# 7 Clinical, Pathological, and Molecular Prognostic Parameters in Glioblastoma Patients Undergoing Chemo- and Radiotherapy

Paolo Tini, Clelia Miracco, Marzia Toscano, Silvia Palumbo, Sergio Comincini, Giovanni Luca Gravina, and Luigi Pirtoli

## Introduction: Prognostic Factors and Clinical Management of Glioblastoma

Glioblastoma (GB) accounts for about 55 % of primary brain tumors, with an incidence of five new cases/100,000 people/year. If untreated, median survival of GB is up to 3 months after diagnosis. The presently available literature uniformly reports the above data, and that multimodal treatment (surgery, radiotherapy—RT, chemotherapy—CHT) significantly improves median overall survival (OS), with about 40 %, 15 %, and 7–8 % outcomes, respectively at 1-, 2-, and 3-years [1–7]. Peak incidence of mortality occurs at the beginning of the second year after diagnosis, thereafter the risk of death halves at 2.5 years. Patients surviving more than 2 years after diagnosis, in fact, have a more favorable probability to survive afterwards, if compared to newly diagnosed cases. However, long-term survival remains poor with a 5-year OS rate barely reaching 5 %. The involvement of a multidisciplinary team in diagnosis, staging, and treatment of GB is mandatory for a correct management of GB. Postoperative RT is the mainstay of postsurgical management: a standard fractionated dose

P. Tini, M.D. (✉)
Tuscany Tumor Institute, Florence, Italy

Unit of Radiation Oncology, University Hospital of Siena (Azienda Ospedaliera-Universitaria Senese), Viale Bracci 11, Siena 53100, Italy
e-mail: paolo-tini@libero.it

C. Miracco
Tuscany Tumor Institute, Florence, Italy

Unit of Pathological Anatomy, Department of Medicine, Surgery and Neurosciences, University of Siena, Siena, Italy

M. Toscano • L. Pirtoli
Tuscany Tumor Institute, Florence, Italy

Unit of Radiation Oncology, Department of Medicine, Surgery and Neurosciences, University of Siena, Siena, Italy

S. Palumbo
Unit of Radiation Oncology, Department of Medicine, Surgery and Neurosciences, University of Siena, Siena, Italy

S. Comincini
Department of Technology and Biotechnology, University of Pavia, Italy

G.L. Gravina
Department of Radiological Sciences-Oncology and Pathological Anatomy, State University of Rome ("La Sapienza"), Rome, Italy


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of 60 Gy is recommended [8], even if altered fractionation schedules are also used, mainly consisting of short-course, hypofractionated RT in older patients. CHT has also acquired a key role in the management of this disease, and the alkylating agent Temozolomide (TMZ), delivered concurrently and sequentially with RT, is presently another standard of treatment [9]. The uncertainty in etiology of GB and the eventually fatal course, have driven research for many years towards an analytic approach of factors conditioning life expectancy. These efforts attempted to individuate both: parameters for a balanced treatment approach in terms of benefit/risk ratio; and characteristics of the natural history of this disease, possibly suitable for new and more effective therapeutic strategies. This contribution may give an overlook of prognostic parameters of GB of the present knowledge of these factors from a clinical point of view.

## Prognostic Parameters

Since the seventies of the past century, different prognostic factors were significantly associated with prognosis of GB, and generally classified as:

1. Patient-related
2. Treatment-related
3. Tumor-related

## Patient-Related Prognostic Parameters

Age at diagnosis, performance, and neurological status have a strong prognostic impact, according to large case-series published over more than 20 years [3, 4, 7, 10–23].

### Age

The Recursive Partitioning Analysis (RPA) by the Radiation Therapy Oncology Group (RTOG), combining the above factors with other patient- and treatment-related parameters for a comprehensive score system [10], indicates age as the best predictor of survival in high-grade gliomas: patients aged 50 or older showed a shorter survival, if compared to younger ones. Old age may be associated with poor prognosis for several reasons. Less aggressive treatments for avoiding toxicity, due to presumably poor physiologic reserves, and comorbidities, may partly account for this evidence. However, phenotypically aggressive GBs occur in old patients, with characteristic molecular profiles [24]. GB, in fact, has two distinct modalities of development. The first one (representing the vast majority) is characteristic of the so-called primary GB, that arises de novo preferentially in old people; while in the second case, an evolution occurs from lower-grade gliomas, which is more frequently observed in younger patients (secondary GB). Oghaki and Kleihues [25, 26] correlated outcome with different biologic behaviors, and younger age of the affected subjects. Primary and secondary GBs, in fact, derive from precursor astrocytic cells through genetic pathways quite different from each other, including gene deletions, mutations, or amplifications [27], as addressed in a following section of this chapter. A SEER (the Surveillance, Epidemiology, and End Results program of the National Cancer Institute, USA) analysis of 34,664 patients affected by GB [28] confirmed the strong prognostic impact of age on survival. In that report, age behaves as a continuous variable in predicting survival, with the most significant decrease found in the group over 50 years, every additional year of age being associated with a significant decreased probability (hazard ratio—HR—1.037). Large series indicate that the mean age of long-term survivors is less than 50 years [29, 30], reported that only 2.2 % of 689 enrolled patients survived more than 3 years, and their mean age was 43.5 years.

### Performance Status

Patients' performance status (PS) represents a well-known quantitative prognostic indicator in GB [31], and some scoring systems are presently in use, taking into account the ability of performing the normal activities of the daily life. The Karnofsky Performance Status (KPS) scale is probably the more widely adopted tool, to this purpose. Karnofsky and Burchenal [32] realized

the necessity for objective and standardized measurements of patients' performance, as a "*method of evaluating a therapeutic agent against cancer* (…) *in the absence of coincident and significant objective evidence of a therapeutic effect.*" Such a methodological approach could be appealing in tumors whose direct apparentness was not easily achievable, that is, the case of GB before the advent of CT and MRI. A growing body of evidence, in fact, subsequently confirmed the prognostic value of KPS in most oncologic settings, and particularly in GB. KPS describes a comprehensive 11-point scale, that quantifies—with 10 % progressive steps—the patient's functional status, with percentage values ranging from 100 % (normal activity, no symptoms) to 0 % (death). The evidence in favor of KPS as a prognostic factor in GB came from the original RTOG RPA classification analysis [10] comprehensive of 1578 patients, enrolled in the RTOG 74-01/ECOG (Eastern Cooperative Oncology Group) 1374; RTOG 79-18; and RTOG 83-02 studies, and from the subsequent validation by the RTOG 90-06 analysis [30]. The prognostic watershed for survival was at the 70 % KPS score level, with a significantly better prognosis for patients showing values above this threshold, as compared to the other ones.

The ECOG PS evaluation, with a different score system, is used for the same purpose. This instrument, proposed by Oken et al. [33], known also as WHO or Zubrod score, is composed of five classes, depicting progressive impairments of clinical conditions from perfect health (score 0) to death (score 5). ECOG scale is probably more suitable for common practice due to the easy use, and to a correspondence with survival as reliable as that of the KPS system.

### Neurological Status

The early RTOG study quoted above significantly correlated the negative prognostic impact of an abnormal mental status on survival of GB patients [10]. Currently, clinicians mostly use the Mini-Mental Status Evaluation (MMSE) to quantify the impairment of mental status in high-grade gliomas [34], also in its "simplified" version, that is, the Folstein's test. It is a relatively short, standardized, and well-validated screening test devised for cognitive impairment and dementia [35]. It includes some easy questions and problems in different domains: the patient is required, for instance, to specify the actual time and place, to repeat lists of words, to address arithmetic issues, to show language use and understanding, and to exert motor skills. Any score greater than, or equal to, 27/30 points indicates a normal status. Scores below this value correspond to severe (≤9 points), moderate (10–18 points), or mild (19–24 points) cognitive impairment. A validation of MMSE score of 27 or higher as a favorable, independent predictor of survival came from a trial by the European Organization for Research and Treatment of Cancer (EORTC) and the National Cancer Institute of Canada (NCIC) [36]. Of notice, other authors reported similar findings in low-grade glioma [37].

## Treatment-Related Prognostic Factors

Resection of the tumor and the following RT and CHT [8] became more and more refined in the last decades, resulting in a progressive improvement of survival outcomes. The already quoted SEER database analysis [28], including 4664 GB patients, showed a progressive trend towards an increased median survival from 1973 to 2008. Patients diagnosed from 2005 to 2006 had a significantly improved survival, when compared to those accrued from 2000 to 2001. Other reports addressed the same subject with comparable results: a large cohort study (1059 patients treated in 18 radiotherapy centers in Italy) [7] also evidenced a significant difference in survival rate favoring patients recruited from 2002 to 2007, compared to those collected by the same study group from 1997 to 2001 in a previous patterns-of-care study [38]. Increased use of MRI imaging, more sophisticated neurosurgical techniques, 3-D conformal RT (3D-CRT) or Intensity-Modulated RT (IMRT), and TMZ CHT, together with an improved supportive management, may be the main factors for these better results, with respect to the past.

## Imaging

Magnetic resonance imaging (MRI) has been the standard of GB workup [39] in the pre- and the postoperative settings over the last two decades, and presently is adopted in current practice both for diagnosis and for therapy planning (surgery and RT). Standard MRI grounds most of the available data on prognosis of GB, in terms of progression-free survival (PFS). Clinical workup usually includes gadolinium-enhanced-T1 and T2 or FLAIR (Fluid Attenuation Inversion Recovery) sequences. In conventional T1-weighted MRI, gadolinium enhancement correlates with cell proliferation, expressed by Ki-67 nuclear staining of glioma cells, and with microvascular density, thus helping to some extent to differentiate high-grade from low-grade gliomas [40]. This sequence is used, in common practice, to drive the extension of surgical removal in GB. T2 and FLAIR sequences, depicts the overall extension of the tumor burden and the surrounding edema, and are used in RT planning, together with the T1 ring enhancement by gadolinium for boost volume contouring. Among the radiological findings, tumor necrosis, mass effect, and edema-surrounding tumor are associated with a significantly shorter survival time in many studies [41, 42]. However, most recent and advanced MRI methodologies may give further information, useful for GB diagnosis, treatment decision, patient outcome prediction, and follow-up monitoring. Diffusion-Weighted Imaging (DWI) evaluates cellular density, that is, a MRI technique providing a measure of the movement of free-water molecules: the higher is cell number in a given volume, the lower is water mobility, in that cell membranes hamper water diffusion. Thus, the Apparent Diffusion Coefficient (ADC) values are useful in differentiating on quantitative grounds, high-grade from low-grade gliomas [43]. Proton MR spectroscopy (MRS) measures brain and tumor metabolites in vivo. Specific GB metabolite MR spectral patterns are not fully univocal, but a significant correlation exists between GB cell proliferation (assessed by Ki-67 labeling index in GB sections) and the Choline/Creatinine–Phosphocreatinine ratio (Cho/Cr), and with the N-acetyl aspartate (NAA)/Cho ratio, out of the spectral peaks. This may be useful not only for the characterization of high-grade gliomas in respect of the normal brain, but also as a guide for biopsy, identifying areas of tumor-most representative of malignancy [44]. GB invasiveness may not be sufficiently evaluated by conventional MRI, in that peritumoral edema may obscure the presence of tumor cells, and is addressed by Diffusion Tensor Imaging (DTI), which measures water movements within the white matter tracts (DTI tractography), that can be displaced (in low-grade gliomas) or interrupted/infiltrated (in high-grade gliomas) by tumor [45]. The high angiogenesis activity, due to VEGF, is characteristic of GB and appears as microvascular density in Dynamic Susceptibility-weighted Contrast-enhanced (DSC) MRI: Microvascular density of Area (MVA) is the corresponding parameter that quantitatively correlates with prognosis [46]. MR-based perfusion studies and particularly tumor blood volume estimates have been shown to provide prognostic information on time to progression or survival [47–50]. Many other MRI methodologies and technical refinements were also developed in recent years, as exhaustively reviewed by some authors [44], and a large quantity of diagnostic features of GB, often related with “functional” parameters of tumor growth besides morphology, are presently achievable, that may help to establish diagnosis and extension of the disease. It is hard to assess the practical impact of these disclosures on the general management of GB, but their use in selected patients may substantially modify the therapeutic plan, for improving therapeutic outcome and limiting treatment-related damages. The same holds true for other functional diagnostic tools, such as radionuclide investigations. Recently, the results of C-Methionine (C-MET) Positron-Emission Tomography (PET) results seemed to show prognostic value for GB, dependent on the uptake by proliferative cells [51]. C-MET uptake highlights also a more extended active tumor volume, with respect to T1 gadolinium-enhanced RMI [52] in relapsing GBs. If confirmed in newly diagnosed cases, C-MET PET could have a relevant role in the

preoperative imaging workup. To date, the widespread use of imaging assessment of GB extension (mainly MRI) may condition subsequent surgical resection and RT planning, thus might have an indirect prognostic role. Presently this assumption is difficult to substantiate, but as a suggestion by the results of the patterns-of-care studies on large series, quoted above [7, 38].

## Surgery

The extension of tumor resection is case-dependent, due to tumor bulk, site, and patient medical conditions. A classification of the amount of removal is usually adopted for prognostic evaluation into three categories, as follows: gross total resection (GTR: in respect of preoperative imaging and intraoperative findings), subtotal resection (STR: a gross complete removal of the tumor is not achieved), and biopsy-only (BO: any attempt to ablative or even cytoreductive surgery is judged impossible or not advisable).

Upfront surgery in newly diagnosed GB patients consists of maximal safe resection, in fact, as a primary goal whenever feasible, in respect of tumor size, shape, proximity to blood vessels or functionally determinant (or "eloquent") brain regions. The anatomical localization of GB in the brain, in fact, may affect patient's survival, in that it may condition the surgical excision [41, 53, 54]. Frontal lobe tumors show better survival, as compared to those located in other sites [55, 56]. Prognosis of the rare cerebellar GBs, with respect to their supratentorial counterparts, was considered by several studies with nonunivocal results [57–59], and probably some favorable outcomes reported in this setting may be related to the young age of these patients [60–62]. Aggressiveness in surgical resection is also dependent on other factors, such as patient's age, KPS, and comorbidity status. The extent of tumor removal is balanced, in fact, considering the operative risk and neurologic dysfunctions. In our experience, 46 % of the patients had GTR, 40 % PR, 11.6 % BO [7]. Most related literature report comparable data. Intraoperative MRI and neuromonitoring have been associated with surgical protocols, for maximal safe resection. With respect to the use of intraoperative MRI, which is expensive and labor consuming, 5-aminolevulinic acid (ALA) tumor-specific fluorescent vital staining helps surgeons to differentiate tumor and healthy brain tissue, with promising results. ALA was the first compound successfully employed to this purpose [63]. Sixty-five percent of ALA-driven resections achieved gross tumor removal vs. 36 % by conventional methods [64]. Sodium fluorescein is another fluorescent compound developed for the same purpose [65].

However, "radical" surgery for GB is an intrinsically abstract concept, given the well-documented infiltrative penetration of tumor cell far beyond contrast enhancement and surgical limits of resection [66]. Most neurooncological literature endorses the benefit or gross GB mass removal, howsoever, and a number of studies clearly indicate that the extensive surgical resection of GB is associated with a significant improvement of the survival outcomes [67]. Lacroix et al. [41] proposed a threshold of 98 % of resected tumor for a significant survival benefit. Sanai et al. [68] attempted a more detailed quantitative analysis of the impact of extent of resection on survival, out of 500 consecutive GB patients, with a significant advantage found after a minimal 78 % resection of the tumor mass, as evident by imaging contrast enhancement. Increasing amounts of resection, even up to the increment from 95 to 100 %, obtained further improvements in survival. Orringer et al. [69] showed that patients with more than 90 % tumor resection achieved an improved 1-year survival with respect to those with a lesser ablation. Chaichana et al. reported a similar finding [70] for every 5 % increment of tumor ablation, over a 70 % threshold of effectiveness of the resection.

Advanced age is a limiting factor for aggressive surgery in GB. Patients older than 65 years, in fact, may be unsuitable for tumor resection because of comorbidities. However, out of fitting cases, Oszvald et al. [71] reported that the overall survival of patients aged over 65 was significantly lower than in younger patients. Notably, the negative impact of age on survival was determinant only in patients undergoing BO, with no significant effect after tumor resection, and an effective

role of surgery on survival was suggested in aged as well as in younger patients.

One can argue from a speculative point of view, whether extensive GB removal may be or not an independent variable in determining prognosis, as the possibility of extended resection may depend, in turn, on inherent tumor aggressiveness. No data are available to this regard from prospective random trials. Furthermore, at the present state-of-the-art, adjuvant RT and CHT have shown a significant effectiveness and this question might have some interest, in that hypothetically RT and CHT might compensate a less-than-optimal resection. These arguments are the subject of a recent study [72], based on a personalized survival model including extension of resection (EOR), age, KPS, and accomplishment of adjuvant RT and TMZ. This multivariate, continuous, no-threshold and nonlinear model provides for the first time an explicit evidence of the independent role of maximum-safe GB resection on prognosis. Further, it shows a significant superiority (20 %, i.e.: a predictive error of 4.7 months) in estimating survival effects by EOR over current methods for prediction of survival, based on thresholds and stepwise increments of effectiveness of tumor ablation. Further, the influence of adjuvant RT and TMZ administration on prognosis is also quantified on a personalized base: due to the nonlinear relationship between the percentage of resected tumor and survival, this study clearly showed that adjuvant therapy exerts a progressively greater effect, with increasing EOR. This holds true both for young and old patients, and for high- and low-KPS cases. Thus, a "cytoreductive" value of tumor debulking in GB, favoring the therapeutic effectiveness of adjuvant RT and CHT, seems to be demonstrated, that is, the same role that surgery may have in many other tumors.

## Radiotherapy

Radiation therapy has a consolidated role in the postsurgical, adjuvant treatment of GB, after the early studies quoted above, and its accomplishment is included in the RPA as a prognostic factor [10]. Postoperative RT is a principal element in the treatment of patients with GB, as shown by different analysis from unselected series, demonstrating improved prognosis. As early as at the seventies of the last century, the addition of RT to surgery increased survival from 3–4 months to 7–12 months, after a random clinical trial [53]. Thumma et al. [28] confirmed the importance of RT in prolonging survival of patients with GB. In their analysis, they found that the "no-radiation" (HR: 3.45) and the "unknown radiation" groups (HR 2.50) showed a marked decreased survival, as compared to the "radiation" group of patients. Filippini et al. [4] showed that RT increased survival with a 39 % reduction in relative risk of dying. External-beam RT should begin within 8 weeks following surgical resection or biopsy. Conventional RT consists of 60 Gy, delivered through limited-field external-beam irradiation, with fractions of 2.0 Gy, 5 days per week, as stated by current guidelines [8]. However, 90 % of the tumors recur at the original site after RT, thus strategies to increase local radiation dose are the subject of clinical radiobiology research for improving patients' outcome. An RTOG–ECOG study randomized 253 patients to 60 Gy whole brain RT vs. 60 Gy plus 10 Gy boost to a limited volume, with no significant advantage on survival in the experimental arm [73]. The dose-escalation RTOG-98-03 phase-I trial failed to demonstrate survival advantage from 3D-CRT, with four dose increments from 64 up to 80 Gy, with 90 % GBs relapsing in the primary site and no advantage from higher doses [74]. Dose-escalation studies with IMRT seemed to show results slightly superior to those normally achievable with standard doses, with a reduction of in-field relapse [75]. However, a systematic, recent review of the studies addressing the subject of RT doses above 60 Gy in the TMZ era, concluded that high-dose treatments do not achieve any substantial prognostic improvement over that of standard dose schedules [76]. Other authors failed to demonstrate a benefit for doses >60 Gy using different RT strategies in newly diagnosed GBs, such as brachytherapy [77] or stereotactic radiosurgery (SRS) [78]. SRS as an initial boost followed by standard volume treatment was the

subject of the prospective RTOG 93-05 trial, failing to show any superiority of this treatment over conventional RT in comparable cases [79]. Differently, Tanaka et al. retrospectively compared patient with GB who received conventional 60 Gy RT vs. 80–90 Gy 3D-CRT and found a survival benefit for high-dose 3D-CRT [80].

In conclusion, the vast majority of the available reports seem to show that RT is a prognostic factor just as a dichotomic parameter: the related survival advantage exists, as compared to surgery alone, but this is not dose-dependent according to a continuous dose-effectiveness function above 60 Gy, as normally happens in solid tumors. This observation poses an intriguing and still unresolved question, from a radiobiological point of view: the issue is widely addressed elsewhere in this book.

## Chemotherapy

Early studies on CHT of GB focused on drugs able to cross the Blood–brain Barrier (BBB) and particularly on nitrosoureas (alkylating agents with this capability) for clinical use, such as Carmustine (BCNU) or Semustine (MeCCNU). RT plus BCNU achieved a modest, not statistically significant improvement of long-term survival, as compared to RT alone, after a Brain Tumor Study Group random trial (BTSG 72-01) out of 358 "malignant glioma" patients [81]. Two independent meta-analyses also suggested that adjuvant nitrosoureas chemotherapy results in a modest increase in survival (from 6 to 10 %-increase in the 1-year survival rate) [82, 83]. Of note, this last study included a relevant percentage (37 %) of patients affected by gliomas of lower grade than GB.

Temozolomide is another alkylating agent able to cross the BBB, and early studies have shown a remarkable activity on recurrent GB [84]. EORTC and NCIC conducted a phase-III trial of RT alone (60 Gy over a period of 6 weeks) vs. concurrent RT-TMZ (75 mg/sm/day for 6 weeks) followed by adjuvant TMZ (150–200 mg mg/sm/day for 5 days, q. 28 days for six cycles), in patients with newly diagnosed GB [9]. Combined RT-TMZ had an acceptable side-effect profile and achieved a significant median survival increase (14.6 months vs. 12.1 months) and the 2-year survival rate was significantly greater, as compared to RT alone (26.5 % vs. 10.4 %), with a 37 % decreased risk of death. Presently, clinicians consider RT plus concurrent and adjuvant TMZ a standard of care for newly diagnosed GB. However, the inclusion of TMZ in postoperative therapy of GB patients is not an independent, favorable prognostic parameter, given that effectiveness of TMZ depends on the methylation status of the O6-methylguanine-DNA methyltransferase (MGMT) promoter. This was demonstrated out of the patients included in a EORTC–NCIC trial [85]. A significant median survival benefit was demonstrated, in fact, for patients undergoing RT-TMZ, whose GB showed a methylated MGMT promoter, as compared to those with the same feature undergoing RT only (21.7 vs. 15.3 months, respectively, $p = 0.007$). Contrarily, the difference between the same treatment groups, out of non-methylated-MGMT GB patients, did not attain statistical significance. The MGMT activity in repairing the drug-induced DNA alkylation causes the lack of effectiveness of TMZ as an adjunct to RT in improving prognosis of GB in the postoperative setting, in fact, a situation prevented by the methylation of the MGMT promoter. This mechanism is the subject of a following section of this chapter, in that prognosis depends also on tumor-related factors, besides TMZ CHT accomplishment.

## Targeted Therapies

Novel perspectives derive in experimental studies from targeted therapies, either alone or combined with traditional RT and CHT [86, 87]. However, clinical trials, had not yet yielded significant results in terms of patient survival improvement. GB is a largely heterogeneous cancer, which partly justifies failure of its treatment [86, 88]. Large-scale omics analyses are unraveling GB pathobiological-altered pathways, which, in the future, might allow for a more comprehensive discovery of prognostic and predictive factors, as well as for novel targets for personalized therapies [88].

## Tumor-Related Prognostic Factors

### Pathology Classification of GB

Histological features of GB are pleomorphic cells, mitotic activity, intravascular microthrombi, necrosis with or without pseudopalisading, and microvascular proliferation, being the last two characteristics necessary for diagnosis. Different histological patterns are recognized, that is, small cell GB, giant cell GB, gliosarcoma, etc. However, these morphological features or categorizations may not have a reliable prognostic value, as life expectancy can be the same for all of them. On the other hand, the previously quoted distinction between "primary" and "secondary" GB, according to the evidence of a precursor lower-grade glioma in the latter, does not imply different morphology features, but has some impact on prognosis. The different aggressive behavior between these two entities is attributable to different genetic pathways in tumor evolution [89]: the former type of GB (95 % of the overall GBs, the most aggressive, arising *de novo* after a short clinical story) shows in many cases (70 %) LOH 10q, and—in 25–36 %—*EGFR* amplification, $p16^{INK4a}$ deletion, *TP53* mutation and *PTEN* mutation. The latter (5 % of the cases) evolves over time, usually in younger patients, from grade II or grade III astrocytoma (with mutated *TP53* in 53–59 %) and mutated IDH1/2 trough one or two subsequent steps, eventually developing LOH 10q (63 %), *EGFR* amplification (8 %), $p16^{INK4a}$ deletion (19 %), *TP53* mutation (65 %), and *PTEN* mutation (4 %) [27, 90]. *EGFR* amplification, IDH1/2 mutation, *TP53* mutation, and *PTEN* mutation rates are distinctive signatures between primary and secondary GBs.

WHO recognizes a "GBM-o" category of GB [91], which has areas of oligodendroglioma and corresponds to anaplastic oligoastrocytoma with mitosis and necrosis, with or without microvascular proliferation. GBM-o may have a better response to therapy and prognosis, as compared to standard GB. However, the identification of GBM-o requires molecular subtyping that discloses the genetic pathway of oligodendroglioma. Loss of heterozygosity 1p/19q correlates with the morphology of oligodendroglioma, and is associated with *IDH* mutation, *MGMT* promoter methylation, G-CIMP phenotype, and a proneural phenotype (see below). Co-deletion of 1p/19q is mutually exclusive with *TP53* mutation. However, GBM-o shows low (≤30 %) rates of 1p/19q co-deletion and genetic heterogeneity, and this marker is useful for differentiation among anaplastic oligodendroglioma, mixed glioma or GBM-o [92].

The Cancer Genome Atlas Network (TCGA) catalogued recurrent genomic abnormalities in GB, which grounded a gene-expression molecular classification of GB into proneural, neural, classical, and mesenchymal subtypes [93]. An aggressive postsurgical therapy (that is, RT with >3 cycles of concurrent chemotherapy, versus a less intensive management), achieved a significantly reduced mortality in the classical (HR = 0.45, $p = 0.02$), and mesenchymal subtype (HR = 0.54, $p = 0.002$), a borderline impact on survival in the neural (HR = 0.56, $p = 0.1$), and no effect on the proneural subtype (HR = 0.8, $p = 0.4$). The proneural subtype is predominant in young age and in secondary GB.

### Biomolecular Factors

We consider henceforth the genetic and molecular signatures that have been most frequently associated with survival outcomes in GB on the grounds of analyses carried out of the pathological samples, taking into account both the prognostic parameters emerging independently from therapy, and those relevant for patients undergoing postoperative standard RT-CHT. We do not attempt here, to consider prognostic biomolecular factors in their relationship with therapy against molecular targets, due to the heterogeneity of data and a present general inconsistency of clinical results with respect to the biological premises. Furthermore, caution is necessary in interpreting the results reported hereafter, in that their significance may largely depend on methodological issues.

#### *MGMT*-Methylation Status

The O6-methylguanine-DNA methyltransferase (*MGMT*) promoter methylation status is a prog-

nostic biomarker in GB undergoing RT-TMZ [85], as outlined before, while its independent predictive power on survival is still uncertain. A meta-analysis study on 2018 high-grade glioma patients included in 20 reports showed that MGMT gene silencing was significantly associated with improved survival in patients undergoing RT-TMZ; this advantage was less significant in those receiving only RT, and null in those receiving neither TMZ nor RT [94, 95] randomly compared elder patients either to receive RT or TMZ: a survival benefit related to *MGMT*-methylation status was evident only for patients receiving TMZ. Contrarily, others demonstrated a better overall survival for high-grade gliomas showing methylation of the MGMT promoter, irrespective of therapy [96]. However, caution is necessary when interpreting all of these results, for several reasons.

First, most studies addressing the above issue, deal with high-grade gliomas in general. However, Anaplastic Astrocytoma (AA, or WHO grade-III glioma, that is included in the high-grade glioma category together with GB) does not show a significant survival advantage after TMZ therapy, in our experience [97]. Some authors evidenced, in fact, that MGMT promoter methylation status does not provide enough information about the sensitivity of AA to alkylating agents [98, 99], and that MGMT expression may be significantly lower in AA than in GB [100]. Thus, including AA in MGMT-methylation status evaluation as a factor for prognosis or response to therapy in GB may be inappropriate.

Second, the method of assessment of the methylation status was not the same in all studies addressed to this subject. Presently, in fact, methylation-specific PCR or pyrosequencing are considered the tests of choice to determine MGMT promoter methylation status, and immunohistochemistry for MGMT protein expression is not recommended [92].

Third, a sample classification according to the methylated and nonmethylated status for a gene, may be dependent on the relationship between the overall CpG island methylation, the CpG methylation at individual sites, and the effectiveness of gene silencing, that is dependent in turn on the location within the gene [101]. In conclusion, MGMT promoter methylation status is a reliable prognostic parameter only in GB patients undergoing a standard course RT-TMZ after surgery, whereas in other settings this role is an investigational subject.

### *IDH1/2* Mutations

Recent genomic studies have addressed Isocitrate Dehydrogenase 1 and 2 genes (*IDH1*, *IDH2*, and *IDH* as a whole) mutations as prognostic factors in GB [102]. These are common in secondary (73.4 %—[90]), but rare (≤10 %) in primary GB, and correlate with young age and longer survival, as compared to $IDH^{wt}$ patients. *IDH* mutation is mutually exclusive with *EGFR* amplification, whereas it is often associated with the methylation of the *MGMT* promoter.

A relatively large series of secondary GB (86 patients), in fact, was recently collected and analyzed [90] for the survival impact of the *IDH* mutation, together with 1p19q co-deletion, *p53* expression, and *MGMT*-methylation status. These authors confirmed that 1p19q co-deletion and *p53* expression were mutually exclusive, and showed that the *IDH* mutation was associated with both the *p53* expression and the methylation of the *MGMT* promoter. *IDH* mutation, 1p19q co-deletion, and *MGMT* promoter methylation were all significantly associated with increased overall and progression-free survival, whereas *p53* expression was not. After TMZ chemotherapy, GB patients with both the *IDH* mutation and the *MGMT* promoter methylation achieved the best survival result, those with no one of the two characteristics the worst, whereas those with the *IDH* mutation alone showed a result intermediate in between, with statistically significant differences. In conclusion, secondary GBs showing *IDH* mutation enjoy a better survival and response to TMZ as compared to the $IDH^{wt}$ counterpart, but whether the relationship between *IDH* mutation and *MGMT* promoter methylation is consequential, or depends on different epigenetic markers is not clarified, so far.

Recent data from the German Glioma Network [103] demonstrate that a high percentage (34 %) of 69 long-surviving (>36 months) primary GB

patients have *IDH1/2* mutations, as compared to 4.3 % out of 257 controls (surviving ≤36 months). This might indicate a prognostic role for the rare *IDH* mutations in primary GB, as suggested also by studies addressing *IDH1* mutation at the clinical onset [104], failing however to show a highly significant correlation with a better clinical outcome at multivariate analysis, when considered in respect to other well-established prognostic factors.

### *PDGFRA* Amplification

Focal amplifications of the locus at 4q12 harboring Platelet-Derived Growth Factor Receptor Alpha (*PDGFRA*) are common in all types of GB, but with a high frequency in the proneural subtype, in which it is associated with high level of *PDGFRA* gene expression, that is, a characteristic signature [93]. However, *PDGFRA* amplification has a negative prognostic impact, when evaluated by FISH out of the rare IDH1-mutant adult de novo GBs [105]: overall median survival was 2179 days in 22 GBs with *IDH1*-mutant/*PDGFR*-no amplification, vs. 480 days in 16 cases with *IDH1*-mutant/*PDGFR*-amplification. This is a statistically significant difference both at the uni- and at the multivariate analysis (log-rank: $p=0.023$, Cox proportional HR: $p=0.01$, respectively).

## EGFR

### EGFR Expression

Epithelial Growth Factor Receptor (EGFR) gene amplification is present in 40–50 % of GBs, being more common in primary than in secondary type, and is a signature of the TGCA classical GB subtype [92]. In general, it has been associated with an aggressive behavior. *EGFR* amplification results in its overexpression [106, 107], in fact, and its downstream signaling pathways enhance many cellular activities, including growth, migration, and survival [108], promoting also resistance to both RT and CHT in clinical and preclinical studies [109, 110]. In other reports, low-*EGFR*-expressing GB patients had a worse response to TMZ-containing adjuvant therapeutic regimens, as compared to those showing either high expression or no expression at all [111].

However, in the clinical setting *EGFR* amplification/overexpression is reported to impact on survival with nonunivocal results: high levels have been associated with a longer median survival [106], or with a worse prognosis in younger patients, in respect of older ones [112, 113]. Some authors suppose a complex relationship between patient's age, *EGFR* amplification, *p53* expression, and survival in GB. The poor survival noted in young patients whose tumors overexpressed EFGR, in fact, correlated also with the co-existent expression of $p53^{wt}$ immunohistochemistry [112]. On the other hand, GB patients undergoing TMZ-containing therapy, showing EGFR amplification, maintenance of PTEN, $p53^{wt}$, and p16 had a relatively favorable prognosis [114]. Others found no significant correlation of EGFR amplification with survival [115].

The combined prognostic impact of *EGFR* expression and components of its downstream pathways, such as the PI3K-Akt-mTOR signaling mechanisms deserve consideration. Autophagy is one of the metabolic pathways inhibited by EGFR, which can act via mTOR or by direct inhibition of Beclin1, a cytoplasmic protein that induces autophagy by binding to the Vps34-Vps15 core [116, 117]. In our experience, low-EGFR and high-Beclin1 expressing GBs (24 patients) have a significantly better median survival (22 months), as compared to other ones (93 patients, median survival: 8 months) showing high-EGFR and both high- and low- Beclin1 expression ($p=0.001$), after standard RT-TMZ [118]. We also experimentally demonstrated that combined EGFR and autophagy modulation impact on IR and TMZ sensitivity in human GB cell lines [119]. In conclusion, probably the EGFR expression level is not a per se reliable prognostic parameter, at the present status of knowledge, but its role in the context of the survival prediction capability of other biological or clinical markers may deserve consideration for further research.

### EGFR Mutations

The most frequent mutant of *EGFR*, expressed in 30–50 % of GB, is the EGFR variant III (EGFRvIII). The deletion of exons 2–7, that is,

the lack of the extracellular domain characterizes EGFRvIII, constitutively activate a high stimulation of the PI3K/Akt/mTOR pathway, and was found to inhibit therapy-induced apoptosis [120]. EGFRvIII enhances repair of DNA double-strand breaks, and is a cause of the resistance to gefitinib [121]. Other genetic alterations of EGFR, such as amplification, may affect both the extracellular domain, with activation of point mutations, and the cytoplasmic domain, with deletions [122]. GBs harboring EGFRvIII are more invasive, as compared to those with $EGFR^{wt}$ [123], but no data demonstrate so far a clear-cut impact on prognosis or on response to therapy.

### Loss of PTEN

Phosphatase and Tensin Homolog (*PTEN*) is a tumor suppressor gene that downregulates the PI3K/Akt/mTOR pathways, thus acting for reduced proliferation, apoptosis, and invasiveness [124]. Its mutation determined a shorter survival in GB patients, as compared to those harboring $PTEN^{wt}$ tumor, in early studies [106]. Presently, in the TMZ era, PTEN loss is not associated with poor survival in GBs undergoing current standard postoperative RT-TMZ [125]. These authors attributed their observation to a high effectiveness of TMZ in PTEN-deficient GB cells, due to their reduced homologous recombination repair activity of DSBs, and to the subsequent autophagy induction, on the ground of previous preclinical studies. In conclusion, loss of PTEN probably is an adverse prognostic marker only in GB patients not undergoing TMZ CHT.

### VEGF Expression

Vascular Endothelial Growth Factor (VEGF) is an angiogenic factor driving neovascularization, which is a hallmark of GB. However, high percentages of both Grade-III astrocytoma—or AA (66.7 %), and Grade IV astrocytoma—or GB (64.1 %) express VEGF, differently from Grade-II astrocytoma (36.8 %), out of a series of 162 cases of primary glial tumors [126]. This study demonstrated a strong correlation between VEGF expression and survival in the whole series, but not within any of the considered tumor grades. To date, no clear evidence exists of a direct relationship between VEGF expression and survival outcome of GB patients, but great scientific efforts address, instead, the relationship of VEGF with GB stem cells, and targeting VEGF with antibodies and TK inhibitors in clinical prospective trials. As a marker of clinical outcome at the present state-of-the-art, VEGF is still "potentially prognostic" [127].

### Loss of Heterozygosity 10q

The allelic deletions on chromosome 10q are frequent in both primary and secondary GB, indicating that the loss of 10q tumor suppressor genes may be important in its tumorigenesis [128], such being also the case of PTEN (10q23), already dealt with. Loss of Heterozygosity (LOH) 10q significantly emerged as a poor prognostic marker in GB, after a study on 97 consecutive patients [129]. Furthermore, in a small patient series from India, LOH 10q was correlated both with a four-fold reduced 1-year survival (not attaining statistical significance), and with age ≥40 years ($p = 0.014$) [130].

### Telomerase mRNA Expression and Activity, and Alternative Lengthening of Telomeres

Telomerase messenger expression (human Telomerase Reverse Transcriptase (hTERT) mRNA) was evaluated by PCR, together with telomerase activity as assessed by Telomeric Repeat Amplification Control (TRAP), in their relationship with survival out of a series of 42 patients (33 GBs, 5 AAs, 4 differentiated astrocytoma, 1 oligoastrocytoma) [131]. Out of the whole series, both overall survival and disease-free interval were adversely affected by hTERT mRNA expression ($p = 0.046$ for both the survival parameters) and by telomerase activity ($p = 0.007$ and 0.008, respectively) at the Kaplan-Maier statistical analysis. The Cox proportional hazard model of overall survival confirmed a significant impact of hTERT mRNA expression and telomerase activity. These authors did not analyze results separately in GB patients. A more recent paper considered the same telomerase-associated parameters for survival [132] out of 100 GB patients, and only those aged ≤60 years, lacking

both telomerase activation and hTERT positivity, showed a significantly better outcome, as compared to the other ones. Therefore, the role of telomerase activation as an independent prognostic factor in GB is not fully demonstrated so far, but deserves further study, taking into account the pathobiological features of GB in younger patients.

Relationship among telomerase activity, alternative lengthening of telomeres (ALT), and other oncogenes, is also worth of investigation. hTERT was found to promote cancer stemness through EGFR, thus inducing tumor progression [133]. In a previous study [134], we found high telomerase activity and reduced telomeres in a group of GB patients overexpressing EGFR, who were characterized by a low survival rate.

Alternative lengthening of telomeres (ALT), a presumed precursor to genomic instability, was found to be driven by mutation in ATRX (α-thalassemia/mental-retardation-syndrome-X-linked) in IDH1 mutant gliomas taking, together with the mutually exclusive del 1p,19q, a favorable prognostic impact [135, 136].

### MAPK and Akt Pathways Members, and *YKL40* Expression

Both Ras signaling pathways members, that is, the Raf/mitogen-activated protein (MAP) extracellular signal-regulated kinase (ERK)/MAP kinase (MAPK), and the phosphoinositide (PI3K)/Akt kinase/mTOR, have been shown as critical determinants of proliferation, invasiveness, and resistance of GB to ionizing radiation (revised by Pelloski et al. [137]). These authors demonstrated by immunohistochemistry, out of a series of 268 GB patients, that a high positive score for p-MAPK correlated with a significantly reduced survival probability ($p=0.003$), as well as many of the Akt cascade-activated members (p-Akt, $p=0.095$; p-mTOR, $p=0.021$; p-p70S6K, $p=0.013$). Low p-MAPK GBs showed a significantly better radiation response, as compared to those expressing high p-MAPK ($p=<0.001$). At multivariate analysis, only p-MAPK showed a significantly increased HR (1.5, range 1.1–2.2, $p=0.009$) as for survival, besides other well-known patient-related prognostic factors (age, PSK).

The aberrant initial Ras signaling has also a relevant interest for identification of prognostic factors in GB. However, Ras mutations are rare (2 %) in GB, according to the TGCA studies [138].

In vitro studies have shown that the chitinase 3-like protein (CHI3L1, or YKL40) may initiate the MAPK and the PI3K signaling cascades in human connective-tissue cells, by phosphorylation of ERK1/ERK2 and Akt, respectively [139]. YKL40 was expressed in 81 % of the cases reported by Pelloski et al., quoted above, and its expression exerted a strong negative impact on survival ($p=0.002$) [137], and is proposed as a possible candidate in regulation of the Ras-dependent pathways. YLK40 concentration can be detected in peripheral blood, as it is secreted both by tumor cells and by tumor-associated circulating macrophages: its concentration seems to correlate with an aggressive phenotype of GB, short survival, and resistance to RT (revised by Conçalves et al. [102]). However, assessment of the prognostic role of serum YLK40 level in GB is still investigational.

### Cytochrome *c* Oxidase

The enzyme Cytochrome *c* Oxidase (CcO) catalyzes the terminal transfer of electrons from cytochrome *c* to oxygen in the respiratory chain. Griguer et al. [140] have recently demonstrated by spectrophotometric determinations that a high CcO activity significantly correlates with reduced overall survival ($p=0.0001$) and progression-free survival ($p=0.0087$), out of a series of 58 primary GB patients, retrospectively evaluated. These authors extensively considered, in this regard, also previous data evidencing that CcO activity reduces Reactive Oxygen Species (ROS), thus facilitating chemoresistance to TMZ through suppression of apoptotic signaling. This series included also patients undergoing therapy before the advent of TMZ, and used for validation an external set of patients not undergoing TMZ CHT. Interestingly, the correlation between CcO activity and survival was not dependent on RT-TMZ treatment accomplishment, and the multivariate analysis indicated CcO activity as a prognostic parameter independent by age, gender,

and MGMT promoter methylation status. CcO activity as a reliable, independent prognostic indicator in glioblastoma, and the hypotheses addressing its role in a mechanism of drug resistance, should be the subject of further research.

### *HOXA9* Gene Expression

Class I homeobox (HB) genes, encoding transcription factors playing a role both in normal development and in tumorigenesis, include *HOXA* genes, mainly activated in GB (revised by Conçalves et al. [102]). Among them, *HOXA9* expression—related to a transcriptional pathway of PI3K—is associated with enhanced cell proliferation, antiapoptotic function, and a worse prognosis in GB [141]: out of two different sets of GB patients, *HOXA9* positivity was significantly an independent factor, with worse overall and progression-free survival. This relationship was even more evident in methylated MGMT GBs, identifying a poor-prognosis set in this category of patients.

### MicroRNAs

Deregulation of some MicroRNAs (miRNAs or miRs) has been detected in GB and is the subject of a dedicated issue in this book, regarding preclinical investigations. A recent study, carried out of 480 GB samples of the TGCA dataset [142], addressed the prognostic role of specific miRNA interactions: high levels of miR-326/miR-130a and low levels of miR-323/miR-329/miR155/miR-210 were significantly associated with favorable OS, while high miR-326/miR-130a and low miR155/miR-210 were associated also with improved PFR. miR-323 and miR-329 were associated with long-term survival. McNamara et al. revised other data on prognostic role of miRNAs in GB patients [127].

### Glioma Stem Cell Markers

A great deal of evidence is growing of the role of GB cells showing stem characteristics (GSC) in tumor initiation and progression, and in conferring an increased resistance to therapy, as compared to their progeny: also this subject is thoroughly addressed elsewhere in this book. However, at the present status of knowledge, an extremely topical issue is whether suitable GSC markers exist that might be useful for identifying prognostic criteria also with respect to resistance to standard CHT and RT, as extensively reviewed in recent papers. Dahlrot et al. [143] have taken into account as many as 27 studies, published in the last decades, addressing also methodological issues: all of the revised papers included immunohistochemistry-based assessment of the investigated markers, and in many instances Western Blot, Confocal microscopy, Immunofluorescence, Immunoblotting, Northern Blot, Real-Time Polymerase Chain Reaction, and Gene-expression analyses. Grade II through IV (GB) cases are included, and the expression level of the CD133 membrane protein and of the filament marker Nestin resulted significantly increased with increasing grade of malignancy; their co-expression had even more influence for a dismal prognosis. Data also suggested trends for a prognostic impact for another surface marker (Podoplanine) and a RNA-binding protein (Musashi-1). Jackson et al. [144] addressed their analysis to the progressive enhancement and gain of GSCs during disease progression and GB recurrence after therapy, considering a possible relationship between GSC markers and the emergence of the more aggressive transcriptional subtype of GB (that is, mesenchymal GB) and of Gliosarcoma (GSM) in recurrences. According to these authors, CD133+ GSCs exhibit transcriptional profiles resembling the "better prognosis" proneural subtype, whereas CD133− GSCs may predominate in mesenchymal GB, and CD133 expression may be downregulated in GSM. Thus, correlating the CD133 expression with prognosis of GB may be misleading. The related literature, in fact, shows contradictory results, but methodological issues may be also determinant in this regard. The quantitative expression of CD133 stem cell antigen mRNA was assessed by RT-PCR in 48 primary GBs by Metellus et al. [145], and high CD133 mRNA expression was shown as a significantly ($p = 0.007$) adverse factor for overall and progression-free survival at multivariate analysis. Contrarily, the CD133 immunohistochemical expression was not a prognostic marker in an analysis out of 68 GB patients, which failed also

in demonstrating a possible correlation between CD133 expression and MGMT protein expression or MGMT promoter methylation status [146].

A suitable approach for identifying useful GSC markers may be addressing the GSC-related gene-expression signatures out of large dataset analyses, such as TCGA. Kim et al. [147] identified stem-like cell-specific gene sets that could be used to divide the tumor samples into several groups, and showed a significantly ($p=0.0051$) improved 2-year overall survival for a group of genes (nestin, SOX2, and EZH2). Their down-regulation corresponded to a significant ($p<0.003$) improvement in 2-year overall survival, that is, 34.3 % compared to 4.1 % for the group with overexpression of the same genes. Sandberg et al. [148] performed a genome-wide analysis of nine enriched populations of GSCs, in a comparison with five populations of stem cells from normal brain, using a functionally validated sphere-forming test. They identified a multiple-gene-expression signature that exists in GSCs, but not in normal brain stem cells, that significantly correlates with survival out of two publically available independent datasets of high-grade gliomas. In this report, the Wnt- and Hedgehog-pathways and the Notch-regulated targets showed altered expression in GSCs. In particular, they identified and characterized alterations of the Wnt-pathway, such as active β-catenin, which was present only in GSCs. Interestingly, a previous report of our group showed a negative impact of high β-catenin positive immunohistochemistry score on GB patients' prognosis, as well as of Gli-1 expression, which is a marker of the Hedgehog pathway activation [149].

In conclusion, at the present state-of-the-art, no GSC-related marker has a reliable role as a prognostic indicator in current clinical practice of GB, in spite of the great deal of preclinical research on this subject, showing intriguing perspectives.

## Conclusions

Present treatment modalities of GB in common practice are still based on the approach "one size fits all," that is, surgery, RT, and TMZ according to widely accepted guidelines, with a more or less grade of aggressiveness of each therapeutic agent resulting from tumor extension and expected patient tolerance. Survival outcomes were substantially stable over the last decade, in spite of substantial improvements in knowledge of the biology of this disease and of technological advances and medical procedure refinements. However, medical community is aware of the extreme complexity of GB since more than 30 years, and attempted to individuate suitable prognostic parameters, which may help to analyze therapeutic results and to drive therapeutic management. In particular, great expectations came from the recent assessment of the genomic landscape [150] and, in general, from the progressively improved understanding of the signal pathways of GB. The strikingly favorable impact of the tyrosine-kinase inhibitor imatinib on prognosis of chronic myeloid leukemia [151] and gastrointestinal stromal tumors (GIST) [152], in fact, has led to a diffuse hope that unveiling biologic prognostic markers of cancers may translate into effective target therapy. Unfortunately, this is not the case of GB so far: clinical research proceeded through prospective trials testing monoclonal antibodies, tyrosine-kinase inhibitors, or other "biological" agents directed against putative determinants of aggressiveness, on the grounds of preclinical results indicating inherent anticancer properties, or radiation- and/or chemotherapy enhancement, with no relevant outcome results [153, 154]. Possible hypotheses for explaining this discouraging scenario include: molecular signaling redundancy; clonal selection (or emergence) of resistant phenotypes under treatment; preclinical studies mainly addressed to tumor initiating or early-growth factors and not to late tumor progression mechanisms; difficulty in penetrating BBB by the drugs, etc. [153].

The trend towards a "personalized medicine" [155], which is more and more frequently implemented in other tumors, appears as presently impracticable in GB, due to its complexity. However, it is "*very reasonable to believe that in the era of individualized medicine, genomically and molecularly driven research in combination with multiple patients specific data* (*clinical, pathological, biological, proteomics,*

*imaging,* etc.)*, will ultimately be successful,*" as stated elsewhere in this book (Meldolesi E et al. Perspective of the Large Databases and Ontologic Models of Creation of Preclinical and Clinical Results). A shift towards new translational approaches is probably necessary. According to the above authors, observational studies can be implemented, grounded on large databases and heterogeneous data collection from multiple sources (i.e., clinical, imaging, laboratory, pathology, genomics, proteomics, other molecular biology data, etc.), without necessarily anticipating the possible study outcome, differently from prospective trials. Numerous information, ontology, and data standardization, "rapid-learning" machine techniques, advanced statistical methods, and external validation of the results, are necessary for this purpose. This approach could also include as a premise the yield of previous prospective trials (Evidence-Based Medicine, EBM), or also might produce hypotheses to be confirmed by random comparisons, but in general some limits of the prospective trials, e.g., selective patients, long time, reliability of results only within a restricted domain, might be overcome.

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