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Title: Cancer subtypes in aetiological research.

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Abstract

Researchers often attempt to categorize tumors into more homogeneous subtypes to better predict prognosis or understand pathogenic mechanisms. In clinical research, typically the focus is on prognosis: the tumor subtypes are intended to be associated with specific responses to treatment and/or different clinical outcomes. In aetiological research, the focus is on identifying distinct pathogenic mechanisms, which may involve different risk factors. We used directed acyclic graphs to present a framework for considering potential biases arising in aetiological research of tumor subtypes, when there is incomplete correspondence between the identified subtypes and the underlying pathogenic mechanisms. We identified two main scenarios: (i) weak effect, when the tumor subtypes are identified through combinations of characteristics and some of these characteristics are affected by factors that are unrelated with the underlying pathogenic mechanisms; and (ii) lack of causality, when the set of characteristics corresponds with a mechanism that is actually not a cause of the tumor of interest. Examples of the magnitude of bias that can be introduced in these situations are provided. Although categorization of tumors into homogenous subtypes may have important implications for aetiological research and identification of risk factors, the characteristics used to classify tumors into subtypes should be as close as possible to the actual pathogenic mechanisms to avoid interpretative biases. Whenever our knowledge of these mechanisms is limited, research into risk factors for tumor subtypes should first aim to causally link the characteristics to the pathogenic mechanisms.

Keywords: cancer subtypes, molecular characteristics, bias, disease classification, aetiological research
Introduction

Clinical and aetiological research often aims to categorize disease entities into more homogeneous subtypes to better predict prognosis, or to improve the understanding of pathogenic mechanisms. Typically, a disease is classified into subtypes that share specific characteristics. In particular, any characteristic associated with prognosis and/or response to treatment may identify subtypes that differ regarding their clinical outcome; however, the same characteristics may not correspond to specific aetiological mechanisms, and vice versa.

Research into the identification of disease subtypes has recently increased due to advances in high-throughput techniques to explore molecular patterns, the availability of large biological databases, and the implementation of collaborative initiatives to obtain and analyze large numbers of tissue samples in a standardized fashion. This increased research activity is tightly linked with the concept of precision medicine which involves the identification of disease subtypes to better target the treatment, estimate prognosis and/or manage clinical follow-up. This concept is having a particular impact on clinical oncology and cancer research [1], but is being adopted also in non-malignant diseases, including, for example, asthma [2], cardiovascular disease [3] and diabetes [4].

A related development is to use disease subtypes in aetiological studies. Through unsupervised cluster analysis, for example, breast cancer researchers have identified subtypes of breast cancer, based on the expression of ESR1 (estrogen receptor), PGR (progesterone receptor), and ERBB2 (HER2) [5]. While this classification is usually adopted for prognosis and to inform therapeutic choices [6], it has been recently proposed to use it for etiological purposes. Epidemiological studies have shown that triple negative breast cancer may have specific risk factors [7].

Traditionally, tumor classification has been primarily based on the anatomical site of origin of the tumor [8]. Then, within each tumor site, stratification is typically carried out on the basis of the cell type of origin and, sometimes, other specific characteristics. It is now becoming increasingly
possible to further subdivide the tumors on the basis of their molecular features [9]. Thus the process of identification of tumor subtypes currently goes from the identification of an organ-specific tumor (e.g. “breast cancer”), to the classification of that tumor into specific subtypes (e.g. “triple-negative breast cancer” or even finer subtypes [10, 11]).

It is debatable whether such tumor subtypes represent subtypes of a single disease, or should be treated as different diseases. We will approach this issue pragmatically assuming that an organ-specific tumor type has been identified, and the issue is whether and how to divide this into subtypes. This approach mimics the current clinical approach to tumor heterogeneity: the characterization of tumor subtypes – often based on molecular analyses of the tumor tissue - logically follows the diagnosis of the tumor as an organ-specific entity. For example, a patient is first diagnosed with a breast cancer and, then, after having the results of immunohistochemistry assays on the tumor tissue, that tumor is further subclassified on the basis of the receptor status.

We support the need to reach a clearer definition of tumors in terms of pathogenic mechanisms. However, in this paper we argue that aetiological research into tumor subtypes may, under some circumstances, be problematic. A better understanding of these limitations is required for appropriate planning and interpretation of aetiological studies based on tumor subtypes. In this paper, we first present a conceptual framework for considering the correspondence between pathogenic mechanisms and the identification of the disease subtypes. We then assess two scenarios in which there is not a direct correspondence between the characteristics and distinct pathogenic mechanisms: (i) weak effect, when the tumor subtypes are identified through combinations of characteristics and some of these characteristics are affected by factors that are unrelated with the underlying pathogenic mechanisms; (ii) lack of causality, when the set of characteristics corresponds with a mechanism that is actually not a cause of the tumor of interest. Finally we
provide some suggestions on how to conduct research to establish the links between the characteristics and the pathogenic mechanisms.

**Tumor subtypes and pathogenic mechanisms**

In the context of aetiological research, tumor subtypes have aetiological value if they identify distinct pathogenic mechanisms. In Fig. 1 we use directed acyclic graph (DAG) to depict the supposed causal relationships between the diagnosis of a tumor, the identification of the tumor subtypes, and the associated pathogenic mechanisms: E denotes an exposure, A denotes a pathogenic mechanism causing an organ-specific tumor T and C is a characteristic that is used alone or in combination with other characteristics to define the tumor subtypes S once the tumor T has been diagnosed. Both of the pathogenic mechanisms (A₁, A₂) lead to the development the tumor T. However tumors caused by the two mechanisms will show different characteristics (C₁ and C₂), which are then used to define the disease subtypes S. It is also possible that the characteristics used to define the tumor subtypes contribute to the initial diagnosis of the tumor, in which case there would be arrows from C₁ and C₂ to T. However, in the current example, we assume that the characteristics that are used to define the subtypes are not used to reach the first diagnosis, but are evaluated after the diagnosis of the tumor.

For simplicity, in Fig. 1 and throughout the article we assume that all variables are binary. It is thus assumed that a tumor can have two subtypes: the first is due to A₁ (and identified through C₁), the second is due to A₂ (and identified through C₂). If it is biologically possible that A₁ and A₂ coexist (as, for example, in the case of intratumor heterogeneity), then the two subtypes can also coexist. A simple example of the scenario described in Fig. 1 is the sub-classification of tumors on the basis of the cell type of origin. For example germ-line testicular cancer can be divided into seminomas and
nonseminomas: these are biological and, potentially, clinical different subtypes, and it is not uncommon that a seminoma and a nonseminoma coexist in the same patient.

In the model proposed in Fig. 1, the subtypes directly correspond to pathogenic mechanisms. The exposures may act specifically on a pathogenic mechanism (E₁ or E₃) or may be shared by the different pathogenic mechanisms (E₂). It should be noted that C₁ and C₂ may be a set of characteristics (rather than single characteristics), but should still be specific for a single pathogenic mechanism.

**Problems of causal interpretation**

It is possible that the assumption of direct correspondence between the characteristics used to define the subtypes and the pathogenic mechanism is not valid. This may affect the interpretability of aetiological studies of tumor subtypes. We discuss two scenarios, which are shown, respectively, in Fig. 2 (hereafter labeled as “weak effect”) and Fig. 3 (hereafter labeled as “lack of causality”)

*Scenario 1: Weak effect*

In the scenario depicted in Fig. 2a, the set of characteristics C₂ is caused by both pathogenic mechanisms A₁ and A₂, thus a subtype is defined by the presence of C₁ and C₂, while the other subtype has only C₂ features. This scenario may apply whenever the tumor subtypes are defined by combinations of characteristics (the definition of breast cancer subtypes, for example, requires the combination of ESR1 and PGR expression with ERBB2 expression). The situation depicted in Fig. 2a would lead to correct causal interpretations. However there may be problems of causal interpretation if, as shown in Fig. 2b, some of the characteristics used to define the tumor subtypes are affected by factors that are unrelated with the pathogenic mechanisms. In Fig. 2b, the exposure
E₄ affects the characteristic C₁ without acting on the pathogenic mechanism A₁. Since C₁ is explained also by E₄, the pathogenic mechanism A₁ is not a necessary and sufficient cause of C₁ and thus its effect on C₁ is weakened (hence the label *weak effect*).

The scenario described in Fig. 2b, in which the characteristic C₁ is caused by both the pathogenic mechanism A₁ and the exposure E₄, may imply that: (i) some individuals have the set of characteristics C₁ for mechanisms that are unrelated with the pathogenic mechanism A₁; or that (ii) the pathogenic mechanism A₁ does not always produce the associated characteristics C₁. These two mechanisms are not mutually exclusive. Their consequence is that individuals who experience the pathogenic mechanism A₂ can have both C₁ and C₂ and be spuriously attributed to the pathogenic mechanism A₁, and, vice versa, individuals who experienced the pathogenic mechanism A₁ can have only C₂ and be spuriously attributed to the pathogenic mechanism A₂. These effects can be seen in Fig. 2b when considering the effect of conditioning on C₁ on its two causes A₁ and E₄. If all variables are binary (0=absent, 1=present): (i) C₁=1 does not imply necessarily that A₁=1, as presence of C₁ could be due also to E₄=1, and (ii) C₁=0 does not imply necessarily that A₁=0 if there is an interaction between E₄ and A₁ that produces C₁=0 when, for example, A₁=1 and E₄=1. It follows that presence of C₁ may occur in absence of A₁ and absence of C₁ may occur in presence of A₁.

It should be noted that the problems described here relate to the underlying biological mechanisms leading to the tumor subtypes, and are therefore different from problems of measurement error and misclassification of the characteristics C₅. They occur even if the characteristics C₅ are perfectly measured.

*Scenario 2: Lack of causality*
A different problem may occur if one of the supposed pathogenic mechanisms leading to the characteristics used to define the subtypes is not a cause of the tumor. The DAG shown in Fig. 3 depicts such a scenario in which \( A_2 \) is not a cause of the disease, and thus there is not an arrow from \( A_2 \) to \( T \). The corresponding characteristic \( C_2 \) could still have a prognostic role, but it would not be a marker of a pathogenic mechanism. For example, methylation in the promoter of the O6-methylguanine methyltransferase (MGMT) gene in glioblastoma affects response to treatment with telozolemide (and it is thus relevant clinically), but this molecular alteration might not be a driver in the pathogenesis of this cancer type. Under this scenario, any risk factor for \( A_2 \) (and thus \( C_2 \)) would be associated with the both disease subtypes defined on the basis of \( C_2 \) (presence or absence of \( C_2 \)) even if this risk factor does not have any aetiological role for the tumor of interest.

**Numerical examples**

In Table 1, we use the scenario of Fig. 2b and consider a tumor caused by two possible pathogenic mechanisms: \( A_1 \) which always causes the characteristics \( C_1 \) and \( C_2 \), and \( A_2 \) which always causes only the characteristic \( C_2 \). We also assume that an exposure \( E_3 \) doubles the risk of \( A_2 \). We assume that the population risk of \( A_1 \) is 1 per 1000 and that the population risk of \( A_2 \) is 1 per 1000 when \( E_3=0 \) and 2 per 1000 when \( E_3=1 \). We assume complete case ascertainment and diagnosis, irrespective of the mechanism involved, and that all variables are measures with no error. We also assume a 10% risk of developing the characteristic \( C_1 \) for reasons that are unrelated with the disease of interest (compared to 100% of subjects who are affected by \( A_1 \)). Finally, we assume that the two mechanisms \( A_1 \) and \( A_2 \) can coexist, even if in this particular example, this assumption has negligible effects because the risks of \( A_1 \) and \( A_2 \) are low.
Using these assumptions, Table 1 gives an example of the weak effect scenario caused when \( C_1 \) can occur also in individuals without the disease of interest. Although the exposure \( E_3 \) affects only the pathogenic mechanism \( A_2 \) (which corresponds to tumor subtype 2 – \( S_2 \)), analyses based on tumor subtypes would suggest that \( E_3 \) also affects \( S_1 \) (which corresponds to the unaffected pathogenic mechanism \( A_1 \)).

In Table 2, we also assume that only 50% of the subjects having the pathogenic mechanism \( A_1 \) will in fact have the characteristic \( C_1 \). This, in combination with the occurrence of \( C_1 \) in subjects without the disease, further contributes to the interpretative problems due the weak effect scenario: the risk ratio for \( S_2 \) (corresponding to the pathogenic mechanism \( A_2 \)) underestimates the effect of \( E_3 \) on \( A_2 \) (RR of 1.67 vs. a true RR of 2.00), while the risk ratio for \( S_1 \) further overestimates the effect of \( E_3 \) on \( A_1 \) (RR of 1.15 vs. a true RR of 1.00).

Finally, Table 3 gives an example of the lack of causality scenario. There are only two possible subtypes (based on the presence or absence of \( C_2 \)) as \( A_2 \) is not causal for the disease, and thus the pathogenic mechanism \( A_1 \) (and \( C_1=1 \)) is a necessary cause. In Table 3 we assume that the population risk of \( A_1 \) is 1/1000 irrespectively of the level of the exposure \( E_3 \), while \( E_3 \) doubles the risk (from 10% to 20%) of another mechanism \( A_2 \) unrelated with the tumor of interest. We also assume that \( A_1 \) always causes \( C_1 \), \( A_2 \) always causes \( C_2 \), no case is left undiagnosed and all variables are measured correctly. Since the characteristic \( C_2 \) is unrelated with any pathogenic mechanism for the disease, when the analyses are stratified on \( C_2 \), the overall group of cases is divided into two groups which are in fact homogeneous in terms of aetiology. An exposure \( E_3 \) affecting \( C_2 \) would thus increase the number of patients labeled as having a “\( C_2 \) tumor subtype”, and thus decrease the
number of patients with a “non-C_2 like tumor”. It follows that E_3 would be incorrectly considered to be a risk factor for the C_2 tumor subtype and a protective factor for the non-C_2 tumor subtype.

**Discussion**

Categorization of tumors into homogenous subtypes can help to identify specific pathogenic mechanisms and thereby specific risk factors [12]. There has recently been increasing interest in the definition and detection of tumor subtypes. This has been given impetus by recent advances in various omics technologies and the resulting increase in the amount of clinically relevant information that is available. Intertumor heterogeneity [13] and molecular pathological epidemiology [14, 15] are fast developing and influential concepts in cancer research. A similar increasing attention to the identification of disease subtypes has been occurring for non-cancer diseases. For example, already in 1995, an influential paper identified three asthma phenotypes based on combinations of age at onset and persistency of wheezing [16]. This classification, as well as subsequent developments including additional clinical characteristics and inflammation markers [17] have been used in a large number of aetiological studies on the assumption that “considering these more homogeneous phenotypes in future studies may lead to a better identification of risk factors for asthma” [18].

As discussed in this paper, the markers used to sub-classify tumors into tumor subtypes should be as close as possible to the actual pathogenic mechanisms. Although this correspondence is crucial to avoid interpretative biases, our knowledge of the pathogenic mechanisms is often too limited to assess whether this important criterion has been achieved. The classification of breast cancer subtypes, for example, based on the expression of the estrogen and progesterone receptors and ERBB2, has been suggested through a cluster analysis; even if its clinical value has been proven,
distinct pathogenic mechanisms for the different subtypes have not yet been demonstrated. In fact, a recent commentary on breast cancer suggests the existence of only two aetiological components (which would correlate with the expression of the ESR1); it argues that, even if the model may seem too simplistic clinically, it is not too simple for aetiological purposes, considering that many molecular alterations may be more linked with tumor progression than with its development [19].

We suggest that aetiological research into tumor subtypes should first aim to connect the pathogenic mechanisms to the relevant characteristics, and then use these characteristics to assess whether the disease subtypes have different risk factors. Biological knowledge is a key factor. For example, when the subtypes are identified on the basis of the cell type of origin, it can be reasonably assumed, solely on a biological basis, that different cell types are involved in different pathogenic mechanisms. They may share risk factors, as, for example, small cell lung carcinoma and lung adenocarcinoma are both affected by smoking [20], but the pathogenic mechanisms remain different as they involve different cell types.

Often, however, biological knowledge is not sufficient to link a characteristic to a pathogenic mechanism and research should be conducted with the primary aim of establishing such a link. There are several options. First, characteristics that are evident at an early tumor stage are more likely to be causally linked to its aetiology than late characteristics. Tumor cells evolve during the tumor lifespan, acquiring new and complex molecular features. If we are however interested in primary prevention and early development of the tumor, molecular characteristics acquired at a later stage are less relevant and may easily be affected by mechanisms that are not related with the risk factors of interest. Thus, studies that have access to pre-diagnostic tissues are highly informative to define tumor subtypes for aetiological studies [21]. Once the subtypes are defined, they can be identified also on the diagnostic tissue, but an initial step involving pre-diagnostic tissue and early molecular characteristics would greatly enhance the potential to validly interpret subsequent studies.
Second, the risk of interpretative bias may be reduced by defining tumor subtypes on the basis of subtype-specific sets of characteristics (i.e. each subtype has different identifying characteristics) instead of combinations of characteristics partially shared by different pathogenic mechanisms. This should be taken into account, for example, when the tumor subtypes are defined on the basis of an unsupervised cluster analysis, and then a set of markers is chosen to characterize each specific cluster. For aetiological research, it is perhaps safer if the characterizing sets of markers do not overlap among clusters. Third, in some instances it is possible to directly test whether a characteristic is causally involved in tumor development. For example, to understand whether gene-specific methylation is causally involved in tumor development, it is possible to study the association between germ-line variants in the DNA methylation machinery genes and cancer incidence [22]. If an association is found, methylation markers are more likely to be causally involved instead of being just epiphenomena. This approach, which is based on the concept of Mendelian randomization, can be carried out almost systematically, at least for characteristics that are known to be affected by germ-line variants.

In conclusion, categorization of tumors into homogenous subtypes may have important implications for aetiological research and identification of risk factors. However, it is essential that the characteristics used to classify tumors into subtypes should be as close as possible to the actual pathogenic mechanisms to avoid interpretative biases. Whenever our knowledge of these mechanisms is limited, research into risk factors for tumor subtypes should first aim to causally link the characteristics to the pathogenic mechanisms.

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**Conflict of Interest:** The authors declare that they have no conflict of interest.

**References**


**Fig. 1.** Simplified diagram showing the relationship between exposures (E), pathogenic mechanisms (A), the organ-specific tumor (T), characteristics (C) and subtypes (S).

**Fig. 2.** Diagram depicting a situation in which the set of characteristics (C) used to define the disease subtypes (S) are not always specifically linked to a given pathogenic mechanism (A). 2a) E denotes an exposure potentially causing at least one of pathogenic mechanisms and T denotes the organ-specific tumor diagnosis. 2b) A further exposure (E_i) causes one of the characteristics used to define the subtypes but it is not a cause of any of the pathogenic mechanisms of interest.

**Fig. 3.** Diagram depicting a situation of lack of causality. The set of characteristics (C) used to define the disease subtypes (S) are linked with pathogenic mechanisms (A) which are (A1) or are not (A2) causes of the disease T of interest. E denotes an exposure potentially causing at least one of pathogenic mechanisms.
Table 1. Example of a weak effect scenario. Probabilities of different combinations of presence of an exposure $E_3$, pathogenic mechanisms $A_1$ and $A_2$, characteristics $C_1$ and $C_2$ and subtypes $S_1$ ($C_1=1$ and $C_2=1$) and $S_2$ ($C_1=0$ and $C_2=1$), when the characteristic $C_1$ may occur also among subjects without the disease. Relationships among the variables are described in Fig. 2b.

<table>
<thead>
<tr>
<th>Exposure $E_3$</th>
<th>Pathogenic mechanism $A_1$, $A_2$</th>
<th>Subtype $S_1$, $S_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$E_3=1$</td>
<td>0.001, 0.002</td>
<td>0.0012, 0.0018</td>
</tr>
<tr>
<td>$E_3=0$</td>
<td>0.001, 0.001</td>
<td>0.0011, 0.0009</td>
</tr>
<tr>
<td>Risk ratio $^c$</td>
<td>1.00, 2.00</td>
<td>1.09, 2.00</td>
</tr>
</tbody>
</table>

$^a$These probabilities are assumed by design (see text for details).
$^b$These probabilities are obtained on the basis of $A_1$ and $A_2$, assuming that $A_1$ always generates $C_1$ and $A_2$ always generates $C_2$ and a 10% risk of developing the characteristic $C_1$ for reasons that are unrelated with the disease of interest (see text).
$^c$Risk ratios are calculated by dividing the disease probabilities in $E_3=1$ for disease probabilities in $E_3=0$. Other approaches are possible, which, however, would give the same results as we assumed a rare disease.
Table 2. Example of a weak effect scenario. Probabilities of different combinations of presence of an exposure $E_3$, pathogenic mechanisms $A_1$ and $A_2$, characteristics $C_1$ and $C_2$ and subtypes $S_1$ ($C_1=1$ and $C_2=1$) and $S_2$ ($C_1=1$ and $C_2=1$), when the characteristic $C_1$ may occur also among subjects without the disease and the pathogenic mechanism $A_1$ does not always produce $C_1$. Relationships among the variables are described in Fig. 2b.

<table>
<thead>
<tr>
<th>Exposure</th>
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<th>Subtype$^b$</th>
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<tr>
<td></td>
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<td>$A_2$</td>
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<tr>
<td>Risk ratio$^c$</td>
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<td>2.00</td>
</tr>
</tbody>
</table>

$^a$These probabilities are assumed by design (see text for details)

$^b$These probabilities are obtained on the basis of $A_1$ and $A_2$ and assuming that $A_1$ always causes $C_2$ and causes $C_1$ in 50% of the affected subjects, while $A_2$ always causes $C_2$. We also assume a 10% probability of $C_1$ in subjects not having $A_1$ (see text)

$^c$Risk ratios are calculated by dividing the disease probabilities in $E_3=1$ for disease probabilities in $E_3=0$. Other approaches are possible, which, however, would give the same results as we assumed a rare disease
Table 3. Example of a lack of causality scenario. Probabilities of different combinations of presence of an exposure $E_3$, pathogenic mechanisms $A_1$ and $A_2$ (the latter is not a cause of the disease of interest), characteristics $C_1$ and $C_2$, and $C_2$-subtype ($C_1=1$ and $C_2=1$) and non $C_2$-subtype ($C_1=1$ and $C_2=0$). The relationships among the variables are described in Fig. 3

<table>
<thead>
<tr>
<th>Exposure</th>
<th>Pathogenic mechanism$^a$</th>
<th>Subtype$^b$</th>
</tr>
</thead>
<tbody>
<tr>
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<td>non $C_2$-subtype</td>
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<tr>
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<td>$A_2$ (not causing the disease)</td>
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</table>

$^a$These probabilities are assumed by design (see text for details)

$^b$These probabilities are obtained on the basis of $A_1$ and $A_2$ and assuming that only $A_1$ causes the disease of interest

$^c$Risk ratios are calculated by dividing the disease probabilities in $E_3=1$ for disease probabilities in $E_3=0$. Other approaches are possible, which, however, would give the same results as we assumed a rare disease.