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BIOLOGICAL AND CLINICAL CONSEQUENCES OF LONGITUDINAL STUDIES IN RODENTS: THEIR POSSIBILITIES AND LIMITATIONS . AN OVERVIEW

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SUMMARY

Rats and mice are used in gerontological research primarily because of their relatively short life spans, ease of handling, and the relatively low costs of production and maintenance under controlled environmental conditions of large numbers of rodents as compared to larger laboratory animal species. They are being used as models for studying intrinsic aging processes, processes that give rise to diseases associated with aging, and the influence of environmental factors on these processes. Contrary to the situation in man, longitudinal studies in rodents can be conducted under well controlled environmental conditions. It has been shown that multiple pathology, the hallmark of aging in man, also occurs in inbred strains of rodents. Some of these lesions are genetically determined and some of them are randomly distributed amongst members of the same inbred strain. Serial killing experiments are necessary to obtain information on the time of development of these lesions in order to interpret properly the outcome of investigations. Furthermore, it has been shown that a considerable variation can exist in the observed maximum ages of the longest-lived animals in cohorts of rats kept under well controlled conditions. For this reason, caution should be exercised in interpreting data from studies which claim maximum lifespan prolongation.

Key words: Longitudinal study; Maximum lifespan prolongation; Multiple pathology, Animal models of aging

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INTRODUCTION

Mammals used in biomedical research act as substitutes for man. In gerontological research, animal models are employed to study the basic mechanisms underlying the aging process, the relationship between aging and disease, and the influence of environmental factors on the aging process. An animal model for gerontological research can be defined as follows: "a living organism on which a normative biological or behavioural aspect of aging can be studied, or on which a spontaneous or induced age-related pathological process can be investigated, and in which the phenomenon in one or more respects resembles the same phenomenon in man" [1]. Furthermore, it has to be realized that animal models are used to establish facts. Once established, the facts may lead to a better understanding of what happens in man or to further experimentation [2].

The aging process must be regarded as an extension of the growth and development phases and can be considered to be a dynamic process. To study this dynamic process one can make use of cross-sectional or longitudinal studies. In cross-sectional studies, groups of various ages are studied and age-related differences are sought. In longitudinal studies, serial prospective measurements are obtained in one group of subjects at specified intervals. Rowe [3] discussed the advantages and disadvantages of the two methods and concluded that longitudinal studies have advantages over cross-sectional studies. However, the drawbacks of longitudinal studies in man are that they are often impractical due to the long lifespan and frequent mobility of humans. In addition, genetic and environmental factors may vary greatly from one person to another, so that the results eventually obtained from such a longitudinal study must be interpreted with caution. On the basis of these arguments and because of legal and ethical constraints on human experimentation it can be stated that animal models represent an essential means of studying the different aspects of aging as outlined above. Depending upon the choice of the animal species, and especially in the laboratory rodents, one can easily perform longitudinal studies under controlled environmental conditions. This will limit the number of variables to a great extent. With regard to the extrapolation of data from animals to man, the fundamental aspects of aging are the same in all mammals.

MICE AND RATS

Mice and rats traditionally have been used in biomedical research. Their use in aging research has steadily increased in recent years, primarily because of their relatively short lifespans, ease of handling in the laboratory, and the relatively low costs of production and maintenance of large numbers of rodents as compared to larger laboratory animal species [for a review see refs. 1 and 4]. In the majority of the studies, long-lived inbred strains of mice and rats are being used. An aged mouse or rat can be defined as one older than the 50% survival age for its strain and derived from a population which exhibits a more or less rectangular survival curve and in which those that die exhibit multiple pathological changes [5].

HEALTH STATUS AND SPONTANEOUS PATHOLOGY

For aging research it is of great importance to have available animals of good quality [6]. Specific-pathogen-free (SPF) animals are mainly used for these studies. The costs of producing and maintaining SPF mice or rats add an extra financial burden to the already expensive experiments. It must be realized that unless the SPF status is specified, the term SPF is rather meaningless as was shown when comparing the microbiological status of four SPF units in Europe [7,8]. No information is presently available on the influence of differing SPF status on the outcome of experiments. Detailed information on husbandry, health status, survival data and spontaneously occurring neoplastic and non-neoplastic lesions of WAG/Rij, BN/BiRij, (WAGxBN)F_1 rats and CBA/BrARij, RFM/UnRij, C57BL/KaLwRij and NZB/Lac mice used in gerontological research at the Institute for Experimental Gerontology TNO has recently been published [5,9–11]. Numerous other publications deal with the pathology of aging rodents [12–21].

It is often not realized that not only the health status, but also environmental factors and the subtleties of the experimental procedure influence the outcome of a study [22,23].

COSTS OF AGED RODENTS

A consideration often overlooked in using rodents for gerontological research is their costs. As has been mentioned before, experiments involving aged mice or rats must be performed with animals that have reached or passed the 50% survival age. This implies that at least half of the animals originally set aside for aging studies will have died before such experiments can begin. Therefore, the cost of maintaining the mice or rats that die during the first part of the their survival curve must be added to that of the aged mice or rats necessary for a particular experiment. The standard price of a mouse or rat is calculated in most laboratory animal facilities by taking into account the so-called capital costs (e.g., depreciation of buildings and equipment), direct recurrent costs (e.g., animal caretakers' salaries, feed, bedding, etc.), indirect recurrent costs (e.g. overhead, equipment service and repair, etc.) and reproductivity per breeding female. The standard price usually is expressed as the cost per animal per day. The cost of maintaining a particular number of unused animals during the first portion of their survival curve can be calculated on the basis of the survival curve, i.e. the length of time that each was present in the colony until the time that animals of desired age are available. Since it is desirable not to disturb the makeup of a group of rodents within a single cage, another factor to be considered is that, as they age, their number per cage decreases. As a result, general personnel and material costs increase, since fewer animals are being housed per unit area and the effective time required for daily care and animal observations increases. Since detailed cost accounting of these various factors is difficult, a formula was derived which aims to combine the above considerations and arrive at an equitable price [5]. The cost of an aged mouse or rat, as calculated at the Institute for Experimental Gerontology TNO, is

derived from the formula:

$$\text{age (days)} \times \text{standard price} \times \frac{100\%}{\%\ \text{survival}}$$

Using this formula costs of aged mice at the 10% survival level vary from DFl. 859.00 to DFl. 1081.00 and for rats from DFl. 4698.00 to DFl. 5569.00 depending on sex and strain (1980 data from the Institute for Experimental Gerontology TNO). It is beyond doubt that aged mice and rats represent a considerable research cost and therefore they must not be used indiscriminately. Preferably, aged rodents should be used as part of a carefully planned and coordinated multidisciplinary research effort.

MULTIPLE PATHOLOGY AS A HALLMARK OF AGING

It has been observed that multiple lesions are found in both aged mice and rats, and that there is considerable individual variability in these age-associated lesions [5,9,24]. This is especially relevant, because the occurrence of multiple pathological changes with individual variability is considered one of the hallmarks of aging in man.

Recently, data have been published by Hollander [23] and Zurcher and Hollander [25] on the outcome of a pilot study into the occurrence of multiple pathological changes in aging rats. In these studies the age-associated pathology was defined as those pathological changes found at necropsy in animals allowed to live out their natural lifespans, provided they did not die from a single disease and that they were derived from a colony which is free of life shortening intercurrent infectious diseases. Such pathological changes are generally grouped as neoplastic and non-neoplastic lesions, the latter including a heterogenous group of lesions with a degenerative, inflammatory or autoimmune basis. The numbers of neoplastic and non-neoplastic lesions per rat were compared in those dying at the 90% survival age with those dying at the 50% or 10% survival age. The data were derived from BN/BiRij (236 female, 74 male), WAG/Rij (101 female, 124 male) and (WAGxBN)F_1 hybrid (68 female, 67 male) rats from the study of Burek [9]. All animals were allowed to live out their lifespans. The husbandry conditions and necropsy protocol have been described in detail [9].

This limited study showed that more lesions per rat were diagnosed with increasing survival age, but the differences were clearer between the 90% and either the 50% or 10% survival groups than between the 50% and 10% survival groups. It appears that the total number of lesions per animal is not much different from that reported in man. Depending on the strain under study, some lesions occurred in a high frequency. The majority of the lesions, however, were observed only in a small percentage of the animals. Furthermore, a conspicuous interindividual variability was found.

It has to be realized that these data were derived from dead or dying rats. The next question which arises is whether such data are of any value in predicting which lesions are present in a living population at a specific age. This question is of importance because

experiments are being carried out with randomly selected, healthy animals and not with dead or dying ones. These lesions, when already present in living animals, may give rise to erroneous conclusions regarding physiological age changes which in fact might be caused by a pathological change of the target tissue studied. Necropsy data can be presented according to age in two different ways: as a percentage of the animals which died spontaneously during a specific age period (prevalence) or as a percentage of those which were at risk during that period (life-table calculation). The two methods and their resulting values differ appreciably. It is important, therefore, to know which of these values approximates the real prevalence in the still living population. An answer to this question can only be given by studying the incidence of lesions in healthy animals killed randomly at different ages [9,23,25]. When lesions are not related to the cause of death of the animal and develop slowly, they can be expected to be present in the living population in a frequency comparable to that found in rats that died or were killed when moribund. However, if they arise shortly before death or kill the animal within a short period, the frequency in the living population will be relatively low and will approximate the value calculated according to the life-table technique. From such a study performed by Burek [9], it appeared that the occurrence of medullary thyroid carcinoma was compatible with it being a slowly growing tumor not causing death within a short period. On the other hand, the pituitary tumor showed a pattern compatible with it being a lesion which, in a majority of cases, arises shortly before death. Because only limited data are available concerning the occurrence of lesions in healthy, living animals, it is obvious that much more information is needed regarding the kinetics with which both neoplastic and non-neoplastic lesions develop in aging mice and rats in order to draw the proper conclusions from experiments.

MULTIPLE PATHOLOGY AND ORGAN FUNCTION

Recently, Zurcher *et al.* [26] have described the possible influence of multiple pathological changes in aging rats on liver function. In this study data from the literature are provided on six strains of rats with reference to changes in the liver and endocrine glands. The major results of this study can be summarized as follows. It appears that only in BN/BiRij and Osborne Mendel (OM) rats, hepatocellular lesions and generalized conditions which may secondarily affect the liver are relatively infrequent. The age-related increase in multiple pathological changes and interindividual variability in inbred strain rats is stressed. By comparing data derived from necropsies of aged animals dying spontaneously with data of rats of the same age which were killed, it could be concluded that the incidence of lesions will be smaller in the killed rats than in the spontaneously dying animals. The difference in the incidences in the two groups will be greater for lesions developing rapidly. Comparing the incidence of endocrine tumors which may affect liver metabolism, it was shown that it will be difficult to exclude a hormonal influence by such tumors in these six strains. Furthermore, it proved to be difficult to demonstrate a relationship between certain age-related morphological changes in the liver and specific

functional disturbances. Nevertheless, this study provides data on the incidence of lesions in the liver and endocrine glands which may assist in the selection of the proper inbred strain for experiments which encompass the liver and/or endocrine glands.

De Koning-Verest [27] studied the decline in acquisition with age of female rats of two inbred strains (BN/BiRij and WAG/Rij) employing the relatively simple drink test. For detailed information concerning the experiments as well as the methodology used, the reader is referred to the publications by de Koning-Verest [27]. Wolthuis *et al.* [28] and de Koning-Verest *et al.* [29]. It can be concluded from this study that both female WAG/Rij and BN/BiRij rats of 30 months of age show a deficit in acquisition when challenged in the drink test in comparison with 3-month-old rats of the same strains. When compared with WAG/Rij rats, BN/BiRij rats clearly acquired the task more slowly but ultimately reached the same level of performance. At the time of this study it was well known that the incidence of pituitary tumors was high in the WAG/Rij rats (95%) and relatively low in the BN/BiRij rats (26%). Taking into account the observation by Burek [9] that these tumors can be considered rapidly growing ones, it seems logical to conclude that in this study, using randomly selected healthy animals, these tumors did not interfere with the outcome. At the time of the study it was overlooked that a form of hereditary retinal degeneration occurs in the WAG/Rij rat [30]. Retrospective examination of eyes of aged BN/BiRij and WAG/Rij rats showed this lesion to be present in the WAG/Rij rats used in this study. Also, it was observed that this lesion could be found in WAG/Rij rats as young as 3 months of age. Because no difference was observed between the two strains in their performance in the drink test, it can be postulated that these retinal lesions did not interfere with the outcome of the study. However, it should be realized that in a more complex test system employing visual parameters, the WAG/Rij rat may not be the strain of choice. In this context, attention should be drawn to a publication by Prieur and Creel [31] concerning the use and misuse of albino rats in biomedical research. These authors eloquently point out that one should carefully select the specific type of animal for use in an experiment. This is contrary to the more common practice of using, for sheer convenience, an inbred strain of rodent which is at hand.

MAXIMUM LIFESPAN

Numerous gerontological studies deal with the rectangularization of the survival curve and the prolongation of the maximum lifespan. Such studies have recently been reviewed by Fries and Crapo [32] and Walford [33]. Because the effects of dietary manipulation and treatment with chemicals or drugs on longevity are measured as a prolongation of the maximum lifespan – i.e. prolongation of the age of the longest lived survivor in a treated group over that of the controls – it seemed warranted to examine how well this endpoint is defined.

To study these questions, cohorts of rats and mice of two inbred strains each, from those maintained at the Institute for Experimental Gerontology TNO during the last 5

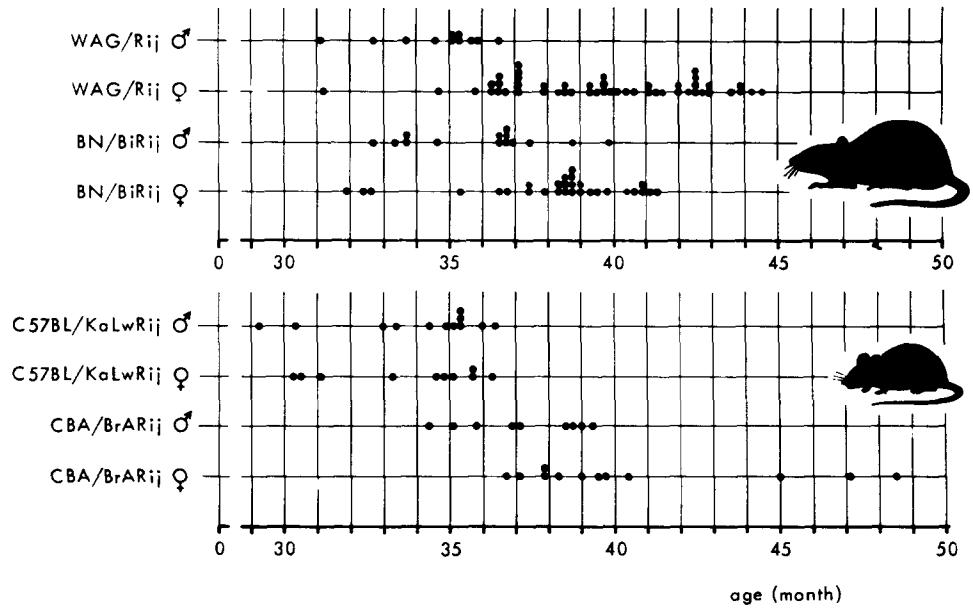

Fig. 1. Maximum lifespan in individual cohorts of male and female WAG/Rij, BN/BiRij rats and C57BL/KaLwRij and CBA/BrARij mice. Each • represents the oldest animal of a cohort.

years were analyzed. The study was designed in such a way that 20–30 animals per sex entered the cohorts at the age of 3 weeks. All were virgin animals originating from an SPF breeding colony and they were kept under clean-conventional conditions as described before [7,9]. The aim of the study was not only to monitor survival characteristics but also the age-associated pathology, so as to ascertain whether constancy existed within each individual strain and sex.

The results of the observed maximum lifespan per individual cohort are depicted in Fig. 1. As can be seen, there is a considerable range of this endpoint in both rats and

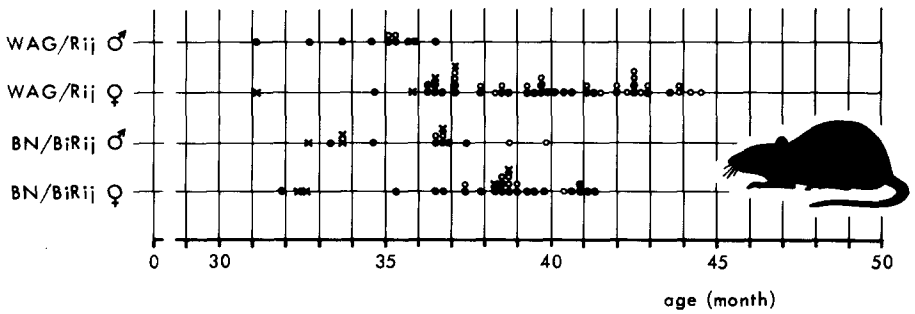

Fig. 2. Maximum lifespan in individual cohorts of male and female WAG/Rij and BN/BiRij rats. Data points represent the oldest animal of a cohort: (○) complete, undisturbed cohort; (●) cohort of which <50% of the animals was withdrawn before the end of the lifespan; (×) cohort of which >50% of the animals was withdrawn before the end of the lifespan (see also text).

TABLE I

MAXIMUM LIFESPAN IN INDIVIDUAL COHORTS OF AGING WAG/Rij AND BN/BiRij RATS

	Mean of maximum ages (ranges) in months			
	WAG/Rij ♀	*BN/BiRij* ♀	*WAG/Rij* ♂	*BN/BiRij* ♂
All cohorts	39.8 (31.2–44.5) ($n = 48$)	38.2 (31.8–41.3) ($n = 29$)	34.4 (30.9–36.5) ($n = 11$)	36.0 (32.8–39.9) ($n = 14$)
>50% of cohort withdrawn before end of lifespan	34.9 (31.2–37.2) ($n = 4$)	35.5 (32.3–38.8) ($n = 4$)	–	34.2 (32.8–36.7) ($n = 4$)
<50% of cohort withdrawn before end of lifespan	39.2 (34.6–43.6) ($n = 25$)	38.6 (31.8–41.3) ($n = 19$)	34.3 (30.9–36.5) ($n = 9$)	35.9 (33.2–37.4) ($n = 6$)
Complete undisturbed cohort	41.5 (37.2–44.5) ($n = 19$)	38.8 (37.4–40.4) ($n = 6$)	34.8, 35.3 ($n = 2$)	38.0 (36.5–39.9) ($n = 4$)

mice. Because during this time some animals were removed from these cohorts for use in other aging studies, the rat data were further analyzed by dividing them into complete, undisturbed cohorts and cohorts from which either more or less than 50% of the animals had been removed before the last survivor died. These data are presented in Fig. 2 and Table I. As can be seen from Fig. 2, the maximum ages of the cohorts from which more than 50% of the animals have been removed tended to be represented within the range of the youngest observed maximum lifespans. The same is the case if one calculates the mean of the maximum observed ages for this group (Table I). Furthermore, in each instance, the highest mean of the maximum observed ages is found in the complete undisturbed cohorts; the cohorts in which less than 50% of the animals were withdrawn before the end of the lifespan had an intermediate age value. More important, however, is the observation that the oldest observed mean of the maximum observed ages is always found in either the complete undisturbed cohorts or in the cohorts from which less than 50% of the animals have been withdrawn before the end of the lifespan. This implies that both types of cohorts can be used to depict the absolute maximum of the lifespan. The fact that this endpoint is not observed in those cohorts from which more than 50% of the animals have been withdrawn before the end of the lifespan may be explained by the greater chance that the potentially longest lived survivor in these cohorts was removed prematurely.

A disturbing finding is the observed range of the maximum ages. Even in case of the complete undisturbed cohorts this may encompass a period as long as 7.3 months, as seen in female WAG/Rij rats, or less as is the case in female BN/BiRij or male BN/BiRij rats. In the latter cases, the ranges are 3.0 and 3.4 months, respectively. The observation that the maximum age of inbred rodents kept under standardized conditions may vary from one cohort to another, and in two inbred rat strains, seriously challenge the value of studies in which by means of dietary manipulations or the addition of chemicals and/or drugs to the diet, a prolongation of the maximum lifespan is observed in the treated group as compared to the control group. Therefore, it seems warranted to have more information concerning the real maximum age of the species, strains and sex under study, as discussed above, before claiming an effect of such treatments. Furthermore, it is not known what effect the cohort size may have on the maximum ages of a particular strain and sex. However, it was found that, for example, the means of the maximum ages of female WAG/Rij rats from complete undisturbed cohorts and those of rats from cohorts in which less than 50% of the animals had been removed, differed significantly from each other (2 tailed Mann-Whitney U-test). This difference could not be explained by cohort size. Therefore, whether other cohort-related effects may influence the maximum age must be analyzed. In a sense, this type of study is as complicated as establishing the maximum lifespan of man under the present-day conditions of life.

An analysis of the mean 50% and 10% survival ages, and the mean of the maximum age of complete undisturbed cohorts of female WAG/Rij rats shows that the midpoint of the ranges observed in these instances increases from 2.65 to 3.45 to 4.20 months, respectively, implying that the maximum fluctuation is found at the maximum lifespan endpoint. It is

a comforting observation that this parameter is relatively low at the 50% survival age, because otherwise a reevaluation of the definition of an old rat or mouse may be necessary.

The above data should be expanded to include other rodent strains kept under the husbandry conditions of a specific investigator to prove or disprove their ultimate value. However, as mentioned above, one should be extremely cautious in claiming maximum lifespan prolongation in an experimental setting.

MODELS OF AGING

In the last decades, several publications have dealt with animal models for studying the physiology and pathophysiology of aging [4,5,8,10]. In these treatises aspects of such studies in both intact animals and in organ systems are dealt with. A thorough review of comparative models of selected problems of the human elderly is provided in a report of the Committee on Animal Models for Research on Aging [1]. It is beyond the scope of this overview, to either review in depth all these described models or to list them exhaustively. Suffice it to say that the value of the rodent as an animal model for aging has been well established. However, it cannot be stressed enough that the selection of an appropriate model for aging studies requires knowledge of both the survival characteristics and the age-associated lesions of the species and strain to be studied. Such information is important to prevent the selection of a rodent strain or species which is short-lived because of a specific disease or condition. Furthermore, knowledge of the age-associated pathology will prevent the selection of a strain in which the target organ to be studied is diseased or the function of which might be seriously hampered by lesions elsewhere in the body.

A selected example will illustrate the above mentioned points. Mice have been employed extensively for studying changes in the immune system with age [24,34]. Strain differences in the behavior of the immune system during aging can be recognized. Furthermore, the longitudinal study of the immune system enables one to examine not only the physiological alterations occurring during aging, but also the pathological changes which might develop as a consequence of such alterations. The CBA/BrARij strain develops essentially no lesions of an immunopathological nature. Accordingly, this strain is an appropriate model for the study of the physiology of the immune system during aging. Information obtained with CBA mice can serve as a reference for the study of mouse strains which display a specific immune dysfunction and/or lesions of the immune system such as, for instance, the C57BL/KaLwRij strain [35]. The latter strain can serve as a valuable model in which to study the mechanisms underlying these lesions, as well as their clinical consequences. Futhermore, both strains can be classified as belonging to the so-called long-lived mouse strains.

CONCLUDING REMARKS

Data have been presented on the definitions of an animal model of aging and an aged animal, on health problems, on survival, on spontaneous incidences of neoplastic and non-

neoplastic lesions in aging rats, on costs of aged mice and rats, on the occurrence of multiple pathological changes in aging rats, on the variability of the occurrence of these lesions in aging rats and on the importance of knowledge of the age-associated pathology for selecting a specific inbred strain of mice or rats for experiments. Furthermore, it has been shown that a considerable variation can exist in the observed maximum age of the longest-lived animal in cohorts of rats kept under identical husbandry conditions. This observation warranted a word of caution in interpreting data concerning maximum life-span prolongation from studies using either dietary manipulations or addition of drugs and/or chemicals to the diet. The value of the laboratory rodent for gerontological research has been established beyond doubt. However, even in this era of well defined inbred strains and husbandry conditions, experimentalists should carefully select their animal model. This is even more important in longitudinal studies, as outlined in this overview, in order to avoid drawing erroneous conclusions. With this in mind, gerontologists can conduct fruitful longitudinal studies on the biological aspects of aging as well as on age-associated diseases and these studies will be a profitable adjunct to longitudinal studies in man. It should be realized that, as is the case in studies in man, limitations exist in rodent studies and careful planning is necessary. An inbred strain of rodents should never be used for longitudinal studies out of sheer convenience because it is at hand.

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