# TMA
# a step ahead in drug discovery

**By Dr CR Camozzi**

Estimation of the clinical importance, market size and therapy-related side-effects are key questions in drug development. The ever-rising costs of drug discovery and development ($800-1,500 million for a successful drug research project) together with demonstrated uncertainty and failure generate a critical need to apply tools and technologies that can effectively reduce the requested investment. This reduction of costs could come through the assertiveness of the most accurate therapeutic indications, predictive therapeutic efficacy model in all potential groups and subgroups of patients, and anticipating the potential side-effects in humans. Large-scale and high-throughput genomic and proteomic studies are generating vast amounts of data that are leading to the identification of drug targets and disease biomarkers. Determination of which genes are truly important for classification in terms of diagnosis, prognosis and therapy represents a critical issue. The first step in attempting to discover disease relevance of a given gene is the elucidation of the precise cells that express it in normal and diseased human tissues. There is a strategic and powerful tool called tissue microarray (TMA) to find out clinically relevant information. TMAs allow thousands of tissue specimens to be examined at once, greatly streamlining drug discovery and clinical diagnosis. Applications of TMA technology attempt to link gene expression data with stages of tumour progression, screening and validation of drug targets, presence of gene mutation and quality control for molecular detection methods.
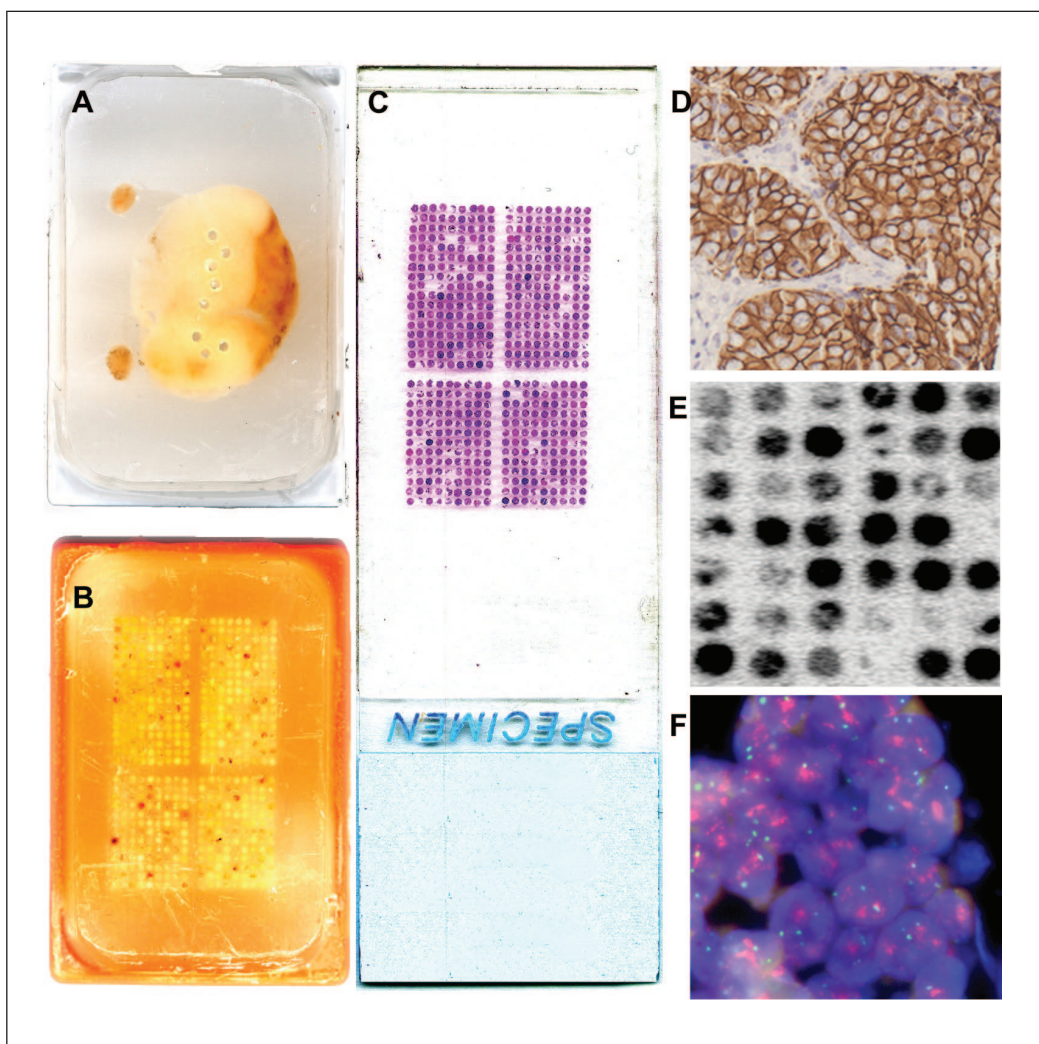
"Seventy per cent of the cost of drugs goes to pay for the research failures and dead-ends," said Michael J. Silverstein, a senior vice-president of the health care practice of the Boston Consulting Group. During drug research and development, the risks of failure are high, as well as the increasing investment demands are not assuring either a successful outcome of drug research projects or the final approval by Health Authorities. In a US published survey in 2001 only 15.2% of drugs in research had been approved for marketing, 1.7% had NDAs or BLAs that were submitted and are still active, 0.9% had NDAs or BLAs submitted but abandoned, 42.2% were terminated in four years or less from the initiation of clinical trials phase, 32% were terminated more than four years after the start of clinical testing and 8% were still in active testing in March 2001. Among the causes of termination of the clinical development, >20% is due to unsatisfactory safety results and >37% because of insufficient clinical efficacy in the selected indications[1,2].

The investment high risk value is exposed by the relation of each year's R&D expenditures and the number of NCE approvals, which were $1 billion in 2000, $743 million in 1999, $839 million in 1998, $568 million in 1997 and $400 million in 1996. Moreover, drug approval has been reshaped by health authorities, like the US FDA (Food and Drugs Administration) and EMEA (European Medicines Evaluation Agency), that prioritise new drugs by therapeutic significance at the time of submission[2].

Risk-value reduction could come through the assertiveness of the most accurate therapeutic indications, predictive therapeutic efficacy model in every group and subgroups of patients and anticipating the potential side-effects in human beings. There is a critical need for tools and technologies

that can increase research's efficiency and reduce the cost.

Considering this reality, a variety of powerful high-throughput technologies are available for the identification of potential new drug targets and bring along the necessary 'certainty'. High-throughput compound screening facilitates the identification of potentially applicable drug substances and identifies the most rewarding drug targets. Thus, an efficient target priority strategy is critical for competitive drug development. While target identification is usually performed in experimental systems like cell lines or animal, target evaluation and priority setting should be optimally based on found leads from patients' tissue.

### TMA technology in drug discovery

When the TMA technology was developed, the vision was to fundamentally speed up tissue analysis in the genomic and proteomic era. For this purpose, the Division of Molecular Pathology (Prof G. Sauter) of Basel University Hospital, Switzerland generated one of the largest tumour tissue archives in TMA technology platform, from more than 20,000 formalin fixed and 2,000 frozen tumour tissues, integrating more than 40,000 years of clinico-pathologic follow up data and precise molecular information. Only such kind of platform enables comprehensive molecular epidemiology surveys of gene products or other molecular features of interest.

TMA technology is increasingly needed for more than validation tests. TMA experience, performed in centres of excellence, is the most powerful strategic tool that allows very early evaluation of drug targets. The simultaneous and rapid analysis of no less than thousands of human tissues discloses the frequency of target gene expression (eg in all human tumour types), associations with disease progression and expression in normal tissues. The early experimental use of human tissue can provide more reliable identification of novel therapies for human diseases both through the direct information it provides on drug targets in human health and disease, and through the insight they provide on the value in establishing efficacy, safety and ADME profiles of new drugs. Comprehensive TMA experiences add value to decision-making for target identification, candidate drug selection and translational activities for biomarkers development.

*In situ* analyses are most helpful to determine which cells express the candidate gene, under what conditions, in which diseases. Almost all research requiring *in situ* tissue analysis can be performed in a TMA format. TMA sections have been extensively used for immuno-histochemistry (IHC), fluorescence *in situ* hybridisation (FISH) or RNA in situ hybridisation (**Figure 1**).

TMAs have mostly been used for cancer research but they can be used in all other fields as well. The following examples reflect typical TMA application in drug discovery.

● **Prevalence TMAs** are assembled from tumour samples of one or several types. These TMAs are useful to determine the frequency of molecular features in tumour entities of interest. Combined with published incidence data, results obtained on such large prevalence TMAs containing virtually all kinds of different tumour types allow a detailed market analysis for potential new drugs. For example, a TMA containing 4,788 different samples from 130 different tumour types has been used for several analyses on DNA and protein level.

● **Progression TMAs** containing samples of different stages of one particular tumour type are instrumental to discover associations between genetic alterations and tumour phenotype. Potential drug targets with an association with tumour progression or metastasis may have higher priority for further development than targets that are not related to advanced tumour stage. An ideal TMA should contain samples of normal breast from patients with and without breast cancer history, different non-neoplastic breast diseases, carcinoma *in situ*, invasive cancers of all stages, grades and histological subtypes as well as metastases and recurrences after initially successful treatment.

● **Prognosis TMAs** contain tumour samples with complete and extensive clinico-pathological follow-up data for the evaluation of the clinical importance of newly detected disease-related genes. Molecular features that are related to poor prognosis may play an active role in tumour progression and, therefore, represent a more suitable target than a gene product. Several studies have demonstrated the high efficiency of large prognosis TMAs to identify associations between molecular features and prognosis. The HER2 protein is a prime example for a gene product, which is strongly related to poor patient prognosis in breast cancer and also constitutes an excellent therapeutic target.

● **Normal tissue TMAs** are especially helpful in the process of drug discovery and development. The expression of target genes in vital normal tissues is a potential danger for new drugs. TMAs composed of normal tissues can massively facilitate the process of normal tissue cross reactivity
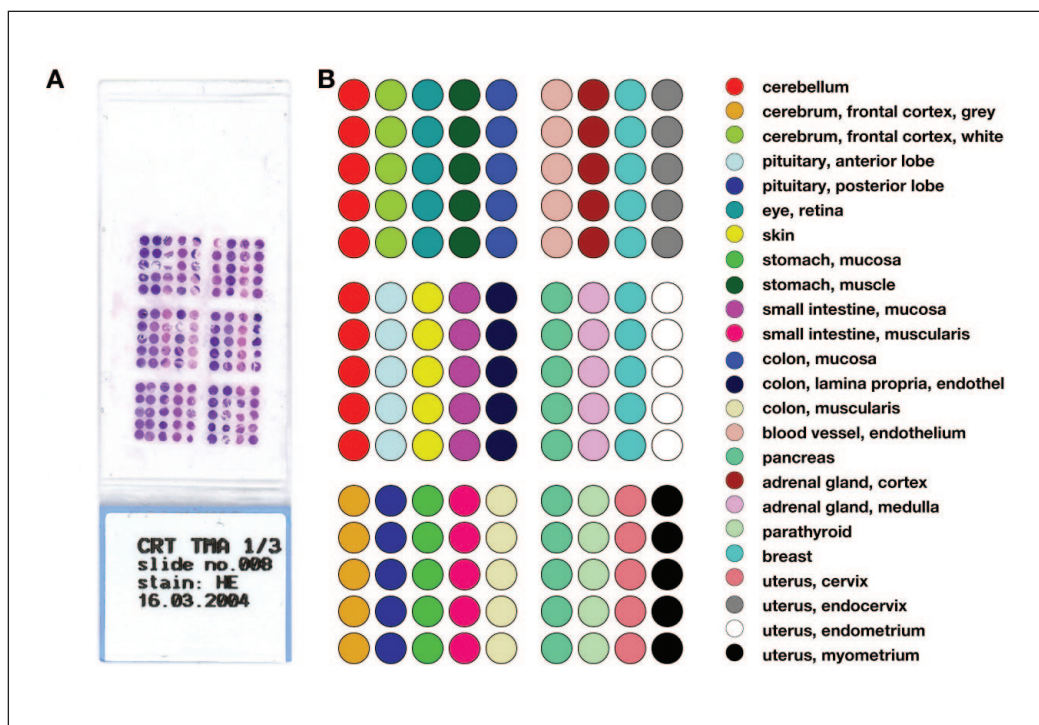
testing required by the US-FDA (Food and Drug Administration). The Division of Molecular Pathology (Prof G. Sauter) of the Basel University Hospital, Switzerland has recently generated the CRT-TMA (Cross Reactivity Testing), a frozen tissue TMA composed by 32 tissues from multiple different donors that reflects the panel of tissues required for new biological drugs like MAbs (monoclonal antibodies). The TMA format (**Figure 2**) especially facilitates extensive cross reactivity testing using multiple different antibody dilutions as requested by authorities.

Each of the described applications has its individual benefits. However, the utmost advantage is the 'all in one' approach, which provides a full picture on the epidemiology of a molecular feature of interest. TMAs make it possible to simultaneously analyse many thousands of tissues under maximally standardised experimental conditions. Large enough TMAs allow immediate analysis of all normal tissues, all different tumour types, associations with tumour stage and clinical outcome in the most important human cancers, as well as heterogeneity analyses between primary tumours and nodal and/or haematogenous metastases. This is important because anticancer drugs are often designed to target tumour metastases. Significant heterogeneity between different metastases would greatly limit the potential utility of such drugs. The added infor-

mation of gene mutations presence in each tumour type reveals the therapeutic power of any gene specific treatment.

### TMAs for economical evaluation

Data on the expression of a given target gene are often available from the literature. HER2, the target gene for trastuzamab (Herceptin), has been analysed in myriad publications. Data are highly controversial with published expression frequencies from <10 to >90%[3]. Published incidence ranges from 4% to 100% HER2-positivity in non-small cell lung cancers (NSCLC). High expression rates, reported in some studies, might have encouraged a costly decision to undertake large but disappointing clinical trials with Herceptin. One study was terminated early because of too little HER2-positive cases and lack of response in other studies. TMAs have been used to investigate target genes for established drug targets like KIT/CD117 (Imatinib; Glivec)[4], epidermal growth factor (EGFR) (Tarceva, Iressa, Cetuximab, Erbitux)[5], or HER2 (trastuzamab; Herceptin). The studies confirmed expression of respective targets in a variety of tumour entities. Glivec, approved by the US FDA, represents a successful example of its application in KIT-positive gastrointestinal stroma tumours (GIST). It exposes the potential clinical benefits and economical impact of including less frequent tumour entities in early epidemiological

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# Microarrays

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target gene evaluations. These demographic results are useful to calculate the potential economical value of a successful drug, as well as to accurately select the patients to be included in clinical trials.

## TMAs for biological evaluation

TMA technology platform is a highly efficient tool to identify associations between molecular features and prognosis. This method has been successfully applied in more than 150 studies. Significant associations were found between oestrogen or progesterone expression, HER-2 alterations and survival in breast cancer patients, between vimentin expression and prognosis in kidney cancer, and between Ki67 labelling index and prognosis in urinary bladder cancer, soft tissue sarcoma and in Hurthle cell carcinoma[5-11]. Another example of bcl2 in breast cancer demonstrates the importance of performing large TMA studies. Ten studies had previously analysed the relationship between loss of bcl2 expression and prognosis in breast cancer. The results were controversial with four studies finding a significant association with poor prognosis and six studies failing to see such an association[12-20]. Prof G. Sauter applies TMAs containing more than 2,000 breast cancers with complete clinical follow up data; therefore he is able to analyse 50% more tumours than all previous studies together (**Table 1**). This extraordinary capability disclosed the answer to whether or not bcl2 expression is related to prognosis in breast cancer. A TMA study analysing 'only' 200-300 tumours cannot be conclusive on this issue. The evaluation of potential clinico-pathological associations can be facilitated by specially designed software (ThresholdFinder™) to systematically evaluate different cut-off levels. The

| AUTHORS | YEAR | NUMBER OF CASES | FOLLOW-UP (YEARS) | ASSOCIATION WITH OVERALL SURVIVAL |
|---|---|---|---|---|
| Silvestrini et al | 1994 | 283 | 6 | yes |
| Joensuu et al | 1994 | 174 | 5 | yes |
| Lipponen et al | 1995 | 140 | 10 | yes |
| Hellemans et al | 1995 | 124 | 7 | no |
| Barbareschi et al | 1996 | 178 | 5 | n/a |
| Van Slooten et al | 1996 | 202 | 4 | n/a |
| Krajewski et al | 1997 | 53 | 53 | no |
| Kapranos et al | 1997 | 90 | 10 | n/a |
| Charpin et al | 1998 | 82 | 10 | no |
| Veronese et al | 1998 | 98 | 5 | yes |
| All previous studies | | 1,424 | | ? |
| Molecular Pathology Basel | | 2,221 | 10 | yes (p<0.0001) |

**Table 1:** Summary of studies investigating the prognostic role of BCL2 expression in breast cancer

**Table 2:** Disadvantages of using multiple samples per tumour

| BREAST CANCER STUDY (P53 IHC ANALYSIS) | 200 PATIENTS | 800 PATIENTS | 800 PATIENTS |
|---|---|---|---|
| | 4 SAMPLES PER TUMOUR | 1 SAMPLE PER TUMOUR | 4 SAMPLES PER TUMOUR |
| Tissue analyses | 800 | 800 | 3,200 |
| Interpretable cases | 194 | 606 | 774 |
| Time for reading | 8h | 8h | 32h |
| Statistical bias issue | yes | no | yes |
| Tissue costs | $3,000 | $3,000 | $12,000 |
| Pathologist costs | $2,000 | $2,000 | $8,000 |
| P value prognosis | p=0.0545 | p<0.0001 | p<0.0001 |
| Rate of positivity | 23.7% | 18.2% | 20.4% |

comparison of the expression of a target gene with previously collected molecular data can also provide further information on the biologic role of target genes.

## TMAs for toxicity evaluation

High expression of a target protein in vital non-diseased tissues is a potential drawback during drug development. The early use of normal tissue TMAs avoids such unpleasant surprise. A cell type specific *in situ* expression analysis enables comparison of the expression level for each individual cell type with the expression in cancer cells, allowing the identification of possible therapeutic windows. Cell type specific comparisons would not be possible if non-*in situ* methods were used. EpCam, a target for several anticancer therapies, is expressed in bile ducts of the liver. Since bile ducts constitute a very small (<1%) but vital component of the liver, comparisons of the expression level in bile ducts and tumours would suggest a comfortable therapeutic window for anti-EpCam drugs. The *in situ* analysis of tissues does reveal, however, the high level of expression in a small compartment of the normal liver. TMAs composed of normal frozen tissues can massively facilitate the process of cross reactivity testing (CRT). CRT-TMA, created by the Division of Molecular Pathology of Basel University, is composed by 32 non-diseased frozen tissues from multiple different donors (**Figure 2**), reflecting the panel of tissues requested by the US FDA for CRT of new biological drugs. This frozen TMA platform allows rapid CRT studies with mul-

tiple different antibody dilutions required by health authorities.

## TMA issues

The value of TMAs for large-scale (thousands of samples) tissue analysis is undisputed. Although the technology is relatively simple and easily applicable, some aspects of the TMA area are controversially discussed, while others are underestimated. These include the importance of tissue heterogeneity, the paramount importance of pathology expertise and the immunostaining set-up.

### Tissue heterogeneity

Initially, it was thought that minute tissue samples might not be sufficiently representative. Many studies have addressed the question as to whether a higher concordance of TMA and large section data can be obtained if multiple samples of each tumour are arrayed. These studies showed that the results obtained on TMA platform can be even more congruent than those performed on large sections[21-34]. Based on Prof G. Sauter's experience of more than five million IHC analyses on TMAs, it can be concluded that analysing just one tissue sample per tumour is not only the least expensive but also the best procedure for research applications, as exposed in **Table 2**.

### Pathology expertise

Extremely qualified pathologist skills are needed for classification of arrayed tissues, experimental design and delivery of staining interpretation are

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critical in TMA experiences. As the molecular data will be compared with clinico-pathological information, the quality of this information is of highest importance for the pathologist analysis and interpretation. A systematic review by a specialised pathologist of all tumours included in a TMA is highly necessary and should include reclassification of all tissues accordingly.

### Immunostaining protocol setup

The difficulties related to the development of a reliable IHC protocol are underestimated. Non-specific positivity is a frequent problem and requires the use of multiple controls. A significant fraction of antibodies cannot be optimised for use on tissue sections. IHC staining results are greatly dependent on antibody selection, antigen retrieval strategy, staining protocol and on minor variables such as the section age. The use of three different antibodies for EGFR resulted in up to five-fold difference in the rate of positivity[4]. Using six-month-old TMA sections it was found a significant decrease of immunoreactivity for multiple antibodies[35].

### Conclusions

A conclusive molecular pathology report, generated throughout TMA experiences, is one of the most powerful elements in the decision-making process before designing and investing in highly expensive clinical research projects. The appropriate application of no less than thousands of well selected and characterised tissues on TMA platform, together with highly standardised laboratory procedures, complete clinico-pathological data, molecular information of each tumour (including respective gene mutations and surrogate markers) and pathologist analysis/interpretation identify suitable indications, progression, prognosis and potential sites of adverse effects of new drugs. Allocation of costly resources for functional and 'drugability' evaluation of the target can then be prioritised according to the results of early TMA analysis. The appropriate application of TMA platform, in hands of a well-qualified pathology centre, provides the necessary certainty on potential therapeutic efficacy and safety of new molecules.                                      DDW

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