proteases.

**GENOMIC-BASED STUDIES**

The most recent studies on biomarkers identification in genomics (Table 4) regard both the determination of prognostic/predictive markers and diagnostic markers and are presented hereafter separated in these two classes. As for proteomic biomarkers, the studies here presented are mainly devoted to identify exploratory biomarkers.

Also the studies based on genomic approaches are potential for identifying PC biomarkers: in future the information provided by genomic approaches should be coupled to proteomic, metabolomic and clinical information to improve the predictive ability of the panels of biomarkers identified.

***Prognostic and/or predictive biomarkers***

Prognostic and predictive biomarkers in genomics (Table 4) are usually determined in tissue samples. Some of the proposed studies include both protein expression and microRNA expression profiles. In these studies, multivariate Cox regression analysis is usually exploited.

Ogura *et al*[68] studied the K-ras mutation status in 242 patients with unresectable PC. The authors focussed on K-ras mutation subtypes, since recent reports indicate that K-ras mutation status acts as a prognostic factor. CA19-9, metastatic stage and mutant-K-ras were negative prognostic factors, indicating a reduced survival. Among the patients who had K-ras mutation subtypes, CA19-9, metastatic stage and the presence of the G12D or G12R mutations were negative prognostic factors for OS.

Hwang *et al*[69] evaluated whether expression of novel candidate biomarkers, including microRNAs, can predict clinical outcome in PDAC patients treated with adjuvant therapy. 82 resected PDAC cases were analyzed for protein expression by immunohistochemistry and for microRNA expression by quantitative Real-Time PCR. Lower than median miR-21 expression was associated with a significantly lower hazard ratio for death and recurrence in the subgroup of patients treated with adjuvant therapy. MiR-21 expression status emerged as the single most predictive biomarker for treatment outcome. No significant association was detected in patients not treated with adjuvant therapy. The results were confirmed in an independent validation of 45 PDAC tissues.

One–fifth of patients with seemingly “curable” PDAC experience an early recurrence and death while some patients with advanced stage tumors are deemed “unresectable” by conventional staging criteria, yet progress slowly. Effective biomarkers that stratify PDAC based on the biologic behaviour are therefore needed. Building on a compendium of 2500 published candidate biomarkers in PDAC[70], Winter *et al*[71] constructed a survival tissue microarray (s-TMA) comprised of short-term (12 mo) and long-term survivors (30 months) who underwent resection for PDAC. The s-TMA acts as a biological filter to identify prognostic markers. 13 putative PDAC biomarkers were identified from the public biomarker repository and tested against the s-TMA. MUC1 and MSLN were highly predictive of early cancer-specific death. By comparison, none pathologic factors (size, lymph node metastases, resection margin status, and grade) reached statistical significance.

Chen *et al*[1] detected metastasis-associated gene 2 (MTA2) expression in PDAC and related it to prognosis. MTA2 mRNA and protein expression were determined by real time q-PCR and immunohistochemistry in primary cancers and their adjacent non-cancerous tissues. MTA2 mRNA and protein expression levels were up-regulated in PC. MTA2 was correlated with poor tumor differentiation, TNM stage and lymph node metastasis. Patients with high expression levels of MTA2 showed lower OS.

***Diagnostic biomarkers***

Diagnostic biomarkers from genomics-based studies (Table 4) are identified both from tissue and serum samples.

To identify biomarkers for early detection, Pedersen *et al*[72] examined DNA methylation differences in leukocyte DNA between PC cases and controls. In phase I, methylation levels were measured at 1505 CpG sites in leukocyte DNA from 132 never-smoker PC patients and 60 never-smoker controls. Significant differences were found in 110 CpG sites. In phase II, 88 of 96 phase I selected CpG sites were tested and validated in 240 PC cases and 240 matched controls. Using penalized logistic regression, a prediction model was built consisting of five CpG sites (IL10\_P348, LCN2\_P86, ZAP70\_P220, AIM2\_P624, TAL1\_P817) that discriminated cancer patients from controls. One CpG site (LCN2\_P86) alone could discriminate resectable patients from controls.

Morse *et al*[73] used complementary assays of mRNA expression profiling of cell-surface genes to determine increased expression in PC versus normal pancreas tissues, and validated protein expression by immunohistochemistry on tissue microarrays. This approach was aimed to the identification of targets for potential use in the molecular imaging of cancer, allowing for non-invasive determination of tumour therapeutic response and molecular characterization of the disease, or in the targeted delivery of therapy to tumour cells, decreasing systemic effects. Expression profiles of 2177 cell-surface genes for 28 pancreatic tumour specimens and 4 controls were evaluated. 170 unique targets were highly expressed in 2 or more of the pancreatic tumour specimens and were not expressed in controls. Two targets (TLR2 and ABCC3) were further validated for protein expression and proved to be potential for the development of diagnostic imaging and therapeutic agents for PC.

Huang *et al*[74] explored specific biomarkers that can differentiate PC–associated diabetes from type 2 diabetes, for the early detection of PC. Peripheral blood samples were collected from 25 patients diagnosed with PC and diabetes, 27 patients with PC without diabetes, 25 patients with diabetes mellitus > 5 years, and 25 controls. 32 samples were used in microarray experiments to find differentially expressed genes specific for cancer–associated diabetes. The results were further validated by quantitative real-time PCR for 101 blood samples. Protein expression of selected genes in serum and tissues was also detected. 58 genes were found to be unique in patients with cancer–associated diabetes (23 up-regulated; 35 down-regulated). 11 up-regulated genes were further validated by RT-PCR and 2 of these-vanin-1 (*VNN1*) and matrix metalloproteinase 9 (*MMP9*) — showed the best discrimination of cancer–associated from type 2 diabetes.

**METABOLOMICS BASED STUDIES**

Just one paper, by Kaur *et al*[75] has recently appeared in literature reporting for the first time the mass spectrometry-based metabolomic profiling of human pancreas in matched tumour and normal tissues. UPLC coupled with TOF-MS was applied to perform small molecule metabolite profiling of matched normal and PC tissues. The resulting multivariate data matrix was pre-processed for spectral alignment and peak detection, followed by normalization of the data to the feature intensities of the internal standard as well as to the total protein concentration. The normalized data were analyzed first by PCA followed by OPLS[31]. The authors also exploited random forest clustering[32] to interrogate the top 50 features with significant alterations in the tumor tissue as compared to the control. The candidate markers were searched against different Databases[76,77] to find compounds that corresponded to the accurate mono-isotopic mass measurements detected by UPLC–TOFMS analysis. The authors report a subset of metabolites which were unequivocally identified and were found to be significantly de-regulated in PC tissues.

The study here reported proves that metabolomic profiling shows a great potential for the identification of biomarkers for PC: certainly, further characterization and validation with large sample size is needed and may help establish the utility of such markers as biomarkers of clinical benefit.

**CONCLUSION**

This review is aimed to present the most recent applications of the –omics approaches (proteomics, genomics and metabolomics) to the identification of biomarkers for PC. Particular attention has been paid to the statistical methods adopted for biomarkers identification, first presenting the main statistical procedures adopted from a theoretical point of view. Then, the most recent applications present in literature were presented separately for non-omics, proteomics, genomics and metabolomics based studies. Within this distinction, studies were presented separately for diagnostic and prognostic/predictive biomarkers and according to the type of marker.

Different statistical approaches are exploited in literature for the identification of markers in PC: the methodologies here presented appear effective and sound. However, it is the authors’ opinion that multivariate methods have to be preferred: with the term multivariate, the authors refer to methods evaluating the relationships between the variables (both predictors and outcomes if several of both are present), in order to provide a pool of markers highlighting synergistic and antagonistic effects. In facts, the biological effect played by a pathology (and PC makes no exception) is the result of a series of different mechanisms both independent from each other or showing relevant interactions. Among all strategies, therefore, multivariate ones, able to point out these relationships, are to be preferred.

Another hint that must be addressed is the risk of identifying false positives, i.e. markers erroneously identified as such: this risk greatly increases when few information is available (*i.e.,* a small number of cases/patients is investigated). Certainly, this problem is deeply related to the problem of experimental design and sample collection: each study should be carefully designed from a statistical point of view before being performed in order to include all possible sources of biological variation; of course, this necessity often clash with the availability of samples, especially when tissue collection is involved. From a statistical point of view, in these cases, characterized by few information, it is very important to apply mathematical tools to validate the models built, thus evaluating the predictive ability of the models: to this respect the use of cross-validation techniques or simulation algorithms is fundamental to identify only statistically significant markers.

Certainly, there is a great gap between the results presented in studies on the identification of candidate markers reported in literature and the actual possibility of exploiting the identified biomarkers at a clinical level. This is due to several aspects, among which the most important are the poor sensitivity/specificity characterizing sometimes the identified pools of markers and the complicated biostatistics design of prospective studies for their validation prior to clinical use.

It is the authors’ opinion that the future perspective in exploratory biomarkers identification has to be searched for in the exhaustive search for potential markers: it is impossible to imagine that a complex pathology acting on a wide range of individuals characterized by a large biological variability could reflect in a very restricted panel of markers. We think that the future will rely on high-throughput techniques and the possibility of combining the results emerging from proteomic, genomic, metabolomic studies coupled to clinical information, to identify exhaustive panels of markers, thus improving the predictive performance of the panels themselves and providing better sensitivity and specificity. Certainly, great attention has to be paid in such studies to the proper evaluation of experimental and biological variability (*i.e.,* a careful selection of experimental design) to provide sound and robust results and to the evaluation of the results through effective multivariate techniques.

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**Table 1 Statistical methods adopted in the identification of biomarkers for pancreatic cancer**

|  |  |
| --- | --- |
| **Type of statistical method** | **Method adopted** |
| Classical mono- and multi-variate methods | *t*-Student test (parametric) |
| Mann-Withney test (non-parametric) |
| T2 Hotelling |
| ANOVA and MANOVA |
| Bayes factors |
| Unsupervised pattern recognition methods | Principal Component Analysis |
| Cluster Analysis |
| Multidimensional Scaling |
| Supervised classification methods | SIMCA |
| Ranking-PCA |
| O-PLS |
| CART |
| Random Forests |
| Methods for determining survival outcomes | Kaplan Meyer functions |
| Cox Regression |
| Other methods | PAM |
| Metropolis algorithm and Monte Carlo Simulation |

**Table 2 Studies based on serum and tissue biomarkers through non-omics techniques**

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| **Ref** | **Type of marker** | **Markers** | **Sample** | **Study group** | **Analytical methods** | **Statistical methods** | **Performance** |
| 41 | P | CA 19-9 | S | Pretreatment Ca 19-9: 115 patients from 5 German centers; 73%treated within prospective clinical trials. Median TTP: 4.4 mo; median OS: 9.4 months.CA 19-9 kinetics during chemotherapy: 69 patients (TTP) and 84 patients (OS). | Elecsys assay | Cox proportional hazards regression; for CA 19-9 kinetics, CA 19-9 was treated as a timevaryingcovariate | Univariate analysis: log (CA 19-9) associated with TTP (HR = 1.24; *P* < 0.001) and OS (HR = 1.16; *P* = 0.002). Multivariate analysis: results confirmed.Log(CA 19-9) kinetics during chemotherapy:significant predictor for TTP in univariate analyses (HR = 1.48; *P* < 0.001) and multivariate (HR = 1.45; *P* < 0.001) and for OS (univariate: HR = 1.34; *P* < 0.001; multivariate:HR =1.38; *P* < 0.001). |
| 42 | P | CA 19-9, CEA, CRP, LDH and bilirubin |  | 291 patients; 253 patients (87 %) received treatmentwithin prospective clinical trials. Median TTP: 5.1 mo. Median OS 9.0 months. | Elecsys assay | Kaplan Meier method and Cox proportional hazards regression | Univariate analysis: pre-treatment CA 19-9 (HR 1.55), LDH (HR 2.04) and CEA (HR 1.89) significantly associated with TTP. Baseline CA19-9 (HR = 1.46), LDH (HR = 2.07), CRP (HR = 1.69) and bilirubin (HR = 1.62) significant prognostic factors for OS.Multivariate analyses: pre-treatment log (CA 19-9) for TTP and log (bilirubin) and log (CRP) for OS had an independent prognostic value. |
| 44 | P | IGFs | S and P | 80 patients received treatment (40 Ganitumab; 40 placebo). | Immunoassays | Kaplan Meier method and Cox proportional hazards regression | Ganitumab associated with improved OS *vs* placebo (HR = 0.49; 95%CI: 0.28-0.87). |
| 45 | P | TROP2 | T | 197 patients;subgroup of 134 patients treated surgically | Immunohistochemistry | Kaplan Meier method and Cox proportional hazards regression | TROP2 overexpression observed in 109 (55%) patients and associated with decreased OS (*P* < 0.01). Univariate Analysis: TROP2 overexpression correlates with lymph node metastasis (*P* < 0.04) and tumour grade (*P* < 0.01). In the subgroup of patients treated surgically, TROP2 overexpression correlated with poor progression-free survival (*P* < 0.01). Multivariate analyses: TROP2 is an independent prognosticator. |
| 46 | P | JAM-A | T | 186 patients; Subgroup of 83 patients treated surgically | Immunohistochemistry | Kaplan Meier method and Cox proportional hazards regression | Low expression of JAM-A observed in 79 (42 %) patients and associated with poor OS (*P* < 0.01). Univariate analysis: low expression of JAM-A correlates with positive lymph node status (*P* = 0.02), the presence of distant metastasis (*P* = 0.05), and tumor grade (*P* = 0.04). In the subgroup of patients with surgically resected PC, low expression of JAM-A correlated with decreased progression-free survival (*P* < 0.01). Multivariate analysis: JAM-A was an independent predictor of poor outcome. |
| 47 | P | TBX4 | T | 77 stage II PDAC tumors | Immunohistochemistry | Kaplan Meier method and Cox proportional hazards regression | 48 cases (62.3%) expressed TBX4 at a high level. No significant correlation between TBX4 expression andother clinicopathological parameters, except tumor grade and liver metastasis recurrence. Survival of patients with TBX4-high expression significantly longer than those with TBX4-low expression (*P* = 0.010). Multivariate analysis: low TBX4 expression independent prognostic factor for OS. TBX4 promoter methylation status frequently observed in PDAC and normal adjacent pancreas. |
| 48 | P | HSP27 | T | 86 patients | tissue microarray (TMA) analysis | Kaplan Meier method and Cox proportional hazards regression | HSP27 expression found in 49% of tumour samples. Univariate analyses: significant correlation between HSP27 expression and survival. Multivariate Cox-regression: HSP27 expression emerged as an independent prognostic factor. HSP27 expression also correlated inversely with nuclear p53 accumulation. |
| 49 | P | dCK | T | 45 patients with resected PDAC received adjuvant gemcitabine based-therapy in multicenter phase 2 studies | Immunohistochemistry | Kaplan Meier method and Cox proportional hazards regression | Median follow-up: 19.95 mo (95%CI: 3.3-107.4 mo). Lymph node (LN) ratio and dCK protein expression significant predictors of DFS and OS in univariate analysis. Multivariate analysis: dCK protein expression the only independent prognostic variable (DFS: HR = 3.48; 95%CI: 1.66-7.31; *P* < 0.001; OS: HR = 3.2; 95%CI: 1.44-7.13; *P* < 0.004). |
| 50 | P | Notch3 and Hey-1 | T | 42 patients who underwent resection and50 patients diagnosed with unresectable PDAC | Immunohistochemistry | Mann-Whitney U test, Wilcoxon test, Cox regression analysis, Kaplan-Meier analysis | All 3 Notch family members significantly elevated in tumour tissue. Significantly higher nuclear expression of Notch1, -3 and -4, HES-1, and HEY-1 (all*P* < 0.001) in locally advanced and metastatic tumours compared to resectable cancers. In survival analyses, nuclear Notch3 and HEY-1 expression significantly associated with reduced OS and DFS following tumour resection with curative intent. |
| 51 | D and P | 21 biomarkers | P | clinically defined cohort of 52locally advanced (Stage II/III) PDAC cases and 43 age-matched controls | Proximity ligation assay | Combination of the PAM algorithm and logistic regression modeling. Biomarkers that weresignificantly prognostic for survival were determined using univariate and multivariate Cox survival models. | CA19-9, OPN and CHI3L1 were found to have superior sensitivity for pancreatic cancer *vs* CA19-9 alone (93% *vs* 80%). CEA and CA125 have prognostic significance for survival (*P* < 0.003). |
| 52 | D | 83 circulating proteins | S | 333 PDAC patients; 144 controls (benign pancreatic conditions); 227 healthy controls.Samples from each group split randomly into training and blindedvalidation sets.Panels evaluated in validation set and in patients diagnosed with colon (33), lung (62) and breast (108) cancers. | bead-based xMAP immunoassays | A Metropolis algorithm with Monte Carlo simulation (MMC) was used toidentify discriminatory biomarker panels in the training set. | Training set (160 PDAC, 74 Benign, 107 Healthy): panel of CA19–9, ICAM-1, and OPG discriminated PDAC from Healthy controls (SN/SP 88/90%), panel of CA 19–9, CEA, and TIMP-1 discriminated PDAC patients from Benign subjects (SN/SP 76/90%). Independent validation set (173 PDAC, 70 Benign, 120 Healthy): panel of CA 19–9, ICAM-1 and OPG demonstrated SN/SP of 78/94%; panel of CA19–9, CEA, and TIMP-1 demonstrated SN/SP of 71/89%. The CA19–9, ICAM-1, OPG panel is selective for PDAC and does not recognize breast (SP 100%), lung (SP 97%), or colon (SP 97%) cancer. |
| 53 | D and P | YKL-40,IL-6, and CA 19.9 | P | 559 patients with PC from prospective biomarker studies from Denmark(*n* = 448) and Germany (*n* = 111) | ELISA and chemiluminescent immunometric assay | Kaplan Meier method and Cox proportional hazards regression | Odds ratios (ORs) for prediction of PC significant for all biomarkers, with CA 19.9 having the highest AUC (CA 19.9: OR = 2.28, 95% CI 1.97 to 2.68, *P* = 0.0001, AUC = 0.94; YKL-40: OR = 4.50, 3.99 to 5.08, *P* = 0.0001, AUC = 0.87; IL-6: OR = 3.68, 3.08 to 4.44, *P* = 0.0001, AUC = 0.87). Multivariate Cox analysis: high preoperative IL-6 and CA 19.9 independently associated with short OS (CA 19.9: HR = 2.51, 1.22–5.15, *P* = 0.013; IL-6: HR = 2.03, 1.11-3.70, *P* = 0.021). Multivariate Cox analysis of non-operable patients: high pre-treatment levels of each biomarker independently associated with short OS (YKL-40: HR = 1.30, 1.03-1.64, *P* = 0.029; IL-6: HR = 1.71, 1.33-2.20, *P* = 0.0001; CA 19.9: HR = 1.54, 1.06-2.24, *P* = 0.022). Patients with preoperative elevation of IL-6 and CA 19.9 had shorter OS (*P* = 0.005) compared to patients with normal levels (45% *vs* 92% alive after 12 mo). |

Type of marker: P: Prognostic/predictive; D: Diagnostic; sample: S: Serum; P: Plasma; T: Tissue.

**Table 3 Proteomic based studies**

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| **Ref** | **Type of marker** | **Markers** | **Sample** | **Study group** | **Analytical methods** | **Statistical methods** | **Performance** |
| 54 | D | Among 2393 unique proteins, 104 proteins significantly changed in cancer | T | 5 patients; matched pairs of tumor and non-tumor pancreas | Tissues treated to obtain cytosol, membrane, nucleus and cytoskeletonfractions. Fractions separated and digested underwent LC-MS/MS. | PLGEM | 104 proteins significantly changed in cancer. Among these, 4 proteins validated that were up-regulated in cancer: biglycan (BGN),Pigment Epithelium-derived Factor (PEDF) Thrombospondin-2 (THBS-2) and TGF-β induced protein ig-h3 precursor (βIGH3). |
| 57 | D | Serum MALDI-TOF features | S | 15 healthy (H), 24 cancer (Ca), 11 chronic pancreatitis (CP) samples | MALDI-TOF | Nonparametricpairwise statistics, MDS, hierarchical analysis | 8 serum features: Ca samples differentiated from H (sn 88%, sp 93%), Ca from CP (sn 88%, sp 30%), and Ca from both H and CP combined (sn 88%, sp 66%).9 features obtained from urine: differentiated Ca from both H and CP combined (sn 90%, sp 90%). |
| 59 | D | Serum SELDI-TOF features | S | 96 serum samples from patients undergoing cancer surgery compared with sera from 96 controls | SELDI-TOF | Mann-Withney test, CART | Data analysis identified 24 differentially expressed protein peaks, 21 of which under-expressed in cancer samples. The best single marker predicts 92% of controls and 89% of cancer samples. Multivariate analysis: best model (3 markers) with Sn 100% and Sp 98% for the training data and Sn 83% and Sp 77% for test data. Apolipoprotein A-II, transthyretin and apolipoprotein A-I identified as markers and decreased at least 2 fold in cancer sera. |
| 60 | D | Serum SELDI-TOF features | S | 57 PC samples were compared to 59 controls | SELDI-TOF | Multivariate decorrelation filtering | Improved classification performances when the presented strategy is compared to standard univariate feature selection strategies |
| 61 | D | Proteins | S | Sera from patients diagnosed with PC compared with age- and sex-matched normal subjects | Protein microarrays | Rank-based non-parametric statistical testing. | A serum diagnosis of PC was predicted with 86.7% accuracy, with a sensitivity and specificity of 93.3 and 80%. Candidate autoantibody biomarkers studied for their classification power using an independent sample set of 238 sera. Phosphoglycerate kinase-1 and histone H4 noted to elicit a significant differential humoral response in cancer sera compared with age- and sex-matched sera from normal patients and patients with chronic pancreatitis and diabetes. |
| 62 | D | Proteins | PDAC cell lines | 435 spots identified from 18 samples from 2 cell lines (Paca44 and T3M4) of control and drug-treated PDAC cells | 2D-PAGE | PCA, SIMCA, Ranking-PCA | Samples were all perfectly classified. Significant proteins were further identified by MS analysis. |
| 63 | D | Proteins regulating the conversion of quiescent to activated PaSC cells | rat PaSC cell line | - | SDS-PAGE and GeLC-MS/MS | QSPEC | Qualitative and quantitative proteomicanalysis revealed several hundred proteins as differentially abundant between the two cell states. Proteins of greater abundance in activated PaSC: isoforms of actinand ribosomal proteins. Proteins more abundant in non-proliferating PaSC: signaling proteins MAP kinase 3 and Ras-related proteins. |

Type of marker: P: Prognostic/predictive; D: Diagnostic; sample: S: Serum; P: Plasma; T: Tissue.

**Table 4 Genomic based studies**

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| **Ref** | **Type of marker** | **Markers** | **Sample** | **Study group** | **Analytical methods** | **Statistical methods** | **Performance** |
| 68 | P | *K*-ras mutation status and subtypes | endoscopic ultrasound-guided fine-needle aspiration specimens | 242 patients | RT-PCR | Kaplan Meier method and Cox proportional hazards regression | Multivariate analysis: CA19-9 C1000 U/mL (HR = 1.78, 95 %CI: 1.28–2.46, *P* < 0.01), metastatic stage (HR 2.26, 95 % CI 1.58–3.24, *P* < 0.01) and mutant-K-ras (HR 1.76, 95 %CI: 1.03–3.01, *P* = 0.04) negative prognostic factors. Among patients with *K*-ras mutation subtypes: CA19-9 C1000 U/mL (HR 1.65, 95%CI: 1.12–2.37, *P* < 0.01), metastatic stage (HR 2.12, 95%CI: 1.44–3.14, *P* < 0.01), and G12D or G12R mutations (HR = 1.60, 95%CI: 1.11–2.28) negative prognostic factors for OS. |
| 69 | P | MicroRNA-21 | T | 82 resected Korean PDAC cases. Subgroup of patients treated with adjuvant therapy (N = 52) | protein expression by immunohistochemistrymicroRNA expression by q RT-PCR | Cox proportional hazards model | Subgroup with adjuvant therapy: lower than median miR-21 expression associated with lower HR for death (HR = 0.316; 95%CI = 0.166–0.600; *P* = 0.0004) and recurrence (HR = 0.521; 95%CI = 0.280–0.967; *P* = 0.04). MiR-21: single most predictive biomarker for treatment outcome. No significant association in patients not treated with adjuvant therapy. Independent validation cohort of 45 frozen PDAC tissues from Italian cases treated with adjuvant therapy: lower than median miR-21 expression confirmed to be correlated with longer OS and DFS. |
| 71 | P | 13 putative PDA biomarkers from the public biomarker repository |  | A survival tissue microarray was constructed comprised of short-term (cancer-specific death ,12 months, *n* = 58) and long-term survivors (30 mo, n = 79) who underwent resection for PDA (total, n = 137) | Immunohistochemical analyses; survival tissue microarray (s-TMA) | Wilcoxon rank sum test | Multivariate model: MUC1 (OR = 28.95, 3+ *vs* negative expression, *P* = 0.004) and MSLN (OR = 12.47, 3+ *vs* negative expression, *P* = 0.01) highly predictive of early cancer-specific death. Pathologic factors (size, lymph node metastases, resection margin status, and grade): ORs < 3 and none reached statistical significance. ROC curves used to compare the 4 pathologic prognostic features (ROC area = 0.70) to 3 univariate molecular predictors (MUC1, MSLN, MUC2) of survival group (ROC area = 0.80, *P* = 0.07). |
| 1 | P | MTA2 mRNA and protein expression | T | 123 PDAC samples and 40 control tissues | qRT-PCR and immunohystochemistry | Kaplan-Meyer curves and Cox analysis | MTA2 mRNA and protein expression levels up-regulated in PC. MTA2 correlated with poor tumor differentiation, TNM stage and lymph node metastasis. Patients with high expression levels of MTA2 showed lower OS. MTA2: independent prognostic factor. |
| 72 | D | Leukocyte DNA Methylation | Blood | Phase I: 132 never-smoker PaC patients and 60 never-smoker healthy controls.Phase II: validation of 88 of 96 phase I selected CpG sites in 240 PaC cases and 240 matched controls. | DNA array | Wilcoxon Rank Sum tests and likelihood penalized logistic regression models | Significant differences found in 110 CpG sites (FDR < 0.05). Phase II: 88 of 96 phase I selected CpG sites validated in 240 PaC cases and 240 matched controls (*P* < 0.05). Prediction model: 5 CpG sites (IL10\_P348, LCN2\_P86, ZAP70\_P220, AIM2\_P624, TAL1\_P817) discriminated PaC from controls (C= 0.85 in phase I; 0.76 in phase II). One CpG site (LCN2\_P86) could discriminate resectable patients from controls (C = 0.78 in phase I; 0.74 in phase II). 3 CpG sites identified (AGXT\_P180\_F, ALOX12\_E85\_R, JAK3\_P1075\_R) where the methylation levels were significantly associated with SNPs (FDR < 0.05). |
| 73 | D | cell-surface targets | T | 28 PC specimens and 4 normal pancreas tissue samples. Expression in normal tissues evaluated by array data from 103 samples representing 28 organ sites as well asmining published data. | Complementary assays of mRNA expression.Immunohistochemistry. qRT-PCR. | - | 170 unique targets highly expressed in 2 or more PC specimens and not expressed in normal pancreas samples. Two targets (TLR2 and ABCC3) further validated for protein expression by tissue microarray based immunohistochemistry have potential for the development of diagnostic imaging and therapeutic agents for PC. |
| 74 | D | Differentially expressed genes | Blood | 25 patients diagnosed with PC and diabetes, 27 patients with PC without diabetes, 25 patients with diabetes mellitus > 5 years, and 25 healthy controls. Results further validated for 101 blood samples. | Microarray and qRT-PCR | - | 58 genes found to be unique in patients with cancer–associated diabetes, including 23 up-regulated and 35 down-regulated genes. 11 up-regulated genes further validated by RT-PCR; 2 of these (*VNN1* and *MMP9*) selected for logistic regression analysis. The combination of *VNN1* and *MMP9* showed the best discrimination of cancer–associated diabetes from type 2 diabetes. The protein expression of MMP9 and VNN1 was in accordance with the gene expression. |

Type of marker: P: Prognostic/predictive; D: Diagnostic; sample: S: Serum; P: Plasma; T: Tissue.

**Figure 1 Example of a dendrogram built using Ward’s linkage method and euclidean distances.** Data refer to samples from two pancreatic cancer cell lines treated or not with Throchostatin-A.



**Figure 2 Example of the graphical representation of the results from Ranking-PCA.** trend of Ind2 *vs* the increasing number of variables added to the model (A); trend of the distance between the two class centroids *vs* the number of variables added to the model (B). Variables on the X-axix are reported in the order in which they are included in the model. Both calibration and cross-validation results are reported.



(a)



(b)