

# Assessment of the General Oxidant Status of Individuals in Non-Invasive Samples

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## 1. Introduction

Determination of the oxidative stress state of a person indicates the risk of suffering many disorders and diseases in humans that are a product of oxidative stress. The oxidant status of an individual is assessed by determining a group of markers in non-invasive samples. Although these biomarkers are formed by oxidation of biomolecules and are supposed to reflect changes in tissues that have been exposed to oxidants, one limitation when measuring these biomarkers in non-invasive samples is that they do not give information about the tissue localization of the oxidative stress, at the least the marker is exported into serum from the tissue. In previous work from our laboratory, we have determined that only a few generic markers of oxidation can be useful to predict the oxidant status of an individual when the markers are measured in non invasive samples. An additional aspect to consider before validating the markers is to determine how stable their levels are for the same individual throughout time. Theoretically, if these markers present a high variability, their utility to study the effects of an eventual intervention would be limited since the effects of the intervention should be clear to be observed over the basal oscillation of the marker. Results from our group show a significant intra day variation of serum biomarkers in many cases. Therefore, it is clear that more than a single measurement will be required to establish the basal status of oxidative stress of individuals and several measurements will be required for a long period of time.

## 2. Aging and its biomarkers

Aging can be defined as the general loss of the optimal body functions of an organism over the years. Although there are several hypotheses that attempt to explain the causes of aging, only a few are widely accepted, which does not mean they are correct. In fact, nowadays, none of these most accepted theories entirely explain the root cause of aging. Until the root cause is known, it will be difficult to design a strategy of intervention to control aging. Also,

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it is important to emphasize that what a theory describes as the cause of aging determines not only the design intervention strategies, but also what type of markers that can be measured to assess the rate of aging.

Among the most popular hypothesis about why we age, the theory of Harman (Harman, 1956), is 50 years-old and it is continuously being revised (Kirkwood, 2005). This theory postulates that the macromolecular damage induced by reactive oxygen species (ROS) is the main causal factor of aging and related diseases. These ERO are formed during the normal metabolism, primarily in detoxification reactions mediated by the microsomal cytochrome P-450 (Ayala and Cutler, 1997; Finkel and Holbrook, 2000; Porter and Coon, 1991) and in mitochondrial electron transport chain (Finkel and Holbrook, 2000). Also, free radicals can be induced exogenously by radiation, smoking, diet, drugs, etc.

An imbalance between oxidants and antioxidants in favour of the oxidants, potentially leading to damage, is termed 'oxidative stress (Sies, 1997). According to free radical theory, the more oxidative stress, the higher the aging rate (biological and pathological aging) and age-related impairments. The intensity of oxidative stress depends on the amount of oxidants, available antioxidants, and the activity of the repair processes, which in turn are responsible for the elimination of oxidized molecules. As soon as oxidant products appear in the serum, urine or breath, they can be used as markers of oxidative stress. Assuming that oxidative stress is a contributing factor to the onset of age-related degenerative diseases (Halliwell et al., 1992; Halliwell, 2006; McCord, 2000), the determination of the oxidative stress state of a person not only allows comparison with the average of a population but it can also indicate the risk of suffering many disorders and diseases in humans that are a product of oxidative stress.

To assess the oxidant status of an individual under normal or pathological conditions or after any kind of intervention, a group of tests for the measurement of oxidative stress in non-invasive samples was developed in the last decade (Butler et al., 2004; Cutler, 2005; Cutler et al., 2005; Johnson, 2006). The idea being to get a complete picture of the oxidative stress state in the body by looking at the levels of markers for the ongoing oxidative damage in serum, urine, saliva and breath samples. Since most of the biological molecules can become oxidized, there are several types of biomarkers for the assessment of oxidative stress. Thus, in a biological sample, we can have oxidized DNA, proteins and lipids products and therefore several markers can be used. So, the first question we are going to analyze in this chapter is which one do we choose by considering that the blood or urinary levels of the marker must reflect tissular oxidative stress and that the marker levels must indicate only oxidation rate and not the rate of a different biological process.

### **3. First question about oxidative stress markers: Which one do we choose?**

Traditionally, the markers of oxidative stress have been classified in three groups, depending on the macromolecule affected: markers of oxidative damage to DNA, lipids and proteins. The attack of free radicals or oxidants to DNA leads to oxidized nucleotides that can be analyzed in serum and urine. Also, the reactions of oxidant compounds with cellular membranes produce lipid peroxides and their derivatives of them and the oxidation of proteins increases the levels of carbonyl groups and the appearance of oxidized amino acids.

According to this, the panel of markers that can be determined is really large. Therefore, the first question that arises is how many of these markers should we measure in order to assess the general status of oxidation of an individual. If the answer is "let's measure all of them", a typical scenario is that for the same person we can get contradictory results so that the marker of DNA oxidation can be high while the marker of lipid oxidation is low. Therefore, a conflict of interpretation is normal when analyzing a panel of markers. Also, the levels of the marker in serum (or urine) must reflect the oxidative damage of the tissues in general. Considering that in vivo oxidative damage is likely to occur in only a few sites or tissues at any given time, high levels of these parameters, for instance in serum, in one individual can be a consequence of: 1. - A generalized increase of oxidative damage in most of the tissues, all of them being able to export the marker into the blood proportionally to the extent of damage; 2.- The increase of oxidative damage in just a particular tissue, this being a dysfunction of the tissue, the origin of a future disease; 3. - An increase of the oxidative damage produced specifically in the circulatory system (Figure 1).

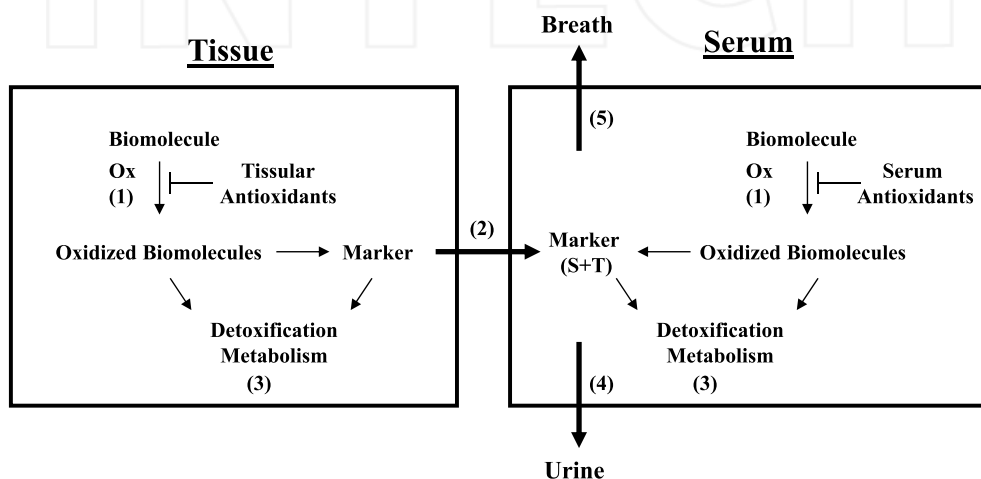


Fig. 1. **Steps involved in the levels of oxidative stress markers.** Step 1: oxidation of the biomolecule by oxidants (Ox). Step 2: Export of the tissular marker into serum. Step 3: Detoxification and metabolism of the marker. "S+T" is the sum of the amount of the marker formed in tissue and serum. Step 4 and 5: Elimination of the marker through urine and breath.

In order to clarify whether the measurement of non invasive oxidative stress marker reflects one of the above possibilities, a study in our laboratory was undertaken a few years ago where a few generic markers of oxidation were determined simultaneously in serum and tissues of six groups of rats treated experimentally to modulate their oxidative stress status (Arguelles et al., 2004). For each marker, the correlation between serum and tissular levels was calculated to test, first, whether changes in serum levels reflect changes in tissular levels and, second, whether these levels change concomitantly in all tissues. According to the scheme shown in Fig. 1., the levels of a particular marker in serum will depend on the rate of steps 1-5. The amount of oxidized biomolecule formed in a tissue or serum will depend on ROS levels and the levels of antioxidants (step 1). If the marker can be exported into

serum from one or several tissues (pathway 2), serum determination of the biomarkers would represent the summation of the amount of modified molecules excreted from the cells and the amount of modified molecules produced in the cardiovascular system at that time ("S+T"). The amount of the marker in the serum would be increased proportionally to the degree of tissular and serum oxidative stress.

Concerning carbonyl groups (CO), a marker of protein oxidation (Levine, 2002), our results indicated that there is no significant correlations between carbonyl groups of serum and tissues, which is not surprising considering the tremendous heterogeneity of protein molecules and environments in the different tissues. It is noteworthy that there is no correlation between serum CO and CO of tissues secreting proteins that act in plasma, such as liver. Due to oxidized proteins being degraded inside the tissue, it is unlikely that they can be exported to the serum once they are degraded (Figure 2). In addition, products of protein oxidation are subject to metabolism and this metabolism can be different in the tissues studied. All these factors may play a role in determining that CO in the different tissues studied and in the serum do not correlate in general. A different situation might occur with oxidized amino acids derived from oxidized proteins, which can appear in the serum and maybe the levels correlate with the tissue levels. In any case, the results of this study showed that the measurement of CO in serum is not useful in predicting the degree of tissular protein oxidative damage and only indicates the oxidative damage to serum proteins. Because carbonyl groups in serum reflects exclusively protein oxidation in the circulatory system they might reflect the risk of cardiovascular disease. This example illustrates that only a good knowledge of the biochemical pathway of the formation of the marker allows us to draw a precise conclusion about the meaning of the different values found in serum.

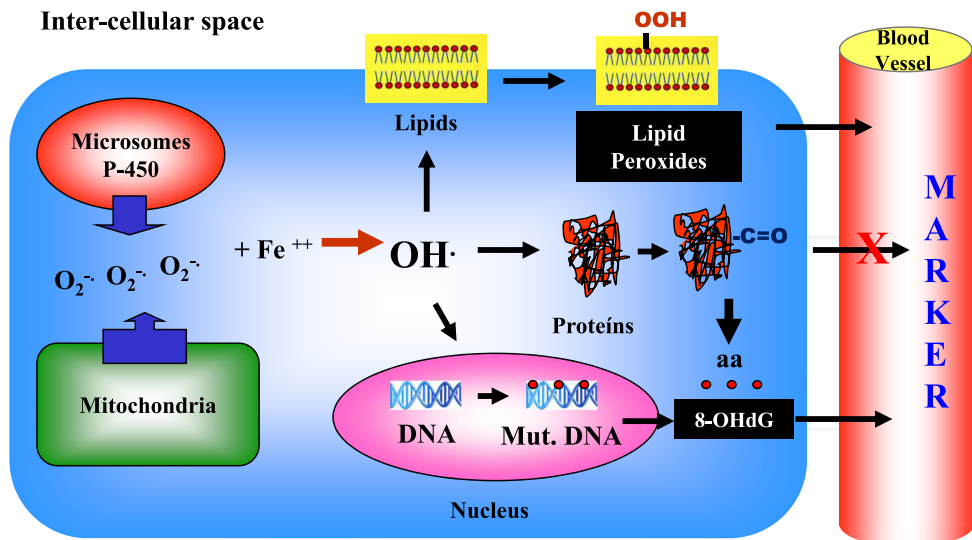


Fig. 2. Effect of free radicals on biomolecules. Lipid peroxides, Hydroperoxides, 8-OHdG and CO groups are formed as a consequence of free radicals attack ( $\text{OH}\cdot$  in this example). Oxidized proteins are not exported into serum because they are degraded inside the cell.

A second oxidative stress marker studied in this animal model was lipid peroxides (LP), which have been suggested as playing a role in the molecular mechanism of several pathological processes (Gutteridge and Halliwell, 2000). Contrary to the results with CO groups, LP changes concomitantly in both tissues and serum. This suggests that the measurement of LP in serum gives an indirect indication about what is happening in the tissues i.e, that it can be used as an indicator of the average amount of free radical damage to lipids in the body tissues at a given time.

Another consideration to take into account is that the biological meaning of the changes of some markers remain to be established when measured in non-invasive samples. For instance, concentration in the urine level of one of the popular markers in the field, 8-hydroxy-2'-deoxyguanosine (8-OHdG), is considered as evidence of a process of oxidative stress in DNA but also as evidence of an optimum repair level of the DNA (Halliwell, 2002). Obviously, this problem does not exist if this marker is directly determined in DNA extracted from tissues.

As can be seen, if one intends to get information about the general status of oxidation of an individual just by looking at the level of markers in the serum the problem is that tissue-specific oxidative damage does not generally cause systemic oxidative stress that can easily be measured in serum. This means that it cannot be assumed that all markers in noninvasive samples are useful for predicting the general oxidative status in all of a person's tissues. Also, oxidative stress in circulation must be interpreted with great caution. Because of the lack of a positive correlation between oxidative stress markers in serum and tissues, whether the risk of a specific disease incidence is more associated with a given oxidative stress level of serum or tissue is yet to be determined. Also, a more comprehensive study using more biomarkers should be performed to select those whose serum levels reflect the oxidative damage in tissues.

#### **4. Second question about oxidative stress markers: Do they change over time?**

An additional aspect to consider before validating the markers is whether these markers remain stable as a function of time for each individual, not only for a long period of time but also during the day. If the source of oxidative stress is both endogenous (mitochondria and detoxification reactions) and exogenous (Finkel and Holbrook, 2000), both fractions can change as a function of many factors such as basal metabolism, medication, diet and habits, for example, smoking (Lesgards et al., 2002; Moller et al., 1996), consequently affecting levels of the markers. Theoretically, if these markers present a high variability, they would not be useful in diagnosing the basal oxidative stress of individuals. Also, their utility to study the effects of an eventual intervention would be limited since the effects of the intervention should be clear to be observed over the basal oscillation of the marker (Figure 3).

To determine the degree of variability throughout the day, and over time, in a previous work from our laboratory (Arguelles et al., 2007), three markers of oxidative stress from healthy volunteers during a period of 51 days were measured. At the same time, the variability in the levels of these markers was studied throughout the day to test whether or not they changed and if so whether the magnitude and trend of this change would be

typical for every person. The aim of this second task was to determine the best moment during the day when the antioxidant or protective supplements should be administered. The results indicated that the levels of these markers can vary greatly within a person during the period studied (51 days). Figure 4 shows the results obtained in three patients. As can be seen, repeated serum lipid peroxide values on nonconsecutive days show serum lipid peroxides of patient 1 to be stable, but this is not the case for patient 3.

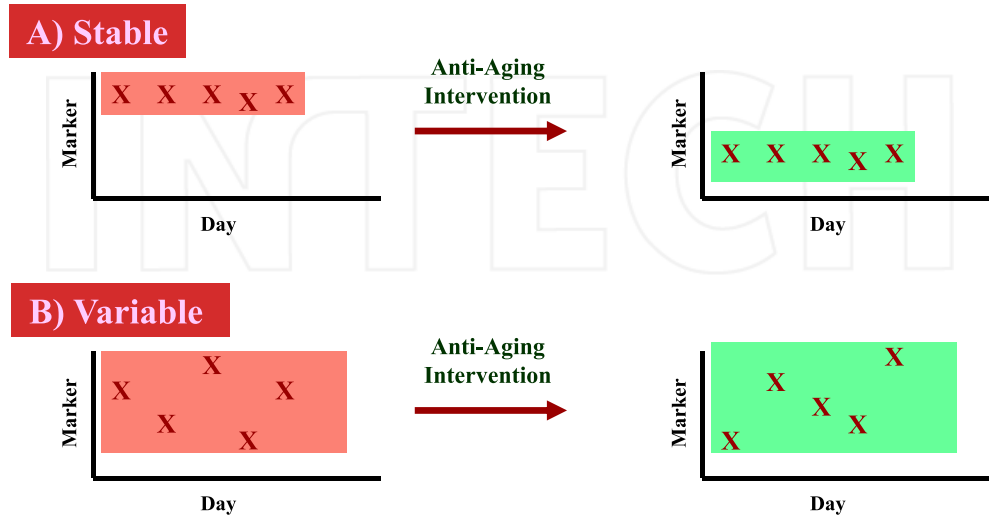


Fig. 3. Variability in the levels of a hypothetical marker. If the markers present a high variability (B), they would not be useful to study the effect of an eventual intervention.

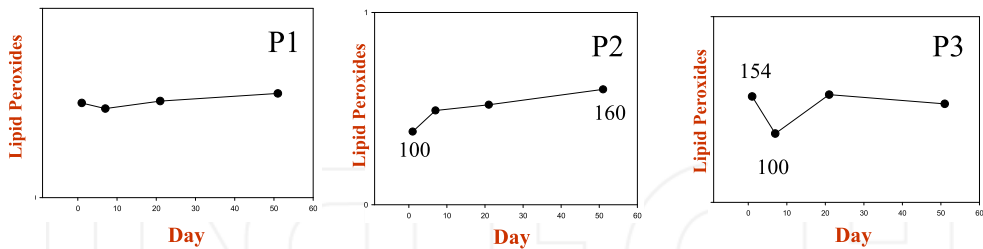


Fig. 4. Change in the morning levels of lipid peroxides in serum of three different subjects during the period studied (51 days). Results are percentage with respect to the lowest value found. (P1-P3)

As mentioned above, the two main endogenous sources of oxidative stress are mitochondrial respiration and detoxification reactions. Therefore, several factors related to basal metabolism and the constitutive content of cytochrome P-450, for example, should affect the oxidative stress of an individual. In this sense, previous works of our group have shown that people with higher amounts of hepatic p-450 present a higher oxidative stress in their proteins (Ayala and Cutler, 1997). Circadian variations in the detoxification reactions have been described and could influence (Reddy et al., 2006). Also, exogenous factors may

contribute to this normal variability of the markers. Thus, both “healthy” and “unhealthy” lifestyle patterns have been described as affecting oxidative stress and antioxidant capacity (Lesgarsds et al., 2002; Moller et al., 1996). In our study, the influence of lifestyle factors was not considered. However, considering the variability found, it would be necessary to take a survey in order to study the relationship between habits and oxidative stress markers.

The results also show a significant intra-day variation of serum biomarkers in most of the subjects, where the general trend is that the levels of oxidative stress markers seem to increase during the day (Figure 5). If this happens every day, it is tempting to speculate that the concentration of the marker starts decreasing during the night until reaching morning values. Since the days of the assays were not consecutive, we cannot affirm that this actually happens because we do not know the night values of the day before the assay. However, we might consider the reparative aspect of sleep. In fact it is described in the paper of Lesgarsds et al. (Lesgarsds et al., 2002) that remaining awake all night was responsible for an important increase in urine level of TBARS. It remains to be studied whether the morning values are affected by the values reached at night the day before and/or other factors that are secreted during sleep.

Day	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10
LP1	↑	↑	=	=	↑	↑	=	=		
LP2	↑		↑	=	↑	↑		↑	=	
LP3	↑	↑	↑	=	↑	↑		↑	=	
LP4	↑	↑	=	=	↑	=	↑	=	=	
CO1	↑	↑	↑	↑	↑	↑	↑	↑		
CO2	↑		↑	↑	↑	↑		↑	=	
CO3	↑	=	=	↑	=	↑		=	↑	↑
CO4	↑	↑	↑	↑	↑	↑	↑	=	=	

Fig. 5. Summary of the Intraday variation of oxidative stress markers in different subjects (P1-P10). Assays for lipid peroxides and carbonyl groups were measured in the same individuals three times daily on four particular days (LP1-4 and CO1-4) over a period of 51 days. The arrows show the intraday changes for each marker.

Also, it is not known whether oxidative stress increases and then the antioxidant defenses decrease as a consequence of consumption of non-enzymatic protective substances, or if the levels of antioxidants diminish first and the consequence is an elevation of oxidative stress. In any case, maybe the important item is the possible delay that can take place between the peak of oxidative stress and the participation of antioxidants, that imbalance being what

cumulatively damages the cells. According to this, if the oxidative stress of an individual increases throughout the day but at the same time the subject has enough level of antioxidants, nothing would happen. But if the increase was not neutralized by the antioxidant systems, the damage to biomolecules would be possible. In fact, it has been suggested, in a new theory of longevity, that the main factor determining lifespan is not the rate of free radical production, but the cell's ability to resist short-term fluctuations in critical metabolites caused by environmental stress (Olshansky and Rattan, 2005). Besides the importance of the total antioxidant defenses, it would be important to know which compounds are the first line of scavenging antioxidant defense in neutralizing the several peaks of oxidative stress that may occur daily and whether these compounds are the same during the 24 h-period.

As to a hypothetical intervention, ideally, it should be carried out before the increase of oxidative stress. Although we do not know the time lag between damage and increase of the markers in the serum and because for many subjects oxidative stress increases at the end of the day, it seems reasonable that the administration of the antioxidant should be distributed throughout the day instead of being administered once a day.

## 5. Conclusion

As a conclusion, we can say that a more comprehensive study using more biomarkers should be performed to select those whose serum levels reflect the oxidative damage in tissues. Also, it is clear that more than a single measurement will be required to establish the status of oxidative stress of individuals along with a study of lifestyle factors. In this way, a customized supplementation strategy can be recommended. Since these values present a great variability, it would be necessary to think about whether the determination of these markers could require the establishment of some indications of lifestyles previous to the assay. The observed variability of the markers does not limit their usefulness in studying the effect of intervention strategies. If the level of oxidative stress varies widely within, and between days, the markers can be useful in assessing the influence of an intervention on minimizing the height of the "oxidative stress peaks". Work is underway to investigate whether variability affects other markers. In addition, it would be interesting to try and establish a relationship between the concentration of the markers and habits because the most important factor in determining the levels of oxidative stress markers still remains to be known. Obviously, this study would require a larger population.

## 6. Acknowledgements

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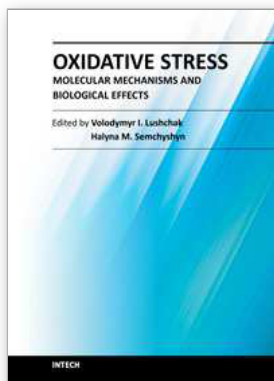


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Since the discovery of free radicals in biological systems researchers have been highly interested in their interaction with biological molecules. Denoted in 1980, and due to fruitful results and ideas, oxidative stress is now appreciated by both basic and applied scientists as an enhanced steady state level of reactive oxygen species with wide range of biological effects. This book covers a wide range of aspects and issues related to the field of oxidative stress. The association between generation and elimination of reactive species and effects of oxidative stress are also addressed, as well as summaries of recent works on the signaling role of reactive species in eukaryotic organisms. The readers will gain an overview of our current understanding of homeostasis of reactive species and cellular processes they are involved in, as well as useful resources for further reading.

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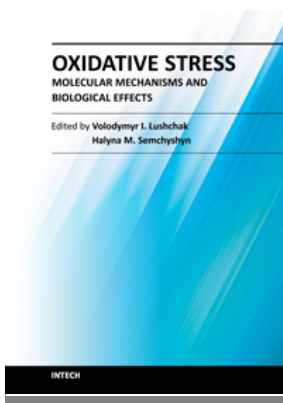
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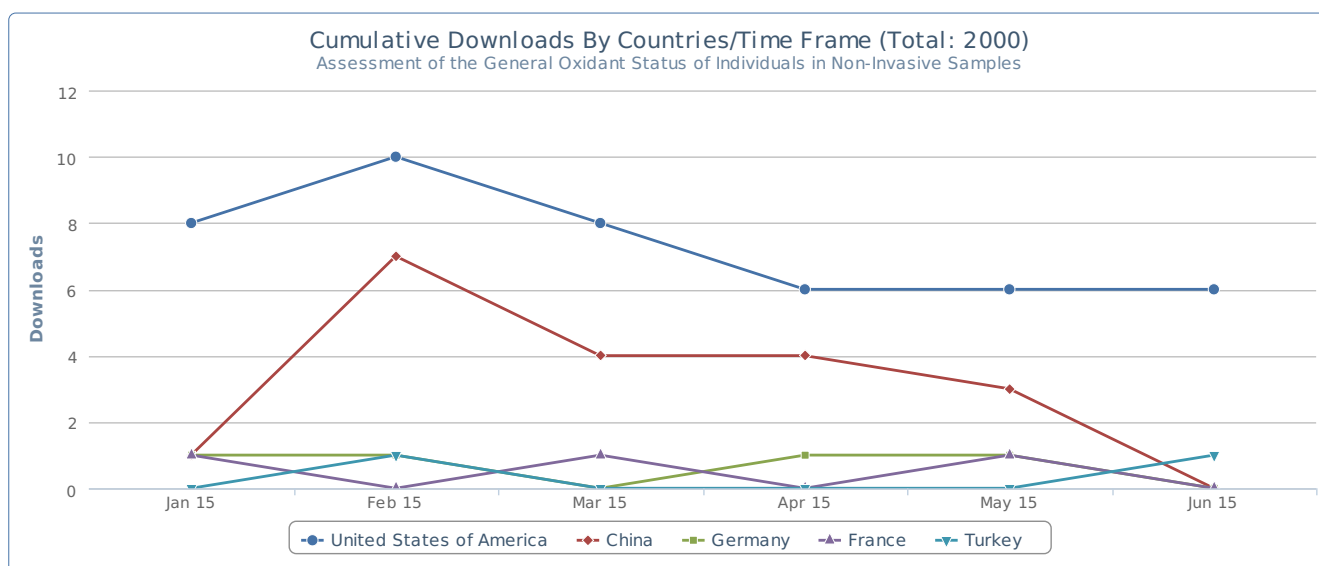
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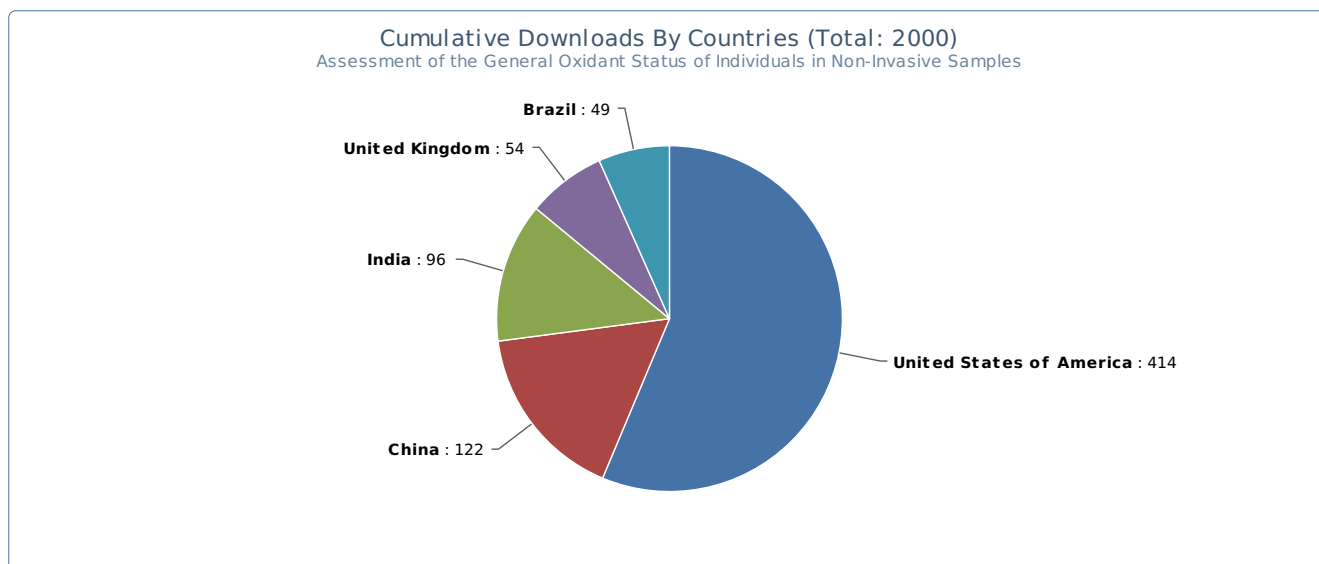
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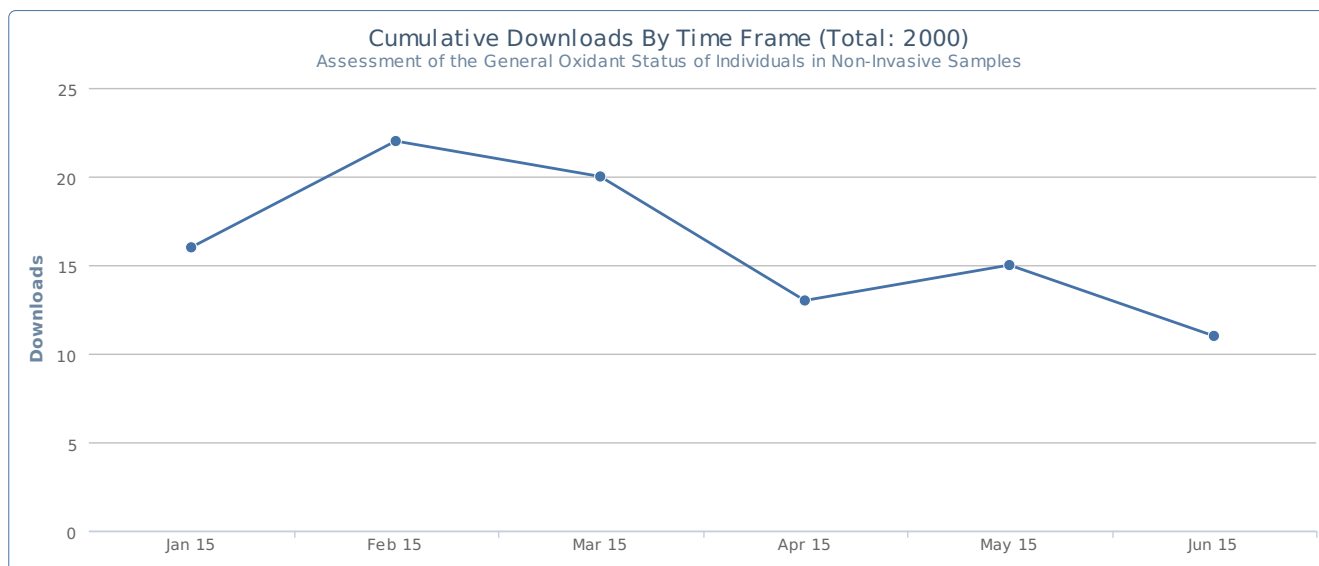
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